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Influence of lactation-induced and condition-dependent mobilization of body fat on bile acid metabolism of high-yielding dairy cows

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Lena Dicks, M.Sc.

aus Kevelaer

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Referentin: Frau Prof. Dr. Dr. Helga Sauerwein
Korreferent: Herr Prof. Dr. Karl-Heinz Südekum

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English abstract

The transition from pregnancy to lactation is characterized by major metabolic, physiological, and hormonal changes. In early lactation, dairy cows experience a negative energy balance (NEB) as the energy required for milk synthesis exceeds feed intake. Thus, body reserves from muscle and adipose tissue are mobilized and used for generating energy through various conversion processes in the liver or mammary gland. Excessive body fat mobilization, particularly in over-conditioned dairy cows, negatively affects liver function and is associated with an increased incidence of metabolic diseases including ketosis and fatty liver. Bile acids (BA) are synthesized from cholesterol in the liver, conjugated with the amino acids glycine or taurine, and stored in the gallbladder. To aid in the digestion of dietary fats and fat-soluble vitamins, they are released into the intestine, where they are modified by the intestinal microbiome before being absorbed through the intestinal mucosa and transported back to the liver via the portal vein. Furthermore, BA are hormone-like signaling molecules found in various tissues, including adipose tissue (AT), being able to trigger receptor-mediated signaling cascades. Changes in BA concentrations, depending on hepatic synthesis, intestinal biotransformation, and tissue metabolism, have been reported to influence glucose-, lipid- and energy metabolism, including body fat content in humans. The aim of this dissertation was to investigate and characterize the influence of lactation-induced and condition-dependent body fat mobilization on the BA metabolism in dairy cows. The experimental design included the examination of a feeding-induced (15-7 weeks before calving) high body condition (HBCS, N = 19) and normal body condition (NBCS, N = 19) in multiparous cows around calving. Using a targeted metabolomics approach, 20 BA were analyzed in the liver, in subcutaneous adipose tissue (scAT) and in serum at -7, 1, 3, and 12 weeks relative to calving. In addition, the mRNA abundance of BA-associated enzymes, transporters, and receptors were examined in the liver and scAT. This study shows that animals with high body condition had lower concentrations of several BA within the liver, serum, and scAT and an increased hepatic mRNA expression of enzymes involved in an alternative, generally less relevant synthesis pathway. These results suggest that an increased mobilization of AT affects the BA metabolism in dairy cows. With the onset of lactation, BA concentrations increased in both groups, which was accompanied by an increased hepatic expression of the key enzyme *CYP7A1*, probably an adaptation to the increased energy demand due to milk synthesis. Although BA have been detected in peripheral bovine tissues in other studies, their origin and function remain largely unexplored. We detected BA in scAT; however, enzymatic conditions (mRNA data) may exclude *de novo* synthesis. The detection of the mRNA abundance of specific transporters and receptors in scAT suggests that BA may play a role in signaling cascades in scAT. This dissertation provides fundamental insights into bovine BA metabolism during the transition from pregnancy to lactation and characterizes condition-dependent and lactation-induced changes.

Kurzfassung

Der Übergang von der Trächtigkeit zur Laktation ist durch metabolische, physiologische und hormonelle Veränderungen geprägt. In der Frühlaktation übersteigt der Energiebedarf für die Milchsynthese die Energiebereitstellung durch die Futteraufnahme was dazu führen kann, dass die Kuh eine negative Energiebilanz (NEB) aufweist. Energiereserven werden aus dem Fett- und Muskelgewebe mobilisiert und unter anderem in der Leber und Milchdrüse verstoffwechselt. Übermäßige Mobilisierung von Körperfett, welche besonders bei überkonditionierten Milchkühen auftritt, kann sich negativ auf die Leberfunktion auswirken und geht mit einer erhöhten Inzidenz für Stoffwechselerkrankungen wie Ketose oder Fettleber einher. Beim Menschen hat sich gezeigt, dass sich Leberfunktionsstörungen auf den Gallensäurestoffwechsel auswirken können. Gallensäuren (BA) werden in der Leber gebildet, mit den Aminosäuren Glycin oder Taurin konjugiert und in der Gallenblase gespeichert. Zur Unterstützung der Verdauung von Nahrungsfetten und fettlöslichen Vitaminen werden sie in den Darm abgegeben und dort durch das Darmmikrobiom modifiziert, über die Darmschleimhaut resorbiert und über die Pfortader zurück in die Leber transportiert. BA können zudem als Signalmoleküle, Rezeptorvermittelt Signalkaskaden auslösen und andere Stoffwechselprozesse, einschließlich des Lipid- und Glucosestoffwechsels, beeinflussen. Ziel dieser Dissertation war es, den Einfluss der laktationsinduzierten und konditionsabhängigen Lipolyse auf den BA-Stoffwechsel bei Milchkühen zu untersuchen. Es wurden Kühe mit fütterungsinduzierter (15-7 Wochen vor der Kalbung) hoher Körperkondition (HBCS, N=19) und normaler Körperkondition (NBCS, N=19) im Zeitraum rund um die Kalbung (-7, 1, 3 und 12 Wochen relativ zur Kalbung) untersucht. Über einen gezielten Metabolomics-Ansatz wurden 20 BA in der Leber, im subkutanen Fettgewebe (scAT) und im Blutserum gemessen. Zudem wurde die mRNA BA-assoziierter Enzyme, Transporter und Rezeptoren in der Leber und im scAT untersucht. Die HBCS-Tiere wiesen eine Vielzahl von BA in niedriger Konzentration auf und zeigten eine erhöhte hepatische mRNA-Expression von Enzymen eines alternativen, normalerweise weniger relevanten Syntheseweges als die NBCS-Tiere. Die Ergebnisse deuten darauf hin, dass die erhöhte Lipolyse den bovinen BA-Stoffwechsel beeinflusst. Mit dem Einsetzen der Laktation konnte bei beiden Gruppen ein Anstieg der BA beobachtet werden, welche mit einer erhöhten Expression des Schlüsselenzyms *CYP7A1* einherging und vermutlich eine Anpassung an den steigenden Energiebedarf infolge der Milchsynthese ist. Obwohl BA bereits in einigen peripheren Geweben bei Milchkühen nachgewiesen worden sind, ist ihre Herkunft und Funktion weitestgehend unerforscht. Unsere Untersuchungen zeigen, dass BA in bovinem scAT vorkommen, wobei die vollständige *de novo* Synthese vermutlich ausgeschlossen werden kann (mRNA-Daten). Der Nachweis der mRNA-Expression spezifischer Transporter und Rezeptoren im scAT lässt vermuten, dass sich BA auf Signalkaskaden im scAT auswirken. Die vorliegende Dissertation liefert fundamentale Erkenntnisse über den BA-Stoffwechsel der Milchkuh in der Übergangszeit zwischen Trächtigkeit und Laktation und charakterisiert konditionsabhängige Veränderungen.

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a.p.	ante partum
AKT	Protein kinase B
APO CII	Apolipoprotein CII
ASBT	Apical sodium-dependent bile acid transporter
AT	Adipose tissue
BA	Bile acids
BAAT	Bile acid-CoA: amino acid N-acyltransferase
BACS	Bile acid CoA synthase
BCS	Body condition score
BFT	Backfat thickness
BHB	ß-hydroxybutyrate
BSEP	Bile salt export pump
BSH	Bile salt hydroxylase
CA	Cholic acids
CCK	Cholecystokinin
CDCA	Chenodeoxycholic acid
CYP27A1	Sterol 27-Hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase
DCA	Deoxycholic acid
DMI	Dry matter intake
ERK	Extracellular regulated kinase
FA	Fatty acids
FAS	Fatty acid synthase
FGF15	Fibroblast Growth Factor 15
FXR	Farnesoid X receptor
GCA	Glycocholic acid
GCDCA	Glycochenodeoxycholic acid
GDCA	Glycodeoxycholic acid
GLCA	Glycolitocholic acid
GUDCA	Glycoursodeoxycholic acid
HBCS	High body condition score

HSL	Hormone-sensitive lipase
LCA	Lithocholic acid
LC ESI MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry
LOD	Limit of detection
MCA	Muricholic acid
MRP2	multidrug resistance protein
NAFLD	Nonalcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NBCS	Normal body condition score
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP1A2	Organic anion transporter Family Member 1A2
OSTα-OSTβ	Organic solute transporters α, β
PCR	Polymerase chain reaction
p.p.	post partum
PPARα	Peroxisome proliferator-activated receptor α
PXR	Pregnane X receptor
RXR	Retinoid x receptor
S1PR2	Sphingosine-1-phosphate receptor 2
scAT	subcutaneous adipose tissue
SHP	Small heterodimer partner
SREBP1c	Sterol regulatory element-binding protein 1c
TBF	Total body fat
TBV	Total blood volume
TCA	Taurocholic acid
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
TGR5	Takeda G protein-coupled receptor 5
TLCA	Taurolitocholic acid
UDCA	Ursodeoxycholic acid

UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry
VDR	Vitamine-D-receptor
VLDL	Very low-density lipoproteins
qPCR	Quantitative polymerase chain reaction

1 Introduction

Modern dairy cows are characterized by high milk yields and a concomitant decrease in resilience of metabolic diseases due to several decades of breeding and single trait selection for milk production (Shanks et al., 1978; Boichard and Brochard, 2012). High milk yields usually go in line with the mobilization of body reserves at the beginning of lactation to compensate the energy demand for milk synthesis which cannot be fully provided by feed intake (Herdt, 2000; Grummer, 2008). Body reserves, mainly from adipose tissue (**AT**) are mobilized as non-esterified fatty acids (**NEFA**) and used for further energy supply by transformation processes in the liver, muscle or in the mammary gland (Grummer, 1993). Thus, circulating NEFA are the main energy source in times of negative energy balance, being available for most body tissues (Katz and Bergman, 1969; Bergman, 1971). However, the liver being the key modulator of blood NEFA, removes large amounts of them (Herdt, 2000). When the capacity for oxidation is exceeded, incomplete NEFA oxidation leads to the accumulation of triglycerides and the production of ketone bodies e.g., acetoacetate or ß-hydroxybutyrate (**BHB**) (Drackley and Drackley, 1999; Herdt, 2000). Over-conditioned cows before calving are more prone to mobilize body fat and thus have a higher risk for metabolic diseases such as ketosis or fatty liver in early lactation (Bernabucci et al., 2005). Therefore, the transition from pregnancy to lactation in dairy cows is accompanied by considerable physiological changes affecting most organs, particularly the liver and AT (Wankhade et al., 2017).

Bile acids (**BA**) are synthesized in the liver and are important in the digestion and absorption of fats and fat-soluble vitamins in the intestine (Armstrong and Carey, 1982; Di Ciaula et al., 2017). Moreover, acting as signaling molecules, BA affect metabolic processes such as the lipid, energy, and glucose metabolism (Chiang, 2009; Lefebvre et al., 2009) and body fat content (Shapiro et al., 2018). In humans it is known that impairments of the liver functions due to diseases such as nonalcoholic fatty liver disease (**NAFLD**) and nonalcoholic steatohepatitis (**NASH**) are related to altered BA concentrations, hepatic BA synthesis, BA transport and intestinal biotransformation (Arab et al., 2017).

Since the cow's lipid mobilization affects the liver metabolism, a profounder knowledge concerning the influence on the BA metabolism in dairy cows is required. The extend of body fat mobilization influences the BA formation in the gut and the fecal BA excretion in dairy cows (Gu et al., 2023). Furthermore, research in humans established that BA are present in AT, indicating evidence for functional BA signaling pathways in adipocytes (Schmid et al., 2019). Since BA have also been detected in organs, tissues, and body fluids outside the enterohepatic

circulation in dairy cows (Tsai et al., 2011; Schmid et al., 2019; Reiter et al., 2021), a more profound knowledge of their action in the transition period, including AT mobilization and metabolism is required.

1.1.1 The Transition Period and Metabolic Challenges in Dairy Cows

The transition period is described as the time of transition from pregnancy to lactation, which is defined as 3 weeks ante partum (**a.p.**) to 3 weeks post-partum (**p.p.**). Homeorhetic mechanisms, physiologic, endocrinologic, and metabolic changes arise, and characterize this time as the most critical and challenging phase of the lactation period (Grummer, 1995). Within the last three weeks of pregnancy, fetal growth and nutrient requirements of glucose and amino acids peak (Bell 1993). The endocrine status changes, dry matter intake (**DMI**) decreases, leading to the mobilization of fatty acids (**FA**) from AT in late gestation and early lactation (Grummer et al., 1990; Grummer, 1995; Drackley and Drackley, 1999). Due to the inadequate feed intake around parturition in contrast to the rapidly increasing milk production, cows get into a negative energy balance (**NEB**; Grummer, 1993; Kessel et al., 2008). Triglycerides from AT are hydrolyzed and released as glycerol and free FA, circulating as NEFA in the blood-stream, which are used to cover the cow's energy requirements (Grummer, 1993; Lafontan and Langin, 2009). The liver either oxidizes NEFA, re-esterifies them into triglycerides, or exports them via very low-density lipoproteins (**VLDL**; Grummer, 1993).

Glucose is the main source for generating lactose in the mammary gland, thus reducing glucose availability for oxidation and energy supply (Bauman and Currie, 1980). Therefore, lipids form the main energy source for the increased energy demands (Lemosquet et al., 2009). In ruminants, glucose is mainly produced by hepatic gluconeogenesis, which is related to the overall tricarboxylic acid cycle activity. Tricarboxylic acid cycle activity depends on the availability of oxaloacetate, and substrates such as propionate, lactate, amino acids, and glycerol. In times of high gluconeogenesis, oxaloacetate availability decreases, and consequently limits the oxidation of FA via the Tricarboxylic acid cycle, inducing an alternative metabolic pathway, i.e., the production of ketone bodies such as acetoacetate and BHB which accumulate in the blood (Grummer, 1993; Goff and Horst, 1997; Bruckmaier and Gross, 2017). Furthermore, if the capacity of the liver reaches its limit to oxidize the FA, triglycerides accumulate in the liver (Grummer, 1993; Drackley and Drackley, 1999). High concentrations of free FA, accompanied by excessively formed ketone bodies, lead to metabolic diseases such as fatty liver syndrome and ketosis (Adewuyi et al., 2005).

As the lactation period progresses, DMI increases and the energy requirement for milk production can be met. However, less milk is also produced towards the end of lactation. Consequently, lipogenesis and fatty acid esterification increases during the positive energy balance, serving as a buffer for the next pregnancy and lactation (Bell and Bauman, 1994; Bell, 1995).

1.1.2 Body Condition in the Transition Period

The metabolic adaptation of dairy cows to the conditions of lactation, going along with the supply of energy reserves for milk production, does not lead to clinical or subclinical diseases in all dairy cows. Although the ability to adapt is individual to each animal (Jorritsma et al., 2003), various risk factors for poor adaptability, including feed, milk yield, parity, and body condition before calving have been established (Komaragiri et al., 1998). Focusing on the pre-calving body condition that is important in the successful transition of dairy cows (Crookenden et al., 2017), energy-dense rations in the period before calving lead to increased body condition and increase body condition losses due to excessive lipolysis after calving (Cardoso et al., 2013). High pre-calving body condition reinforce the excessive mobilization of body fat, reduce the ability to oxidize FA in the liver, and lead to increased concentrations of ketone bodies in the circulation (Goff and Horst, 1997; Busato et al., 2002). Circulating ketone bodies in the plasma inhibit DMI even more after calving, which increases the NEB (Rukkwamsuk et al., 1999; Laeger et al., 2013). The degree of NEB also affects the degree of immunosuppression and the ability to fight disease in the periparturient period (Bernabucci et al., 2005; Goff, 2006). Moreover, a high prepartum body condition is associated with an increased risk of metabolic diseases such as ketosis (Gillund et al., 2001), milk fever (Roche and Berry, 2006), and fatty liver (Drackley and Drackley, 1999). Since the excessive mobilization of body fat affects animal health, productivity, and reproduction, the assessment of body fat reserves is included in management (Bewley and Schutz, 2008). Also, physiological changes can be classified by scoring body condition and thus provide an indication for management (Bewley and Schutz, 2008). In most countries, the body condition score (**BCS**) is usually measured on a 5-point scale with 0.25 percentage points, where 0 is considered as severely under-conditioned and 5.0 extremely over-conditioned (Edmonson et al., 1989). As the determination of BCS is a rather subjective assessment, other methods like the determination of body fat by measuring subcutaneous back fat thickness (**BFT**) via ultrasonography have been developed to determine the body condition in dairy cows (Butler-Hogg et al., 1985; Bewley and Schutz, 2008).

1.1.3 Adipose Tissue

In general, AT is the body's largest energy reservoir (Lafontan and Langin, 2009); and based on the distribution in the body it is classified as visceral or subcutaneous AT (**scAT**) which differ in their cellular composition, metabolic function, and the capacity to secrete adipokines (Coelho et al., 2013). In early lactation, AT metabolism is subject to endocrine regulation, whereby the cell structure of the AT changes (Collier et al., 1984; Smith and McNamara, 1990, McNamara, 1990; Vernon, and Pond, 1997; Chilliard et al., 2000). Hormone-sensitive lipase (**HSL**) hydrolyzes stored triglycerides in adipocytes, providing free FA and glycerol as an energy source in early lactation (Vernon, and Pond, 1997). Moreover, AT communicates with other tissues and reacts to stimuli of the lipid metabolism, the immune system, blood pressure, and hormone balance (Ottaviani et al., 2011; Häussler et al., 2022). Besides triglycerides, AT contains signaling molecules, so-called adipokines, such as leptin, adiponectin, resistin, visfatin, apelin, or growth factors which are produced and secreted in AT (Funcke and Scherer, 2019) and enable the tissue to communicate with other organs such as the liver, or muscle (Romacho et al., 2014). Lipophilic steroid hormones can be stored in AT and locally converted, depending on the enzymatic composition (Deslypere et al., 1985; Diamanti-Kandarakis, 2007; Schuh et al., 2022). Thus, AT is involved in the regulation of various processes through an interplay of endocrine, paracrine, and autocrine mechanisms (Mohamed-Ali et al., 1998), effecting whole-body physiology (Rosen and Spiegelman, 2006).

1.2 Bile Acids

In 1928, Heinrich O. Wieland reported in his Nobel Prize lecture about the novel discoveries of BA research and described that the BA glycocholic acid (**GCA**) and taurocholic acid (**TCA**) were discovered and isolated for the first time by A. Stecker in 1848, in oxen bile (Wieland, 1928). To date, at least 84 unconjugated BA have been identified in vertebrates (Hofmann et al., 2010), although not all are of physiological relevance (Ticho et al., 2019). In general, BA have an amphiphilic structure, consisting of a lipophilic steroid backbone with a hydrophilic side chain, ensuring that the BA form micelles and ultimately support the intestinal digestion and absorption of dietary fats and fat-soluble vitamins (De Aguiar Vallim et al., 2013). Short-chain FA can be absorbed without the aid of BA, whereas long-chain FA need BA for absorption (Tomkin and Owens, 2016). Conjugation with glycine or taurine lowers the pKA and increase the water solubility at a physiological pH and structural changes to the hydroxyl groups at position 3, 6, 7. or 12 on the steroid backbone change the hydrophilicity and solubility, which in turn can highly influence the extend of lipid absorption (Ticho et al., 2019).

Additionally, there are interspecies variations in the metabolism of BA, including BA composition and conjugation specificity, synthesis, signaling pathways, intestinal microbiota composition, and existence of a gallbladder (Angelin et al., 2012). The human BA pool mainly contains cholic acid (**CA**) (~ 40%), chenodeoxycholic acid (**CDCA**) (~ 40%) and deoxycholic acid (**DCA**) (~ 20%) with the majority being glycine-conjugated. In rodents, the BA pool contains mainly CA (~ 60%) and the murine-specific BA α-muricholic acid (**MCA**) and β-MCA (~ 40%), predominantly in taurine-conjugated form (Chiang, 2017). The bovine BA pool contains mainly CA (68-86%), with a predominance for glycine conjugation (Hofmann et al., 2018).

1.2.1 Bile Acids Synthesis in the Liver

In humans, it is assumed that the liver secretes 200-600 mg BA per day (Chiang 2013). The majority of the BA are formed via the classical/neutral pathway in the liver (Chiang and Ferrell, 2018), which is based on the formation of neutral sterols (Monte et al., 2009), forming the primary BA CA and CDCA. There are at least 17 enzymes involved in the formation of BA, located in the endoplasmic reticulum, mitochondria, peroxisomes, and the cytosol (Chiang, 2013). The rate-limiting enzyme in this process and within the classical/neutral pathway is cholesterol 7α-hydroxylase (**CYP7A1**) (Monte et al., 2009), whose transcription is highly regulated by feedback mechanisms exerted by BA and their involvement as ligands of a variety of nuclear receptors (Russell, 1999; Lu et al., 2000; Russell, 2003). Via the alternative/acidic pathway, cholesterol can be modified by enzymatic processes in different tissues, where the precursor molecules must subsequently be transported to the liver for further synthesis. Sterol 27-hydroxylase (**CYP27A1**), a mitochondria cytochrome P450 enzyme, initiates the alternative pathway (Chiang 2013). In humans, about 70% CDCA and 30% CA are formed from the alternative pathway of BA (Chiang, 2017), which only produces 9% of the total BA in human hepatocytes (Duane and Javitt, 1999). Nevertheless, this pathway is considered to be more important in patients with liver diseases (Crosignani et al., 2011; Chiang, 2013; Lake et al., 2013). For example, oxysterol 7a-hydroxylase (**CYP7B1**) gene expression, which is considered to be the marker enzyme for the alternative pathway, was upregulated in humans with NASH (Lake et al., 2013).

Figure 1 provides an overview of the involved enzymes within the BA pathways. Regardless of the pathway, BA are conjugated to the amino acids glycine or taurine by BA CoA synthase (**BACS**) and BA-CoA: amino acid N-acyltransferase (**BAAT**) to build the conjugated

primary BA TCA, GCA, taurochenodeoxycholic acid (**TCDCA**), glycochenodeoxycholic acid (**GCDCA**) which are stored in the gallbladder (Di Ciaula et al., 2017).

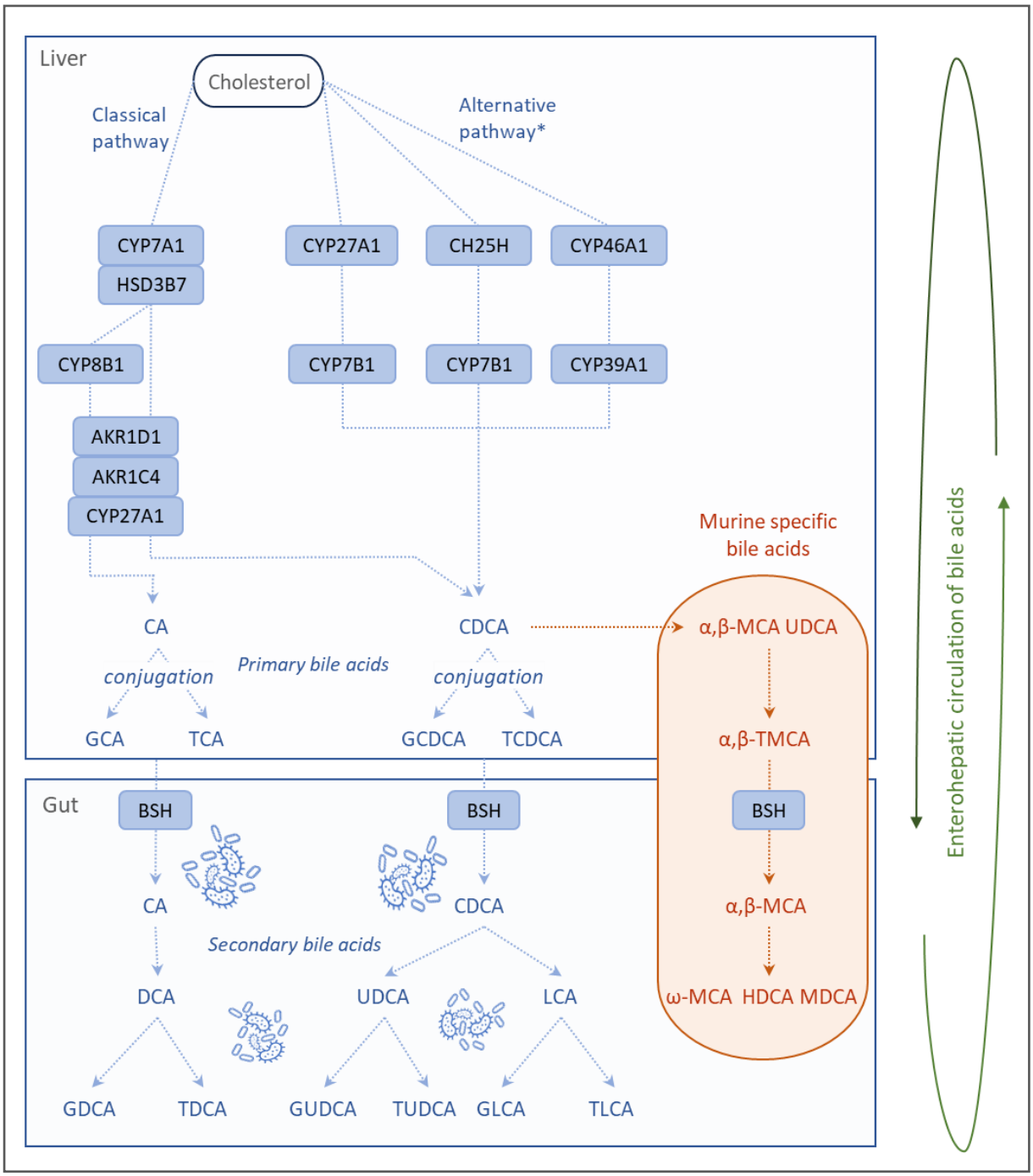

Figure 1: Schematic illustration of bile acid synthesis: * BA-related enzymes are ubiquitously expressed and not limited to the hepatic alternative pathway. Involved enzymes: CYP7A1: Cholesterol 7alpha-Hydroxylase; HSD3B7: 3 Beta-Hydroxysteroid Dehydrogenase Type 7; CYP8B1: Sterol 12-Alpha-Hydroxylase; AKR1D1: Aldo-Keto Reductase Family 1 Member D1; AKR1C4: Aldo-Keto Reductase Family 1 Member C4; CYP27A1: Sterol 27-Hydroxylase; CYP7B1: Oxysterol 7-Alpha-Hydroxylase; CH25H: Cholesterol 25-Hydroxylase; CYP46A1: Cholesterol 24-Hydroxylase; CYP39A1: Cytochrome P450 Family 39 Subfamily A Member 1; BSH: Bile Salt Hydrolase Bile acids: cholic acid (CA); chenodeoxycholic acid (CDCA); taurocholic acid (TCA); glycocholic acid (GCA); taurochenodeoxycholic acid

(TCDCA); glycochenodeoxycholic acid (GCDCA); deoxycholic acid (DCA); lithocholic acid (LCA); ursodeoxycholic acid (UDCA); taurodeoxycholic acid (TDCA); glycodeoxycholic acid (GDCA); taurolithocholic acid (TLCA); glycolitocholic acid (GLCA); glycoursodeoxycholic acid (GUDCA); tauroursodeoxycholic acid (TUDCA); α-muricholic acid (αMCA); β-muricholic acid (βMCA); tauromuricholic acid (sum of alpha and beta) (α, β-TMCA); ω-muricholic acid (ωMCA); hyodeoxycholic acid (HDCA); murideoxycholic acid (MDCA). Self-designed illustration, based on Chiang et al. (2004), Poland et al. (2021), and Chiang and Ferrell (2019).

1.2.2 Bile Acids in the Intestine

In humans, after food intake, the entero-hormone cholecystokinin (**CCK**) stimulates the contraction of the *Musculus sphincter oddi* at the gallbladder, to release BA into the intestine where they are first deconjugated by the bile salt hydroxylase (**BSH**; Hofmann and Hagey, 2014). The reabsorption of BA through intestinal epithelial cells towards the portal blood starts in the small intestine, whereas the bacterial modification including 7α-dehydroxylation to form the secondary BA (DCA, lithocholic acid (**LCA**) ursodeoxycholic acid (**UDCA**)) starts in the distal small intestine (Hofmann, 1984; Di Ciaula et al., 2017). Deconjugation and dehydroxylation improve the absorption by increasing the BA hydrophilicity and pKA (Ridlon et al., 2006). BA are again conjugated with glycine or taurine, to form the secondary conjugated BA (taurodeoxycholic acid (**TDCA**), glycodeoxycholic acid (**GDCA**), taurolitocholic acid (**TLCA**), glycolitocholic acid (**GLCA**), glycoursodeoxycholic acid (**GUDCA**)). Intestinal microbiota, performing various biotransformations of BA (Ridlon et al., 2016), are highly influenced in population, composition, and metabolic activity through the diet (Le Chatelier et al., 2013; Zhang et al., 2018; Singh et al., 2019). Also, metabolic changes in obese humans altered the intestinal microbiota in microbial richness and composition (Le Chatelier et al., 2013) and affected the fecal excretion of BA (Haeusler et al., 2016). In healthy individuals 90-95% of BA return to the liver at the terminal ilium via the portal vein (Yang et al., 2003; Chiang, 2004; Chen et al., 2019a; Chen et al., 2019b) and BA being not absorbed, enter the colon, and are subsequently excreted with the feces (Ferrebee and Dawson, 2015).

In the portal vein-liver junction, not all BA are equally absorbed, and a small fraction is not reabsorbed and recycled but enters the large bloodstream via the *Vena cava* (Hofmann, 2009). Disruptions in the absorption of BA by blocking the intestinal uptake, stimulate the hepatic *de novo* synthesis of BA (Ferrebee and Dawson, 2015). The circulation of BA between the liver and intestine is called the enterohepatic circulation of BA, which can occur up to 10 times per day (Stamp and Jenkins, 2008).

1.2.3 Bile Acid Transport through the Enterohepatic Circulation

The transport of BA through the compartments of the enterohepatic circulation and the associated maintenance of BA homeostasis is essentially controlled by various BA transporters in humans. Controlling the secretion of BA from the liver into the biliary canaliculi, the liver contains the ATP-dependent bile salt export pump (**BSEP**) and multidrug resistance protein (**MRP2**; Trauner and Boyer, 2003; Ferrebee and Dawson, 2015). In intestinal epithelial cells, conjugated BA are luminally absorbed via the apical sodium-dependent BA transporter (**ASBT**) and are basolaterally released via the organic solute transporters (**OSTα-OSTβ**; Alrefai and Gill, 2007). About 95% of the BA are reabsorbed in the terminal ileum in the brush border, whereas some unconjugated BA can already be reabsorbed in the upper intestine via passive diffusion (Chiang, 2013). The BA pass the enterocytes at the basolateral membrane, reach the blood stream and then the liver where they are taken up into the hepatocytes (Chiang 2013). BA in the portal vein are largely bound to albumin for transportation and are dissociated via a conformational change of the albumin by contact with the basolateral membrane of the hepatocytes (Horie et al., 1988; Meier, 1995). During the first passage of the portal blood through the liver lobules, the extraction rate of the conjugated BA varies between 75 and 90% (Meier, 1995; Trauner and Boyer, 2003) and is mainly mediated by the Na^+-taurocholate co-transporting polypeptide (**NTCP**) and the solute carrier organic anion transporter Family Member 1A2 (**OATP1A2**; Trauner and Boyer, 2003; Ferrebee and Dawson, 2015). Gene expression of NTCP can be regulated in various physiological challenges like pregnancy (Arrese et al., 2003) or in the case of high accumulations of BA to reduce their flux into the hepatocytes (Anwer, 2004). When the hepatic BA concentration increases, BA spill over into the sinusoid blood, pass through the kidney and are back transported to the liver via systemic circulation (Chiang, 2009). Figure 2 provides an overview of the involved BA transporters within the enterohepatic circulation.

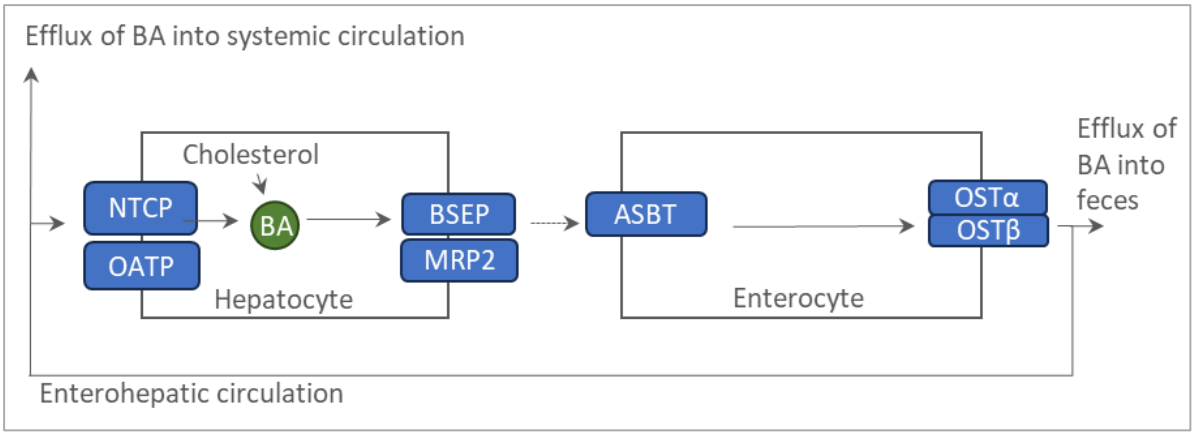

Figure 2: Bile acid transporters within the enterohepatic circulation. BA: Bile acids; NTCP, SLC10A1: Na+-taurocholate co-transporting polypeptide; OATP: Solute Carrier Organic Anion Transporter; OSTα, SLC51A: solute carrier family 51 subunit alpha; OSTβ, SLC51B: solute carrier family 51 subunit beta; MRP2, ABCC2: Multidrug Resistance-Associated Protein; BSEP, ABCB11: Bile Salt Export Pump. Self-designed illustration, based on Alrefai et al. (2007) and Cai et al. (2022).

1.2.4 Regulation of Bile Acid Synthesis

The BA metabolism is subject to negative feedback mechanisms, which can be regulated within the enterohepatic circulation by inhibiting the biosynthesis of primary BA or by modulating the flux of BA in hepatocytes or the intestine (Hofmann, 2009). In the hepatocytes, free BA, or bound to the Farnesoid X receptor (**FXR)** or Retinoid X receptor (**RXR**) - FXR heterodimer, induce the synthesis of the transcription factor small heterodimer partner (**SHP**), which then acts on the promoter of the rate-limiting enzyme CYP7A1, inhibiting BA synthesis (Chiang, 2004). Also, BA binding to the FXR induces the expression of the SHP which subsequently inhibits NTCP, preventing the excessive BA uptake into the hepatocytes in times of BA accumulations (Denson et al., 2001). The transcription of CYP7A1 can also be suppressed by activating the Pregnane X receptor (**PXR**), thereby inhibiting BA synthesis (Li and Chiang, 2005; Pavek, 2016). The Vitamin-D-receptor (**VDR**), present in enterocytes, can also be activated by BA, which leads to the release of fibroblast growth factor 15 (**FGF15**) and in turn has a suppressive effect on CYP7A1 in the liver (Schmidt et al., 2010).

The membrane-bound Takeda G-protein coupled receptor 5 (**TGR5**) has been identified as BA receptor as well (Maruyama et al., 2002), being able to reduce the hepatic protein expression of CYP8B1 which is necessary for the *de novo* synthesis of CA (McGavigan et al., 2017). In TGR5 knock-out mice a 21-25% decreased BA pool as compared to wild-type mice was reported, supporting its role in BA homeostasis (Maruyama et al., 2006). However, the mechanisms of TGR5 influencing the BA pool composition are not fully understood (Holter et

al., 2020). Figure 3 shows how the main BA receptors (FXR and TGR5) control the BA biosynthesis and transport.

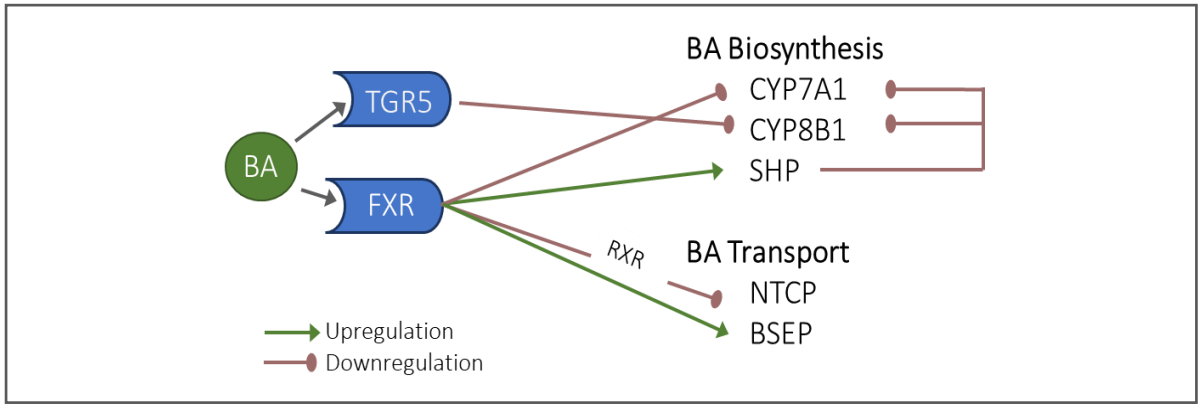

Figure 3: Bile acid controlling biosynthesis and transport. BA: Bile acids; FXR, NR1H4: Farnesoid X Receptor; TGR5, GPBAR1: Takeda G protein–coupled receptor 5; CYP7A1: Cholesterol 7alpha-Hydroxylase; CYP8B1: Sterol 12-Alpha-Hydroxylase; SHP: Small Heterodimer Partner; NTCP, SLC10A1: Na+-taurocholate cotransporting polypeptide; BSEP, ABCB11: Bile Salt Export Pump. Self-designed illustration, based on Chiang and Ferrell (2020), Distrutti et al. (2015), and Lefebvre et al. (2009).

1.2.5 Role of Bile Acids in Metabolic Regulation

Disruptions of BA metabolism is associated with the pathogenesis of diseases (Li and Chiang, 2012, 2014; Pandak and Kakiyama, 2019). Acting as signaling molecules, BA interact with the gut microbiota affecting the pathophysiology of obesity, type 2 diabetes, and NAFLD in humans (Molinaro et al., 2018; Chávez-Talavera et al., 2019). Obese humans had increased BA synthesis and impaired BA transport (Haeusler et al., 2016). Plasma BA correlated positively with the body mass index and negatively with the cognitive restraint of eating in obese humans (Prinz et al., 2015). Diet-induced obesity in mice decreased hepatic BA (Hatori et al., 2012; Suzuki Y et al., 2013; Liu et al., 2015) and increased plasma BA (Ma and Patti, 2014). However, fasting BA concentrations were not affected by obesity, whereas obese humans had a higher fecal excretion, suggesting a higher BA removal from the body (Gälman et al., 2003). Also, the diet (Ridlon et al., 2014; Ridlon et al., 2016), and physiological changes due to pregnancy and lactation (Zhu et al., 2013; Fan et al., 2021) can alter the BA pool and the composition of BA in humans and murine species. These structural changes due to BA modifications can influence the specificity for binding to BA receptors (De Aguiar Vallim et al., 2013). Thus, considering that BA can affect different physiological processes based on their structure, BA profiles have to be considered individually.

The identification of FXR and TGR5 as BA targets, points to their involvement in major signaling pathways of energy homeostasis, as well as glucose and lipid metabolism (Houten et al., 2006; Keitel et al., 2008; Nguyen and Bouscarel, 2008; Thomas et al., 2008; Lefebvre et al., 2009; Ma and Patti, 2014; Taoka et al., 2016; Vítek and Haluzík, 2016). Increasing BA concentrations correlated strongly with improvements in glucose and lipid metabolism (Patti et al., 2009; Pournaras et al., 2012). Hepatic BA can bind to the FXR, activating the FXR-SHP signaling pathway, suppressing the lipogenic transcription factor sterol regulatory element-binding protein 1c (**SREBP1c**), and influencing lipid metabolism (Watanabe et al., 2004). Additionally, BA activation of FXR increased the expression of apolipoprotein CII (**APO CII**), further activating lipoprotein lipase and stimulating the hydrolysis of triglycerides in VLDL and chylomicrons (Kast et al., 2001). In mice, FXR binding enhances the transcription of fatty acid synthase (**FAS**; Matsukuma et al., 2006), and FXR activation reduces plasma and hepatic triglyceride levels (Watanabe et al., 2004; Zhang et al., 2006). Furthermore, the expression of peroxisome proliferator-activated receptor alpha (**PPARα**), playing a key role in lipolysis, is induced via the FXR, activated by BA (Pineda Torra et al. 2003). Likewise, BA stimulate energy expenditure via TGR5 in brown AT in mice and skeletal muscle in humans (Watanabe et al., 2006). In humans, TGR5 expression in AT correlates positively with obesity (Svensson et al., 2013), and BA acting on TGR5 can stimulate the release of glucagon-like peptides in the portal vein, regulating the glucose homeostasis (Ayewoh and Swaan, 2022). Conjugated BA binding to the sphingosine-1-phosphate receptor 2 (**S1PR2**) activates the extracellular signal-regulated protein kinase (**ERK**)1/2 and the protein kinase A (**AKT**) pathways which in turn affect the hepatic glucose, lipid, and BA metabolism (Studer et al., 2012; Kwong et al., 2015). Moreover, in recent years, an increasing number of membrane and nuclear receptors have been identified that can be activated by BA. The receptors involved have been detected in organs of the enterohepatic circulation but also in other organs, such as AT (Zhang et al., 2003; Schmid et al., 2019). Figure 4 schematically illustrates the enterohepatic circulation of BA. The BA pool and the BA composition can be influenced by environmental and individual factors, which in turn can affect the binding and signal transduction through BA receptors.

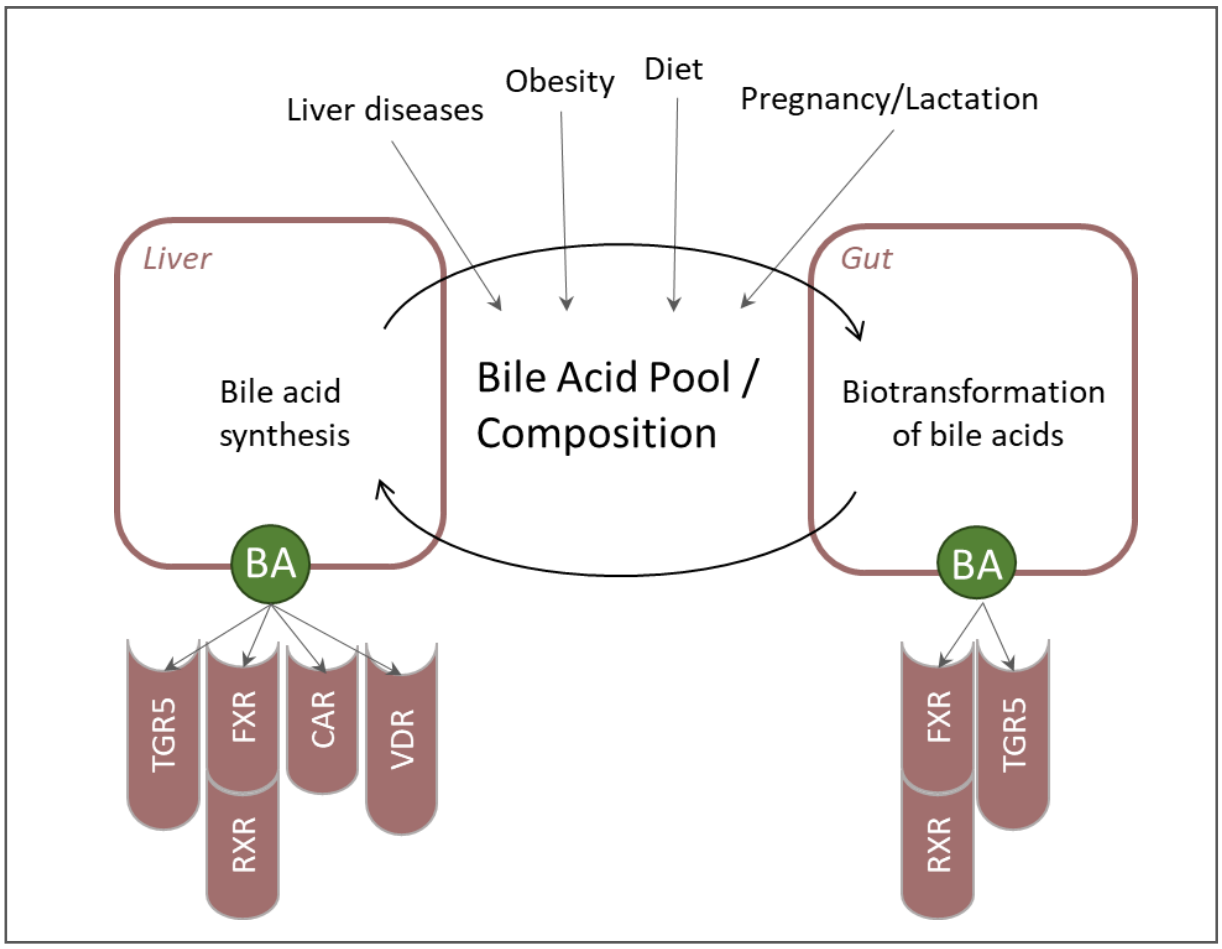

Figure 4: Schematic illustration of the enterohepatic circulation of bile acids. The bile acid pool and composition can be influenced by environmental and individual factors, affecting the binding and signal transduction through bile acid receptors. BA: Bile acids; FXR, NR1H4: Farnesoid X Receptor; TGR5, GPBAR1: Takeda G protein–coupled receptor 5; VDR: Vitamin-D Receptor; RXR, NR2B1: Retinoid X Receptor Alpha; CAR: Constitutive Androstane Receptor. Self-designed illustration, based on Zhu et al. (2013), Di Ciaula et al., (2017), Ahmad and Haeusler (2019), Chiang and Ferrell (2020), Fiorucci et al. (2021), and Gu et al. (2023).

1.2.6 Bile Acids in Tissues outside of the Enterohepatic Circulation

Due to the spillover of BA into the systemic circulation, BA can be detected in several tissues, organs, and body fluids outside of the enterohepatic circulation (Blaschka et al., 2019; Schmid et al., 2019; Reiter et al., 2021). In 2014, 17 BA could be detected for the first time in human AT, providing insights into the endogenous role of BA and their role as biomarkers (Jäntti et al., 2014). Findings of conjugated BA, which are unable to cross cell membranes by passive diffusion in peripheral tissues pointed to the existence of an active transport of BA into the cells or the possibility of BA conjugation within the cells (Jäntti et al., 2014). Also, the expression of BA receptors like the FXR and TGR5 in tissues outside of the enterohepatic circulation, point to a role throughout the body and not just exclusively within the enterohepatic

circulation (Swann et al., 2011). Furthermore, significantly higher concentrations of BA in human follicular fluid compared to serum could indicate *de novo* production of BA in this tissue (Smith et al., 2009).

1.2.7 Bile Acids in Cattle

Since the discovery of BA, little but steady research has been carried out on these metabolites in cattle. However, with the findings that BA can also act as signaling molecules and influence other metabolic processes (Shapiro et al., 2018), the field of research is experiencing an enormous upswing. In dairy cows, BA concentrations and compositions of the individual BA were determined in the compartments of the enterohepatic circulation, including the liver (Tsai et al., 2011; Reiter et al., 2021), bile (Sheriha et al., 1968), and blood from the portal vein (Reiter et al., 2021). Furthermore, BA have been discovered in follicular fluid, urine (Sanchez et al., 2014; Blaschka et al., 2019), feces (Sheriha et al., 1968), serum (Washizu et al., 1991; Pacífico et al., 2020; Reiter et al., 2021; Ghaffari et al., 2024), udder, muscle, heart, lung, bone, tongue, esophagus, omasum, abomasum, spleen, and kidney (Reiter et al., 2021). The BA in the plasma of dairy cows were investigated considering diurnal and individual variations of BA (Abdelkader and Ropstad, 1989) or the hepatic conjugation patterns with taurine and glycine (Vessey et al., 1977). Grain feeding in Angus beef cattle increased intestinal secondary BA and was accompanied by an increase in bacterial taxa being responsible for the conversion of BA, compared to grass feeding (Liu et al., 2020).

The hepatic uptake and conjugation of BA in plasma from cows with fatty livers were altered (West, 1990), and dairy cows with a fatty liver syndrome also had increased plasma concentrations of bile components, i.e., bilirubin and BA (Bobe et al., 2004). However, the large variability of serum BA after feeding was not correlated with liver fat content (Garry et al., 1994). The extraction rate of the liver for BA from portal and hepatic venous blood was not affected by different fat portions in the diet in healthy animals (Mohamed et al., 2002), whereas cows with fasting-induced hepatic lipidosis had an decreased BA extraction, probably caused by hepatic triglyceride accumulation causing liver impairment (Mohamed et al., 2004). Excessive lipolysis induced an increased excretion of BA in the feces, indicating that the BA metabolism is also affected by lipid mobilization in dairy cows (Gu et al., 2023).

Taken together, the aforementioned studies in cattle mainly characterized the BA concentration in different tissues and the relation with dietary and hepatic fat. Even though studies in mice and humans strongly point to the involvement of BA in several metabolic pathways such as lipid and glucose metabolism and disruption during liver disease, data is scarce in transition

dairy cows. The liver BA concentrations in the transition period, as affected by different body condition around calving have not been investigated so far. The existence of BA in various tissues outside the enterohepatic circulation has been demonstrated (Washizu et al., 1991; Blaschka et al., 2019; Pacífico et al., 2020; Reiter et al., 2021; Ghaffari et al., 2024); however, the function of the metabolites in these tissues is still not clear. Moreover, it is also still unclear whether BA can be synthesized *de novo* in extrahepatic tissues or whether they are transported into them.

2 Objectives and hypotheses

Bile acids (**BA**) are involved in several metabolic processes besides aiding the digestion of fats and fat-soluble vitamins. However, basic information concerning the abundance of BA in tissues within the enterohepatic circulation and other tissues like adipose tissue (**AT**) was lacking in dairy cows. Furthermore, the physiologically challenging time of transitioning from pregnancy to lactation, as well as different body condition around calving and their effects on the BA concentration in the liver, serum, and subcutaneous AT (**scAT**) have not been investigated previously. Therefore, the present study aimed to fill the gap of knowledge pursuing the following objectives:

I. To investigate the composition of BA in serum, liver, and scAT of cows with high (**HBCS**) and normal (**NBCS**) body condition around calving.
II. To investigate the mRNA abundance of BA synthesizing enzymes, transporters, and receptors in the liver to characterize differences in the formation, transport, and receptor expression in cows with different body conditions around calving (HBCS vs. NBCS).
III. To investigate the mRNA abundance of BA synthesizing enzymes, transporters, and receptors in scAT to characterize the role of BA in scAT in HBCS and NBCS cows around calving.

We hypothesized that cows with different body condition around calving would differ in their BA profiles in serum, liver, and scAT and would show differences in the mRNA abundance of BA-associated enzymes, receptors, and transporters within the liver. Furthermore, we hypothesized that the mRNA of BA-associated enzymes, receptors, and transporters would be present in scAT and their abundance would differ between HBCS and NBCS cows.

3 Manuscript I

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Bile acid profiles and mRNA expression of bile acid-related genes in the liver of dairy cows with high versus normal body condition

Lena Dicks[1], Katharina Schuh-von Graevenitz[2], Cornelia Prehn[3], Hassan Sadri[4†], Morteza H. Ghaffari[1], Susanne Häussler[1*]

[1] Institute of Animal Science, Physiology Unit, University of Bonn, 53115 Bonn, Germany

[2] Department of Life Sciences and Engineering, Animal Nutrition and Hygiene Unit, University of Applied Sciences Bingen, 55411 Bingen am Rhein, Germany

[3] Helmholtz Zentrum München, German Research Center for Environmental Health, Metabolomics and Proteomics Core, 85764 Neuherberg, Germany

[4] Department of Clinical Science, Faculty of Veterinary Medicine, University of Tabriz, 516616471 Tabriz, Iran

*Corresponding author: susanne.haeussler@uni-bonn.de

†H. Sadri was a visiting scientist at the Institute of Animal Science, Physiology Unit, University of Bonn, 53115 Bonn, Germany, at the time the research was done.

ABSTRACT

Bile acids (BA) play a crucial role not only in lipid digestion but also in the regulation of overall energy homeostasis, including glucose and lipid metabolism. The aim of this study was to investigate BA profiles and mRNA expression of BA-related genes in the liver of high versus normal body condition in dairy cows. We hypothesized that body condition and the transition from gestation to lactation affect hepatic BA concentrations as well as the mRNA abundance of BA-related receptors, regulatory enzymes, and transporters. Therefore, we analyzed BA in the liver as well as the mRNA abundance of BA-related synthesizing enzymes, transporters, and receptors in the liver during the transition period in cows with different body conditions around calving. In a previously established animal model, 38 German Holstein cows were divided into groups with high body condition score (BCS) (HBCS; n = 19) or normal BCS (NBCS; n = 19) based on BCS and backfat thickness (BFT). Cows were fed diets aimed at achieving the targeted differences in BCS and BFT (NBCS: BCS < 3.5, BFT < 1.2 cm; HBCS: BCS > 3.75, BFT > 1.4 cm) until they were dried off at wk 7 before parturition. Both groups were fed identical diets during the dry period and subsequent lactation. Liver biopsies were taken at wk −7, 1, 3, and 12 relative to parturition. For BA measurement, a targeted metabolomics approach with LC-ESI-MS/MS was used to analyze BA in the liver. The mRNA abundance of targeted genes related to BA-synthesizing enzymes, transporters, and receptors in the liver was analyzed using microfluidic quantitative PCR. In total, we could detect 14 BA in the liver: 6 primary and 8 secondary BA, with glycocholic acid (GCA) being the most abundant one. The increase of glycine-conjugated BA after parturition, in parallel to increasing serum glycine concentrations may originate from an enhanced mobilization of muscle protein to meet the high nutritional requirements in early lactating cows. Higher DMI in NBCS cows compared to HBCS cows was associated with higher liver BA concentrations such as GCA, deoxycholic acid (DCA), and cholic acid (CA). The mRNA abundance of BA-related enzymes measured herein suggests the dominance of the alternative signaling pathway in the liver of HBCS cows. Overall, BA profiles and BA metabolism in the liver depend on both, the body condition and lactation-induced effects in periparturient dairy cows.

Key words: bile acids, body condition, liver, periparturient period

INTRODUCTION

The transition from late gestation to early lactation is a critical period for dairy cows, characterized by complex and significant physiological and metabolic adaptations as they overcome the challenges of transitioning from gestation to the demands of milk production (Ghaffari et al., 2024a). During this challenging period, cows often have a negative energy balance (NEB), in which the energy requirements of lactation exceed the energy derived from feed intake, resulting in increased mobilization of body energy reserves (Drackley et al., 2001). This metabolic state mainly involves the mobilization of energy stores from adipose and muscle tissue to meet the increased demand during lactation (Grummer, 1993; Sadri et al., 2023). As a result, this leads to an overload on the metabolic capacity of the liver, as evidenced by an enhanced release of free fatty acids (FFA) from adipose tissue, leading to an accumulation of lipids and an increase in the production of ketone bodies, particularly β-hydroxybutyrate (BHB). Such changes can affect liver function and the general health of dairy cows (Goff and Horst, 1997; Drackley, 1999; Bobe et al., 2004).

Over-conditioned cows around calving face challenges due to a higher NEB, leading to increased lipolysis, reduced feed intake, and a greater risk of liver disease and metabolic disorders (Roche et al., 2009; Ghaffari et al., 2023). Recent metabolomics studies reveal that these cows have higher levels of acylcarnitine and long-chain acylcarnitines in muscle and serum during early lactation, suggesting incomplete fatty acid oxidation (Ghaffari et al., 2019a; Ghaffari et al., 2020; Sadri et al., 2020). In addition, a study by Ghaffari et al. (2021) using microfluidic quantitative PCR found increased gene expression related to hepatic mitochondrial fatty acid oxidation and ketogenesis in over-conditioned cows, indicating potential β-oxidation impairment and increased acylcarnitines in circulation.

Moreover, the presence of fatty liver in cows is often signaled by increased levels of liver enzymes and bile components such as bilirubin and BA in the plasma, pointing to an impaired hepatic BA extraction rate (West, 1990; Rehage et al., 1999; Mohamed et al., 2004). The liver plays a pivotal role in these metabolic processes related to BA metabolism, synthesizing BA through both, the classical and alternative synthesis pathways. While the classical pathway is confined to the liver, the alternative pathway, involving the 27-side-chain hydroxylation of cholesterol by mitochondrial sterol 27-hydroxylase (CYP27A1), is active in various other tissues (Myant and Mitropoulos, 1977; Armstrong and Carey, 1982; Lund et al., 1996). Notably, in cases of chronic liver diseases in humans, the alternative pathway frequently dominates over the classical pathway (Crosignani et al., 2007). Following their synthesis, primary BA such as

cholic acid (CA) and chenodeoxycholic acid (CDCA) undergo conjugation with glycine or taurine, leading to the formation of primary conjugated BA. Primary conjugated BA are then excreted into bile canaliculi via ATP-dependent mechanisms like the bile salt export pump (BSEP; ABCB11) or multidrug resistance proteins (MRP2; ABCC2), and stored in the gallbladder (Trauner and Boyer, 2003; Ferrebee and Dawson, 2015). Hormonal stimuli, particularly from cholecystokinin (CCK), induce the gallbladder to release BA into the intestine, aiding in the emulsification of dietary lipids and fat-soluble vitamins (Di Ciaula et al., 2017). The gut microbiome further converts these primary conjugated BA into secondary forms. In humans, about 90-95% of these BA are reabsorbed and returned to the liver via the portal vein, largely through passive diffusion or transporters like the Na^+-taurocholate co-transporting polypeptide (NTCP; Yang et al., 2003; Chiang, 2004). Intriguingly, in patients with non-alcoholic fatty liver disease (NAFLD), BA uptake in the liver is diminished compared to healthy individuals, suggesting impaired BA reabsorption in fatty liver conditions (Jahnel et al., 2015).

In addition to their role in emulsifying dietary fats and vitamins, BA act as signaling molecules regulating glucose, lipid, and energy metabolism through the activation of specific receptors (Lefebvre et al., 2009). Recent targeted metabolomics studies on serum and liver (Ghaffari et al., 2024a,b) have revealed significant changes in BA profiles and concentrations in healthy dairy cows with normal body condition during the transition period. These changes indicate dynamic alterations in BA synthesis, lipid digestion, and absorption.

With this background, we aimed to investigate liver BA as well as the BA-related enzymes, transporters, and receptors in the liver of periparturient dairy cows with different body conditions. We hypothesized that differently conditioned cows with varying degrees of lactation-induced lipolysis would have different liver BA profiles and different expressions of BA-related genes within the liver. This study will demonstrate that condition-dependent and lactation-induced lipolysis affects BA metabolism in dairy cows and will provide further insights into BA metabolism by analyzing metabolomics and mRNA data simultaneously.

MATERIALS AND METHODS

The animal experiment was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany, in compliance with European guidelines for the protection of experimental animals. The study was authorized by the local animal welfare authority (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany [G 14-20-071]).

Basic trial

A comprehensive description of the experiment has been published previously (Schuh et al. 2019). Briefly, the experiment lasted from wk 15 before calving to wk 14 after calving and involved 38 German Holstein cows that were divided into two treatment groups based on their body condition score (BCS) and backfat thickness (BFT) from the previous lactation. Cows were assigned to two treatment groups to achieve either a high (HBCS) or normal (NBCS) BCS and BFT until dry-off at wk 7 antepartum (a.p.) (see Figure 1A). To emphasize the differences between BCS and BFT, the cows received different diets from wk 15 a.p. to wk 7 a.p. (HBCS: 7.2 NE_L MJ/kg dry matter (DM); NBCS: 6.8 NE_L MJ/kg DM). The HBCS group (n = 19) had BCS > 3.75 (3.82 ± 0.33) and BFT > 1.4 cm (2.36 ± 0.35 cm), while the NBCS group (n = 19) had BCS < 3.5 (3.02 ± 0.24) and BFT < 1.2 cm (0.92 ± 0.21 cm). From drying off to calving, all cows received a ration with the same energy density (6.8 MJ NE_L/kg DM) for ad libitum intake. In addition, the animals received the same total mixed ration (7.2 MJ NE_L/kg DM) after calving.

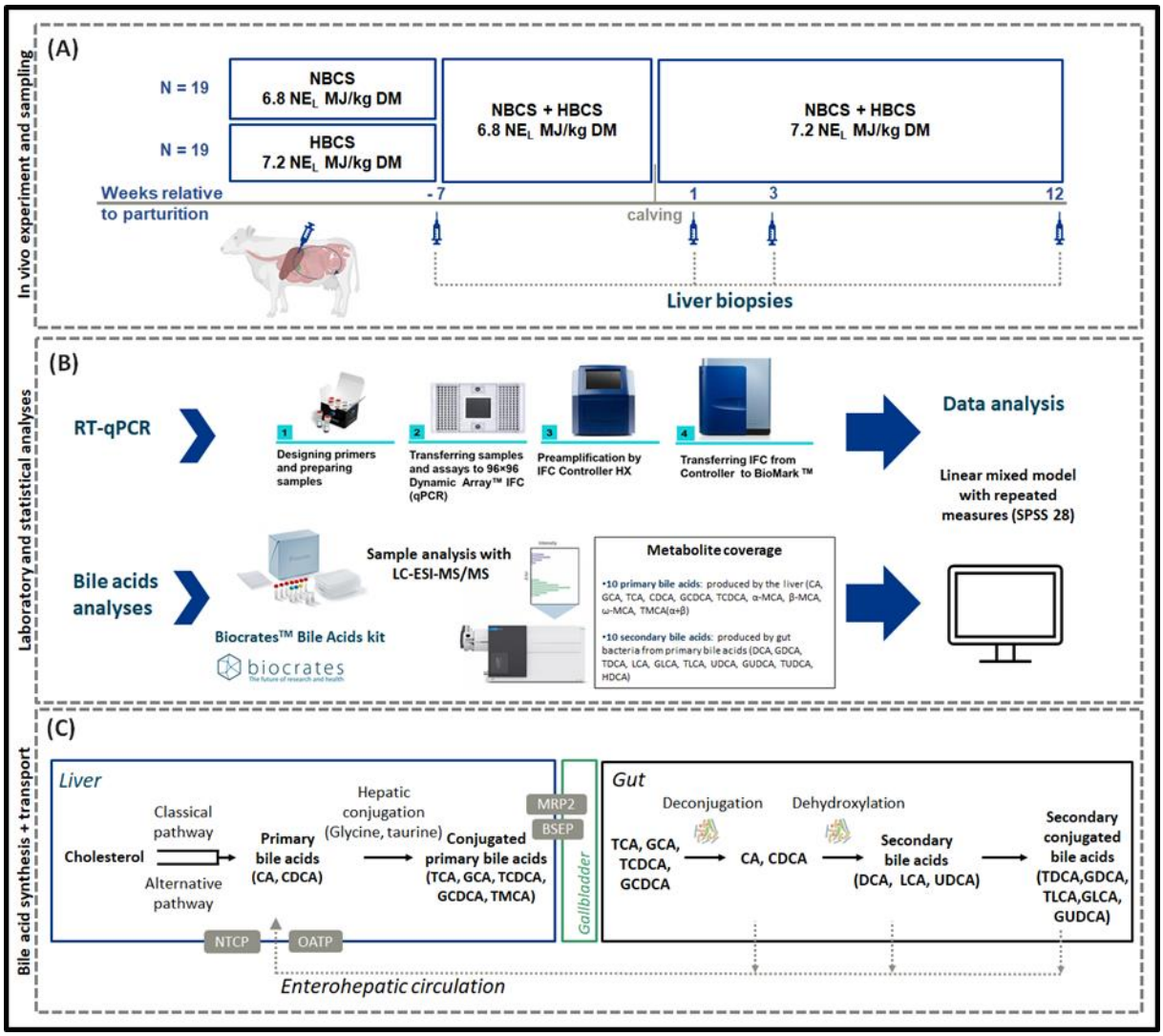


Figure 1. Schematic representation of the study and the analytical workflow. Timing of treatment and data collection during the study period from wk 15 before the expected calving date to wk 12 after calving. (A) The baseline experiment consisted of a high BCS (HBCS) group and a normal BCS (NBCS) group as described by Schuh et al. (2019). (B) Summary of the procedure for the quantitative PCR (qPCR)-based microfluidic array using the BioMark HD 96 × 96 system (Fluidigm) and bile acid (BA) measurements. Liver samples were analyzed using the Biocrates^{TM} Bile Acids Kit (biocrates life sciences ag, Innsbruck, Austria). IFC = Integrated Fluidic Circuit. LC-ESI-MS/MS = Liquid chromatography-electrospray ionization tandem mass spectrometry. (C) Schematic representation of the BA synthesis and transport within the liver, gallbladder, and the gut. Abbreviations: cholic acid (CA), chenodeoxycholic acid (CDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA) taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), taurolithocholic acid (TLCA), glycolitocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), α-muricholic acid (αMCA), β-muricholic acid (βMCA), tauromuricholic acid (sum of alpha and beta) (α, β-TMCA), ω-muricholic acid (ωMCA), hyodeoxycholic acid (HDCA), murideoxycholic acid (MDCA). NTCP, SLC10A1: Na^+-taurocholate cotransporting polypeptide; OATP: Solute Carrier Organic Anion Transporter; MRP2, ABCC2: Multidrug Resistance-Associated Protein; BSEP, ABCB11: Bile Salt Export Pump. Parts of the Figure were created using BioRender.com (JU260ULEB0).

Sampling and BA analyses

Liver biopsies were collected at late pregnancy in wk 7 a.p., and early lactation in wk 1, 3, and 12 postpartum (p.p.; see Figure 1A) and were taken by a liver puncture at the 11th and 12th intercostal space using a 14-gauge biopsy needle (Dispomed Witt oHG, Gelnhausen, Germany). Liver samples were taken before the presentation of fresh feed after the morning milking. The samples were rinsed with 0.9% NaCl solution, immediately frozen in liquid nitrogen, and stored at -80°C until further analysis.

Liver samples were analyzed using the Biocrates^{TM} Bile Acids Kit (biocrates life sciences ag, Innsbruck, Austria; Figure 1B). This standardized assay includes sample preparation and LC-ESI-MS/MS measurements. The assay enabled the simultaneous quantification of 20 different BA, including CA, CDCA, deoxycholic acid (DCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), hyodeoxycholic acid (HDCA), lithocholic acid (LCA), alpha-muricholic acid (MCA(a)), beta-muricholic acid (MCA(b)), omega-muricholic acid (MCA(o)), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), tauromuricholic acid (sum of alpha and beta) (TMCA(a+b)), tauroursodeoxycholic acid (TUDCA), and ursodeoxycholic acid (UDCA). Compound identification and quantification were based on scheduled multiple reaction monitoring measurements (sMRM). The method of Biocrates^{TM} Bile Acids kit has been proven to be in conformance with the EMEA-Guideline "Guideline on bioanalytical method validation (July 21^{st,} 2011") (European Medicines Agency, 2011), which implies proof of reproducibility within a given error range. The assay procedures of the Bile Acid kit and the results of an inter-laboratory ring trial have been described in detail previously (Pham et al., 2016; McCreight et al., 2018).

In brief, frozen bovine liver samples were weighed into homogenization tubes containing ceramic beads (1.4 mm). For metabolite extraction, 3 µL of ethanol/phosphate buffer (85/15 v/v; 4 °C) per 1 mg liver was added and homogenized using a Precellys 24 homogenizer (PEQLAB Biotechnology GmbH, Germany) 4 × for 20 s at 5,500 rpm and -4 °C, with 30 s pause intervals to ensure constant temperature, followed by centrifugation at 10,000 × *g* for 5 min. Subsequently, the freshly prepared supernatants were used for quantification of metabolites.

For assay preparation, 10 µL of internal standard solution in methanol were pipetted onto the filter inserts of a 96-well sandwich plate using a Hamilton Microlab STAR^{TM} robot (Hamilton Bonaduz AG, Bonaduz, Switzerland). After drying the filters for 5 min at RT with

an ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, U.K.), 10 µL of blank, calibration standards, quality control samples, and tissue homogenate supernatants were pipetted into the wells of the plate and the filters were dried again for 5 min. For extraction of metabolites and internal standards, 100 µL methanol were added per well and the plate was shaken for 20 min at 650 rpm. The metabolite extracts were eluted to the lower deep well plate by a centrifugation step (5 min at 500 × *g* at RT). The upper filter plate was removed, the extracts were diluted with 60 µL ultrapure water, and the plate was shaken for 5 min at 450 rpm and finally placed into the cooled auto sampler (10 °C) for LC-MS/MS measurements.

Liquid chromatography separation was performed using 10 mM ammonium acetate in a mixture of ultrapure water/formic acid (99.85/0.15 v/v) as mobile phase A and 10 mM ammonium acetate in a mixture of methanol/acetonitrile/ultrapure water/formic acid v/v/v/v 30/65/4.85/0.15 as mobile phase B. The BA were separated on the UHPLC column for the Biocrates™ Bile Acids kit (Product No. 91220052120868) combined with the precolumn SecurityGuard ULTRA Cartridge C18/XB-C18 (for 2.1 mm ID column, Phenomenex Cat. No. AJ0-8782). Mass spectrometric analyses were performed using an API 4000 triple quadrupole system (SCIEX Deutschland GmbH, Darmstadt, Germany) equipped with a 1260 series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and an HTC-xc PAL autosampler (CTC Analytics, Zwingen, Switzerland) and controlled by the Analyst 1.6.2 software. Data analysis for quantification of metabolite concentrations and quality assessment was performed using the MultiQuant 3.0.1 software (SCIEX) and the MetIDQ™ software package. Data correction, including normalization of plate effects, was based on the Biocrates protocol for normalization.

Serum samples

Weekly blood samples were taken from the vena coccygea from wk 7 a.p. until wk 12 p.p. to analyze BHB and NEFA in serum, as previously described (Schuh et al., 2019, see Supplemental Figures S1 and S2). Furthermore, the amino acids glycine and taurine were previously measured in serum by liquid chromatography-electrospray ionization tandem mass spectrometry measurements by targeted metabolomics using the Absolute IDQ p180 kit (Biocrates life sciences ag, Innsbruck, Austria) and have been previously described in detail (Ghaffari et al., 2019b).

Primer design and quantitative real time PCR

Extraction of mRNA and cDNA synthesis were performed as previously described (Webb et al., 2019). After homogenization of the tissue using the Precellys 24 system (VWR/Peqlab Biotechnologie, Erlangen, Germany), total RNA was extracted from the liver using TRIzol (Invitrogen/Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Subsequently, the RNA was purified using spin columns according to the Qiagen kit protocol (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany). The concentration and purity of total RNA were quantified at 260 nm and 280 nm using the Nanodrop 1000 (peQLab Biotechnology GmbH, Erlangen, Germany). For cDNA synthesis, a reverse transcription reaction of 250 ng total RNA per 20 µL reaction volume was performed using RevertAid Reverse Transcriptase (Thermo Scientific GmbH, Dreieich, Germany) according to the manufacturer's instructions with a Multicycler PTC 200 (MJ Research Inc, Watertown, MA).

Bovine specific primer pairs used in this study were designed using the National Center for Biotechnology Information (NCBI) Primer Blast. In addition, eight reference genes [low-density lipoprotein receptor-related protein 10 (*LRP10*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), RNA Polymerase II (*POLR2A*), eukaryotic translation initiation factor 3, subunit K (*EIF3K*), marvel domain containing 1 (*MARVELD1*), hippocalcin-like 1 (*HPCAL1*), emerin (*EMD*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*)], which have previously been identified as stable in bovine tissues (Saremi et al., 2012), were also investigated. Primer pairs and characteristics are shown in Table 1. The selected primer pairs had an ideal melting temperature of 59°C and were evaluated by reverse transcription quantitative real-time PCR (RT-qPCR) on pooled cDNA samples from the liver using a Bio-Rad CFX96 touch real-time PCR detection system (Bio-Rad Europe GmbH, Leipzig, Germany). The RT-qPCR procedure consisted of an initial denaturation at 90°C for 3 min followed by 40 cycles of amplification at 95°C for 30 sec, 59°C for 60 sec, and 72°C for 60 sec. For the subsequent RT-qPCR experiments, only primer sets with PCR efficiencies between 90 and 110% and an R^2 > of 0.985 were used. The analysis was performed in accordance with the guidelines of the Minimum Information for Publication of Quantitative RT-PCR Experiments (MIQE) (Bustin et al., 2009).

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

Gene	Target	Sequence 5'- 3'*	Primer	Ass. No	(bp)
Enzymes					
CYP7A1		F	CTACCCAGACCCGTTGACTT	NM_001205677	270
		R	GGTAAAATGCCCAAGCCTGC		
HSD3B7		F	CCCAGGAGACACAGAAGACC	NM_001034696.1	74
		R	CGGCCATACCTGGCTGC		
CYP8B1		F	GGGAAGGCTTGGAGGAGC	NM_001076139.2	142
		R	GGAGGTGATGAGGAGCCAGA		
AKR1D1		F	ACTCGGAACCTAAATCGACTCC	NM_001192358.1	103
		R	TTCTGGTAGAGGTAGGCCCC		
CYP27A1		F	GGCTGGAGTAGACACGACAT	NM_001083413.2	201
		R	GGGACCACAGGATAGAGACG		
CYP7B1		F	ACAATTGGACAGCCTGGTCT	XM_025001826.1	220
		R	ACTGGAAAATAGCAGCCCATCT		
CH25H		F	ACGCTTGAGGTGGACTTGAG	NM_001075243.1	375
		R	AATCTGAGTCACTGCCCAGC		
CYP46A1		F	TTTCCTTCTAGGGCACCTCC	NM_001076810.1	96
		R	CCGTACTTCTTAGCCCAATCC		
BAAT		F	ACCTGCCTTTCAGAGTGGAG	XM_015472664.1	90
		R	CTGGCCCAAGGACCTTAGTAT		
STAR		F	AAGACCCTCTCTACAGCGAC	NM_174189.3	471
		R	CGTGCTCCGCTCTGATGAC		
TSPO		F	CCTCGTCGTCGCTGAACTTT	NM_175776.2	145
		R	GTACCAGCGGAAACCCTCTC		
Transporter					
SLC10A1	*NTCP*	F	GCTATGTCACCAAGGGAGGG	NM_001046339.1	272
		R	GGGGAAGGTCACATTGAGGA		
SLC10A2	*ASBT*	F	TTTCCTTCCAGCGTCAGCAT	XM_019971692.1	566
		R	TATACCACGTACACTGCCAGG		
SLC51A1	*OST α*	F	CCCAGCTTTTGAGAGCCATC	NM_001025333.2	676
		R	GGTGAACAAGCAATCTGCCC		
SLC51B	*OST β*	F	AGCAGACCAGACGAGTCCT	NM_001077867.2	261
		R	TTCCAAGGAGTTGCGTCCTC		
ABCC2	*MRP2*	F	GATGAGGCCACAGTCAATGAG	XM_024985942.1	81
		R	CACGTCCTCTGGGATTTCCT		
ABCB1	*MDR1*	F	GCGGCTCTTCAAGACTCAGTG	XM_024991021.1	137
		R	AGATCCATCGCGACCTCGG		
ABCB11	*BSEP*	F	GCACTGAGTAAGGTTCAGCA	NM_001192703.3	241
		R	TCTCAAGTAAGGCATCTTCGG		
ABCB4	*MDR3*	F	TGGGGCCGGACACTCT	XM_024991318.1	395
		R	TTAGCTTGGCTGCTGCTGA		
OATP1A2	*SLCO1A2*	F	TCAGAAGAACGACCCTTTATGACT	NM_174654.2	198
		R	TGCCAACAGAAACATCTTCAACT		

Table 1. continued

Gene	Target	Sequence 5'- 3'*	Primer	Ass.No	(bp)
Receptors					
NR1H4	*FXR*	F	AAGCCCGCTAAAGGTGTACT	NM_001034708.2	298
		R	TGATTCTCCCTGCTGATGCT		
GPBAR1	*TGR5*	F	GACCTCAACGGTCAGGACAC	NM_175049.3	126
		R	GGCATGCATGACTGTAGGTG		
NR1I2	*PXR*	F	GCGGCATGAGAAAAGAGATGAT	NM_001103226.1	998
		R	AGCCAGTCAGCCATTTGTG		
S1PR2	*S1PR2*	F	GATCGGCCTAGCCAGCATCA	NM_001081541.1	650
		R	AAGATGGTCACCACGCAGAG		
VDR	*VDR*	F	CACCCGCAGGACCAGAGTC	NM_001167932.2	701
		R	GAGAAGCTGGTTGGCTCCAT		
CHRM2	*CHRM2*	F	ACCTCCAGACCGTCAACAAT	NM_001080733.1	139
		R	CAAAGGTCACACACCACAGG		
NR2B1	*RXRα*	F	CCATTTTCGACAGGGTGCTG	NM_001304343.1	171
		R	CCAGGGACGCATAGACCTTC		
NR0B1	*SHP1*	F	TCCTCTTCAACCCTGACGTG	XM_002685759.5	173
		R	GCTGGGTGGAATGGACTTGA		
NR1I3	*CAR*	F	GAACAACGGAGGCTACACAC	NM_001079768.2	197
		R	TGTTGACTGTTCGCCTGAAG		
Reference genes					
YWHAZ		F	CCACCTACTCCGGACACAG	NM_174814.2	464
		R	GACTGGTCCACAATCCCTTTC		
EIF3K		F	CCAGGCCCACCAAGAAGAA	NM_001034489	125
		R	TTATACCTTCCAGGAGGTCCATGT		
HPCAL1		F	GCCGGCTTCCTTTTGTCTTT	NM_001098964	216
		R	CTAGACCATGCCCTGCTCC		
POLR2A		F	CTATCGCAGAACCCACTCACC	NM_001206313.2	91
		R	CACAGCGGGAAGGATGTCTG		
GAPDH		F	GAAGGTCGGAGTGAACGGATTC	NM_001034034.2	153
		R	TTGCCGTGGGTGGAATCATA		
MARVELD1		F	TCGGTGCTTTGATGTCTTGC	NM_001101262.1	71
		R	CAATCCACGGGCACTTCCTA		
LRP10		F	TTTTCCCGAATCCTGCCTGT	NM_001100371.1	73
		R	ACAGGCCTCTGTAAGGTGC		
EMD		F	GCCAGTACAACATCCCACAC	NM_203361.1	155
		R	CGCCGAATCTAAGTCCGAGA		

CYP7A1: Cholesterol 7alpha-Hydroxylase; *HSD3B7*: 3 Beta-Hydroxysteroid Dehydrogenase Type 7 Hydroxylase; *CYP8B1*: Sterol 12-Alpha-Hydroxylase; *AKR1D1*: Aldo-Keto Reductase Family 1; *CYP27A1*: Sterol 27-Hydroxylase; *CYP7B1*: Oxysterol 7-Alpha-Hydroxylase; *CH25H*: Cholesterol 25-Hydroxylase; *CYP46A1*: Cholesterol 24-Hydroxylase; *BAAT*: Bile Acid Coenzyme A:Amino Acid N-Acyltransferase; *STAR*: Steroidogenic Acute Regulatory

Protein; *TSPO*: Translocator Protein; *NTCP, SLC10A1*: Na+-taurocholate cotransporting polypeptide; *SLC10A2, ASBT*: Apical Sodium-Dependent Bile Acid Transporter; *OSTα, SLC51A1*: solute carrier family 51 subunit alpha; *OSTβ, SLC51B*: solute carrier family 51 subunit beta; *MRP2*, *ABCC2*: Multidrug Resistance-Associated Protein; *MDR1*, *ABCB1*: Multidrug Resistance Protein 1; *BSEP*, *ABCB11*: Bile Salt Export Pump; *MDR3*, *ABCB4*: Multiple Drug Resistance 3; *OATP1A2*, *SLCO2A1*: Solute Carrier Organic Anion Transporter Family Member 1A2; *FXR*, *NR1H4*: Farnesoid X Receptor; *TGR5*, *GPBAR1*: Takeda G protein–coupled receptor 5; *NR1I2*, *PXR*: Nuclear Receptor Subfamily 1, Group I, Member 2; *S1PR2*: Sphingosine-1-Phosphate Receptor 2; *VDR*: Vitamin D Receptor; *CHRM2*: Cholinergic Receptor Muscarinic 2; *RXRα, NR2B1*: Retinoid X Receptor Alpha; *SHP1*: Small Heterodimer Partner; *CAR*: Constitutive Androstane Receptor; *YWHAZ*: Tyrosine 3-Monooxygenase/ Tryptophan 5-Monooxygenase Activation Protein Zeta; *EIF3K*: Eukaryotic Translation Initiation Factor 3 Subunit K; *HPCAL1*: Hippocalcin Like 1; *POLR2A*: RNA Polymerase II Subunit A; *GAPDH*: Glyceraldehyde-3-Phosphate Dehydrogenase; *MARVELD1*: MARVEL Domain Containing 1; *LRP10*: LDL Receptor Related Protein 10; *EMD*: Emerin.
*F = forward; R = reverse.

RT-qPCR

The mRNA abundance of 27 genes in liver tissues from 38 cows at four-time points was measured by RT-qPCR using the Biomark HD 96.96 system (Fluidigm Co., San Francisco, CA; Figure 1B). The details of the technique and measurements were previously described (Alaedin et al., 2021). Primers were measured in triplicate using the Biomark HD RT-qPCR system and 96.96 integrated fluidic circuits (IFC) prepared according to the protocol "Fast Gene Expression Analysis Using EvaGreen on Biomark HD for Biomark" by Fluidigm. To remove the technical run-to-run variation, three inter-run calibrators were added to each IFC. The Biomark HD real-time PCR reader was used with the "GE Fast 96 × 96 PCR + Meltv2" protocol for subsequent gene expression (GE) analysis. Quality control of the melting and amplification curves was performed using Fluidigm Real-Time PCR Analysis Software (V4.5.2). The qBASE^{plus} software (V3.3, Biogazelle, Ghent, Belgium) was used for calibration between runs to adjust for inter-run variations. The stability of reference genes, including *LRP10, GAPDH, POLR2A, EIF3K, MARVELD1, HPCAL1, EMD,* and *YWHAZ,* was analyzed by qBASE^{plus} software. The geNorm^{plus} function was used to determine the optimal number of reference genes for the normalization of the data.

Statistical Analyses

Statistical analyses of BA concentrations in the liver and mRNA abundance of BA-associated enzymes, receptors, and transporters were performed using a linear mixed model with repeated measures (IBM SPSS version 28). All residuals were tested for normality using the Kolmogorov-Smirnov test. Data that did not meet the assumptions for the normality of the

residuals were log-transformed (base 10). The model consisted of treatment group, time, and interaction of the treatment group and time as the fixed effects and cow as the random effect. Time (wk relative to parturition) was classified as repeated measures. The most appropriate covariance structure was selected based on the indices of the Akaike information criterion and an autoregressive type 1 covariance structure and identity (scaled identity matrix) were selected as best fit. Multiple comparisons were performed using the Bonferroni correction. Correlations were calculated using the Spearman correlation (IBM SPSS version 28). The correlation coefficients were categorized as very strong ($1.0 \geq r > 0.9$), strong ($0.9 \geq r > 0.7$), moderate ($0.7 \geq r > 0.5$), weak ($0.5 \geq r > 0.3$), and very weak to zero correlation ($r \leq 0.3$). The threshold of significance was set at $P \leq 0.05$; trends were declared at $0.05 < P \leq 0.10$.

RESULTS

BA Concentrations in Liver

A total of 14 BA was detected in the liver. Regardless of the treatment and the time point, the conjugated BA GCA was detected with the greatest concentration (53% of the total liver BA concentration). The mean percentage of each BA in relation to the total BA measured in the bovine liver samples is shown in Figure 2.

In the liver, 81% of the total BA concentration consisted of primary or primary conjugated BA. In addition, 70% of liver BA concentrations were glycine-conjugated BA (both primary and secondary BA). The BA CDCA and LCA were detected above the limit of detection (LOD); however, as these were single values per time point and treatment, the data could not be statistically analyzed. The BA concentrations in the liver from wk 7 a.p. to wk 12 p.p. in HBCS and NBCS cows are shown in Figure 3.

Irrespective of time, CA concentrations tended to be greater in NBCS cows (1.27-fold; $P = 0.053$) and GCA and DCA concentrations were greater (1.14-fold; $P = 0.03$ and 1.34-fold; $P = 0.04$, respectively) in NBCS cows than in HBCS cows. In addition, HBCS cows had 1.76-fold ($P = 0.03$) greater concentrations of MCA(b) than NBCS animals. Irrespective of treatment, CA concentrations at wk 1, 3, and 12 p.p. were 1.4-fold ($P = 0.05$), 2.11-fold ($P < 0.001$), and 2.12-fold ($P < 0.001$) greater than before calving. In addition, CA concentrations at wk 1 p.p. also differed from all other time points, with a 1.4-fold ($P = 0.05$) greater concentrations compared to wk 7 a.p. and 1.53-fold ($P = 0.003$) and 1.54-fold ($P = 0.005$) greater concentrations at wk 3 and 12 p.p., respectively. Furthermore, the lowest concentrations were observed for GCA a.p., which increased 1.51- ($P < 0.001$), 1.82- ($P < 0.001$), and 1.41-fold ($P < 0.001$) at wk 1, 3, and 12 p.p., respectively. The concentrations of GDCA were 1.23- ($P = 0.04$), 1.40-

(P = 0.004), and 1.39-fold (P = 0.002) greater at wk 3 p.p. than at wk 7 a.p., as well as wk 1 and 12 p.p. The TCDCA concentration was 1.56- (P = 0.002), 2.09- (P < 0.001), and 2.19-fold (P < 0.001) greater at wk 1 p.p. than at wk 7 a.p., as well as wk 3 and 12 p.p. Also, the concentrations of TMCA(a+b) were 1.46- (P = 0.005), 1.83- (P < 0.001), and 1.54-fold (P < 0.001) greater at wk 1 p.p. than wk 7 a.p., wk 3 and 12 p.p., respectively. The concentrations of TUDCA were 2.01- ($P \leq 0.001$), 2.42- (P < 0.001), and 3.50-fold (P < 0.001), greater at wk 1 p.p. than at wk 7 a.p., wk 3 and 12 p.p., respectively. Interactions between treatment and time were detected for CA, GUDCA MCA(b), and TLCA. The NBCS animals had 2.83- (P = 0.008) and 1.41-fold (P = 0.05) greater CA concentrations than the HBCS animals at wk 7 a.p. and wk 12 p.p. The HBCS animals had 1.47-fold (P = 0.05) greater GUDCA concentrations than the NBCS animals at wk 3 p.p. For MCA(b), the HBCS animals had 1.82-fold greater (P = 0.01) concentrations than the NBCS animals at wk 3 p.p. At wk 1 p.p., the NBCS animals had 1.31-fold greater (P = 0.006) concentrations of TLCA than the HBCS cows.

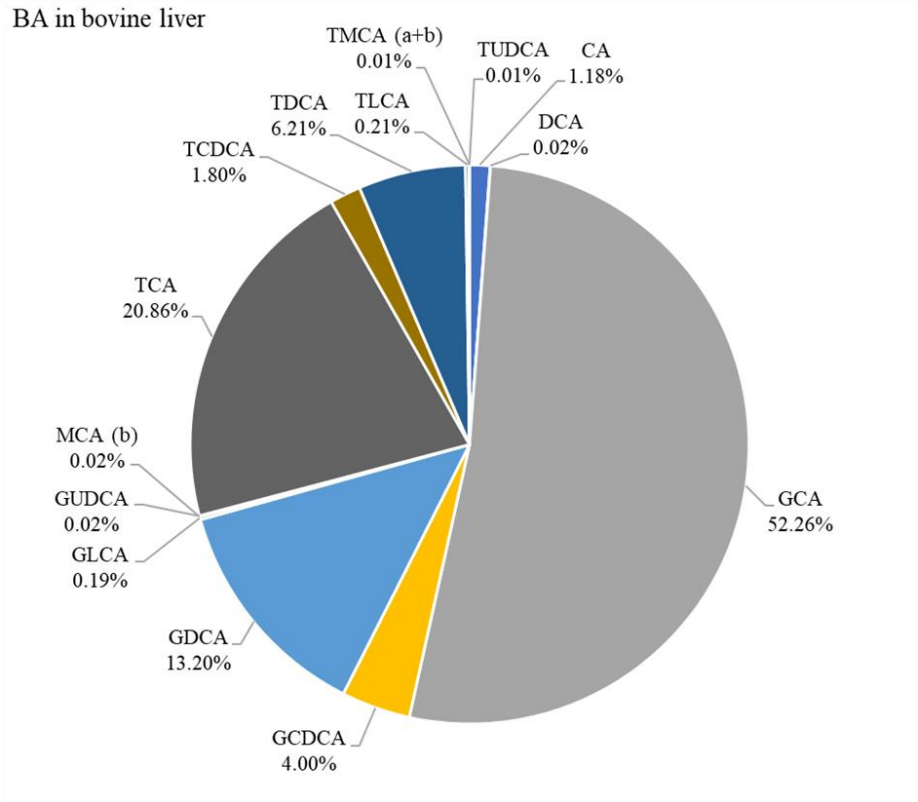


Figure 2. Mean percentage (%) of total bile acids in liver across all timepoints. Abbreviations: cholic acid (CA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), taurolithocholic acid (TLCA), glycolithocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), β-muricholic acid (β- MCA), tauromuricholic acid (sum of alpha and beta) (TMCA(α+β)).

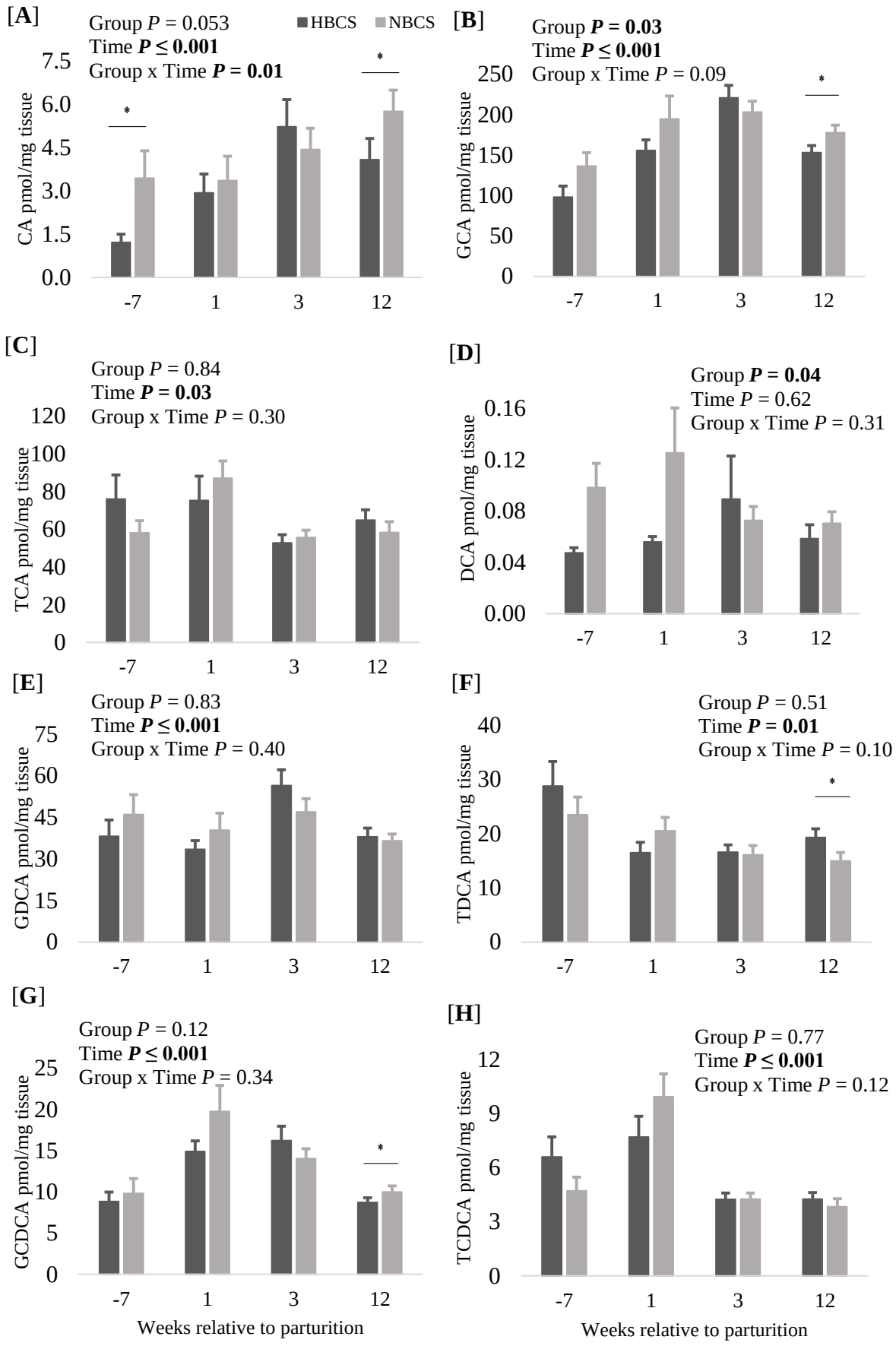

[A]
Group P = 0.053
Time P ≤ 0.001
Group x Time P = 0.01
HBCS
NBCS
CA pmol/mg tissue
[B]
Group P = 0.03
Time P ≤ 0.001
Group x Time P = 0.09
GCA pmol/mg tissue
[C]
Group P = 0.84
Time P = 0.03
Group x Time P = 0.30
TCA pmol/mg tissue
[D]
Group P = 0.04
Time P = 0.62
Group x Time P = 0.31
DCA pmol/mg tissue
[E]
Group P = 0.83
Time P ≤ 0.001
Group x Time P = 0.40
GDCA pmol/mg tissue
[F]
Group P = 0.51
Time P = 0.01
Group x Time P = 0.10
TDCA pmol/mg tissue
[G]
Group P = 0.12
Time P ≤ 0.001
Group x Time P = 0.34
GCDCA pmol/mg tissue
Weeks relative to parturition
[H]
Group P = 0.77
Time P ≤ 0.001
Group x Time P = 0.12
TCDCA pmol/mg tissue
Weeks relative to parturition

Figure 3.

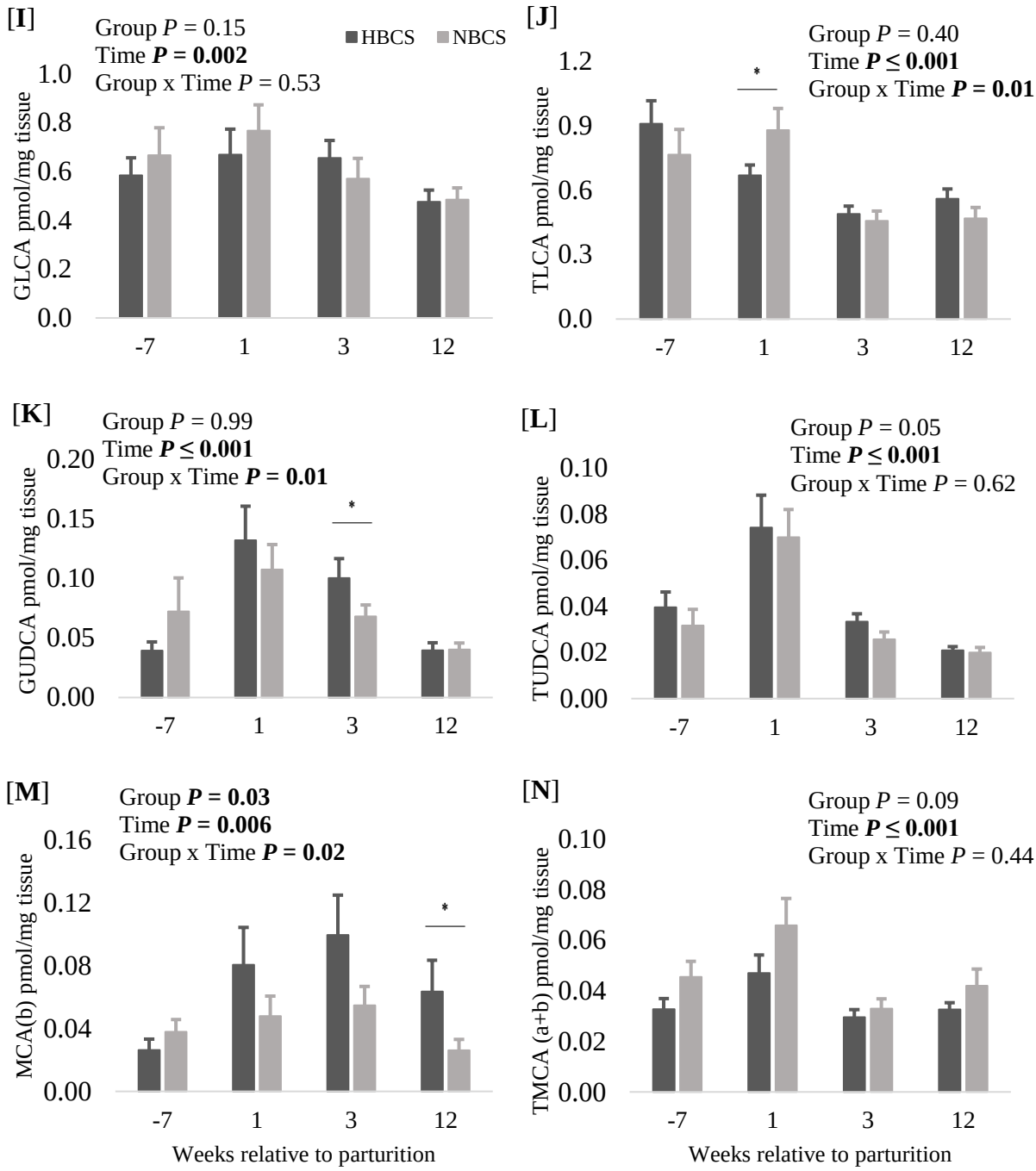


Figure 3 continued. Concentration of bile acids (pmol/mg tissue) in liver from cows with normal (NBCS) versus high body condition score (HBCS) at wk 7 ante partum (a.p.) and wk 1, 3, and 12 post partum (p.p.). Values are given as means ± SEM. Significant differences ($P \leq 0.05$) between the groups are indicated by asterisks. Cholic acid (CA), glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), deoxycholic acid (DCA), glycodeoxycholic acid (GDCA), glycolitocholic acid (GLCA), taurodeoxycholic acid (TDCA), taurolitocholic acid (TLCA), glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), β-muricholic acid (MCA(β)), tauromuricholic acid (sum of alpha and beta) (TMCA (α+β)). Asterisks indicate significance * $P < 0.05$.

mRNA abundance of BA-related enzymes

The mRNA abundance of enzymes related to BA metabolism in the liver is shown in Table 2. Regardless of treatment, cholesterol 7-alpha-hydroxylase (*CYP7A1*) mRNA abundance was lowest a.p. and increased 1.50- ($P = 0.008$), 1.30- ($P = 0.028$) and 1.85-fold ($P < 0.001$) at wk 1, 3, and 12 p.p., respectively. Regardless of treatment, translocator protein (*TSPO*) had the highest mRNA abundance 1 wk p.p., being 1.43- ($P = 0.03$), 1.37- ($P = 0.04$) and 1.43-fold ($P = 0.001$) higher compared to wk 7 a.p., wk 3 and 12 p.p. Regardless of time, the mRNA abundance of oxysterol 7-alpha-hydroxylase (*CYP7B1*) was 1.27-fold ($P = 0.01$) higher in HBCS cows than in NBCS cows, while the mRNA abundance of cholesterol 25-hydroxylase (*CH25H*) was 1.33-fold higher ($P = 0.01$) in NBCS cows than in HBCS cows. An interaction between treatment and time was found for the mRNA abundance of aldo-keto reductase family 1 (*AKR1D1*) 7 wk before calving, with HBCS cows having 1.02-fold ($P = 0.005$) higher values than NBCS cows. An interaction between treatment and time was also seen at wk 3 p.p. for steroidogenic acute regulatory protein (*STAR*) with a 2.65-fold higher ($P = 0.03$) mRNA abundance in HBCS cows.

Table 2: mRNA abundance of enzymes related to bile acid metabolism in the liver from cows with normal (NBCS) and high body condition score (HBCS) at wk 7 ante partum, as well as wk 1, 3 and 12 p.p.. *P*-values written in bolt are statistically significant ($P \leq 0.05$) Data are given as mean ± SEM

		Weeks relative to parturition								***P*-value**		
		-7		**1**		**3**		**12**		**Group**	**Time**	**Group x Time**
		HBCS	NBCS	HBCS	NBCS	HBCS	NBCS	HBCS	NBCS			
	mean	1.97	1.45	2.62	2.54	2.12	2.36	3.33	3.02	0.43	< **0.001**	0.39
CYP7A1	SEM	0.30	0.18	0.35	0.37	0.29	0.28	0.39	0.37			
	N	18	17	14	17	16	15	17	14			
	mean	0.83	0.90	1.11	1.43	1.25	1.00	0.99	0.70	0.54	< **0.001**	0.11
HSD3B7	SEM	0.12	0.09	0.18	0.17	0.19	0.10	0.14	0.07			
	N	13	15	12	16	11	14	9	13			
	mean	6.69	6.15	8.57	6.69	6.92	6.27	8.09	7.63	0.30	0.20	0.89
CYP8B1	SEM	1.22	0.95	1.38	0.71	0.99	0.86	0.90	1.08			
	N	7	9	7	8	8	10	6	8			
	mean	8.48	8.28	7.20	7.64	7.06	8.64	8.41	8.61	0.12	< **0.001**	**0.005**
AKR1D1	SEM	0.36	0.45	0.38	0.44	0.37	0.44	0.43	0.36			
	N	19	17	14	17	16	15	17	14			
	mean	4.39	3.82	4.49	3.90	3.99	3.98	4.49	4.19	0.07	0.46	0.48
CYP27A1	SEM	0.22	0.24	0.33	0.17	0.23	0.23	0.19	0.31			
	N	19	17	14	17	16	15	17	14			
	mean	2.69	2.05	2.12	2.01	2.49	1.54	2.52	2.35	**0.01**	0.25	0.40
CYP7B1	SEM	0.20	0.27	0.25	0.26	0.29	0.20	0.31	0.33			
	N	18	13	12	15	12	15	15	11			
	mean	1.24	1.32	0.85	1.46	0.94	1.43	0.92	1.05	**0.01**	0.48	0.23
CH25H	SEM	0.19	0.21	0.17	0.20	0.15	0.18	0.13	0.18			
	N	15	15	12	13	15	13	16	11			
	mean	0.39	0.37	0.31	0.41	0.33	0.26	0.46	0.33	-	-	-
CYP46A1	SEM	0.04	0.04	0.03	0.11	0.04	0.09	0.11	0.04			
	N	9	4	10	6	10	3	5	5			
	mean	2.30	2.09	2.25	2.41	2.28	1.79	1.74	1.63	0.27	**0.01**	0.19
BAAT	SEM	0.23	0.21	0.34	0.21	0.18	0.24	0.13	0.28			
	N	17	15	13	14	16	13	15	12			
	mean	0.32	0.42	0.47	0.56	0.41	0.36	0.38	0.35	0.89	**0.002**	0.17
TSPO	SEM	0.04	0.05	0.07	0.11	0.08	0.06	0.09	0.05			
	N	19	17	13	17	16	15	16	14			
	mean	0.65	1.14	0.97	0.93	1.26	0.48	0.70	1.09	0.60	0.80	**0.02**
STAR	SEM	0.11	0.32	0.41	0.35	0.26	0.13	0.18	0.23			
	N	11	9	8	5	10	6	12	9			

CYP7A1: Cholesterol 7alpha-Hydroxylase; *HSD3B7*: 3 Beta-Hydroxysteroid Dehydrogenase Type 7 Hydroxylase; *CYP8B1*: Sterol 12-Alpha-Hydroxylase; *AKR1D1*: Aldo-Keto Reductase Family 1; *CYP27A1*: Sterol 27-Hydroxylase; *CYP7B1*: Oxysterol 7-Alpha-Hydroxylase; *CH25H*: Cholesterol 25-Hydroxylase; *CYP46A1*: Cholesterol 24-Hydroxylase; *BAAT*: Bile Acid Coenzyme A: Amino Acid N-Acyltransferase; *TSPO*: Translocator Protein; *STAR*: Steroidogenic Acute Regulatory Protein.

BA transporters in the liver

The mRNA abundance of BA transporters is shown in Table 3. Irrespective of grouping, hepatic mRNA abundance of *NTCP* was increased 1.21-fold ($P < 0.003$), 1.27-fold ($P < 0.001$), and 1.27-fold ($P < 0.001$) at wk 1, 3, and 12, respectively, compared to a.p. values. The mRNA abundance of *MRP2* was 1.28-, 1.35-, and 1.21-fold higher before calving ($P \leq 0.001$) than at wk 1, 3, and 12 after calving. NBCS cows had a 1.10- ($P = 0.004$) and 1.04-fold higher ($P =$

0.04) mRNA abundance of *BSEP* 7 wk a.p. and at wk 12 p.p. compared to HBCS cows. A treatment effect was observed for solute carrier organic anion transporter family member 1A2 (*OATP1A2*) at all p.p. time points, with NBCS cows showing 1.29- ($P = 0.03$), 1.57- ($P = 0.02$) and 1.44-fold ($P = 0.04$) higher mRNA abundance than HBCS cows. Irrespective of treatment, mRNA abundance of *BSEP* was 1.21- ($P = 0.003$), 1.33- ($P < 0.001$), and 1.31-fold ($P < 0.001$) higher at wk 12 p.p. compared to wk 7 a.p., 1 and 3 p.p., respectively. The mRNA abundance of solute carrier family 51 subunit beta (*OSTβ*) tended to be 1.12-fold higher ($P = 0.081$) in HBCS compared to NBCS before calving.

Table 3. mRNA abundance of transporters related to bile acid metabolism in the liver from cows with normal (NBCS) and high body condition score (HBCS) at wk 7 ante partum, as well as wk 1, 3 and 12 p.p.. *P*-values written in bolt are statistically significant ($P \leq 0.05$) Data are given as mean ± SEM.

		Weeks relative to parturition								***P*-value**		
		-7		**1**		**3**		**12**		**Group**	**Time**	**Group x Time**
		HBCS	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**			
***NTCP* (*Gene: SLC10A1*)**	mean	19.94	18.71	24.83	22.40	23.90	25.41	25.69	23.35	0.06	< **0.001**	0.39
	SEM	0.93	1.23	1.54	1.77	1.48	1.62	1.16	1.62			
	N	19	17	14	17	16	15	17	14			
***OST-β* (*Gene: SLC51B*)**	mean	2.14	1.91	1.63	1.84	1.56	1.96	2.28	2.34	0.45	**0.004**	0.08
	SEM	0.18	0.17	0.22	0.19	0.21	0.16	0.19	0.32			
	N	19	17	14	15	16	15	16	14			
***MRP2* (*Gene: ABCC2*)**	mean	13.71	13.35	10.38	10.77	9.32	10.70	11.51	10.74	0.87	< **0.001**	0.10
	SEM	0.60	0.72	0.66	0.68	0.53	0.68	0.58	0.60			
	N	18	17	14	17	16	15	17	14			
***MDR1* (*Gene: ABCB1*)**	mean	0.43	0.49	0.46	0.54	0.49	0.51	0.44	0.43	0.96	0.73	0.87
	SEM	0.05	0.09	0.07	0.11	0.07	0.09	0.06	0.09			
	N	18	17	14	16	16	13	14	13			
***BSEP* (*Gene: ABCB11*)**	mean	11.25	10.19	9.49	9.93	9.00	10.84	13.21	12.72	**0.02**	< **0.001**	**0.03**
	SEM	0.71	0.71	0.63	0.68	0.46	0.51	0.64	0.85			
	N	19	17	14	17	16	15	17	14			
***MDR3* (*Gene: ABCB4*)**	mean	1.84	0.39	2.56	2.98	0.83	2.50	0.98	1.62	-	-	-
	SEM	0.52	-	2.26	0.94	0.42	1.62	0.02	0.55			
	N	3	1	2	4	3	3	2	3			
***OATP* (*Gene: SLCO1A2*)**	mean	15.18	19.32	14.00	18.15	12.71	19.99	13.87	19.97	**0.02**	0.58	0.69
	SEM	2.01	1.94	2.67	1.45	1.89	2.23	1.90	2.60			
	N	18	17	14	17	16	15	17	14			

NTCP, *SLC10A1*: Na+-taurocholate cotransporting polypeptide; *SLC10A2*, *ASBT*: Apical Sodium-Dependent Bile Acid Transporter; *OSTα*, *SLC51A1*: solute carrier family 51 subunit alpha; *OSTβ*, *SLC51B*: solute carrier family 51 subunit beta; *MRP2*, *ABCC2*: Multidrug Resistance-Associated Protein; *MDR1*, *ABCB1*: Multidrug Resistance Protein 1; *BSEP*, *ABCB11*: Bile Salt Export Pump; *MDR3*, *ABCB4*: Multiple Drug Resistance 3; *OATP1A2*, *SLCO2A1*: Solute Carrier Organic Anion Transporter Family Member 1A2.

BA receptors in the liver

The mRNA abundance of BA receptors in the liver is shown in Table 4. An interaction between treatment and time was found for constitutive androstane receptor (*CAR*) *(NR1I3)* mRNA abundance, which was 1.22-fold ($P = 0.04$) higher in HBCS than in NBCS cows before calving.

Table 4. mRNA abundance of receptors related to bile acid metabolism in the liver from cows with normal (NBCS) and high body condition score (HBCS) at wk 7 ante partum, as well as wk 1, 3 and 12 p.p.. *P*-values written in bolt are statistically significant ($P \leq 0.05$) Data are given as mean ± SEM.

		Weeks relative to parturition								***P*-value**		
		-7		**1**		**3**		**12**		**Group**	**Time**	**Group x Time**
		HBCS	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**			
***FXR* (*Gene: NR1H4*)**	mean	1.63	1.72	1.82	1.66	1.63	1.63	1.77	1.58	0.42	0.82	0.39
	SEM	0.06	0.12	0.13	0.09	0.07	0.07	0.08	0.10			
	N	19	17	13	17	16	15	17	14			
***TGR5* (*Gene: GPBAR1*)**	mean	3.25	3.07	3.70	4.38	3.53	3.21	2.85	3.10	0.46	**0.02**	0.42
	SEM	0.28	0.28	0.63	0.43	0.39	0.33	0.32	0.24			
	N	18	15	13	16	15	15	17	14			
***S1PR2* (*Gene: S1PR2*)**	mean	-	-	-	0.61	-	1.75	-	-	-	-	-
	SEM	-	-	-		-		-	-			
	N	-	-	-	1	-	1	-	-			
***VDR* (*Gene: VDR*)**	mean	-	1.28	1.28	-	-	-	-	0.60	-	-	-
	SEM	-	0.91	-	-	-	-	-				
	N	-	2	1	-	-	-	-	1			
***CHRM2* (*Gene: CHRM2*)**	mean	1.95	2.67	1.82	2.40	2.33	2.82	1.75	2.19	0.14	0.20	0.84
	SEM	0.36	0.60	0.27	0.36	0.39	0.55	0.24	0.50			
	N	18	16	12	13	14	14	16	13			
***RXRα* (*Gene: NR2B1*)**	mean	1.98	2.04	2.21	2.02	2.18	2.11	2.14	1.96	0.76	0.23	0.76
	SEM	0.14	0.17	0.17	0.13	0.11	0.13	0.13	0.13			
	N	19	17	14	17	16	15	17	14			
***CAR* (*Gene: NR1I3*)**	mean	3.74	3.05	2.86	3.13	3.09	3.25	3.49	3.43	0.51	**0.012**	**0.010**
	SEM	0.17	0.20	0.18	0.19	0.15	0.20	0.17	0.21			
	N	19	17	14	17	16	15	17	14			

FXR, *NR1H4*: Farnesoid X Receptor; *TGR5*, *GPBAR1*: Takeda G protein–coupled receptor 5; *NR1I2*,; *S1PR2*: Sphingosine-1-Phosphate Receptor 2; *VDR*: Vitamin D Receptor; *CHRM2*: Cholinergic Receptor Muscarinic 2; *RXRα*, *NR2B1*: Retinoid X Receptor Alpha; *CAR*: Constitutive Androstane Receptor.

Relationship between hepatic BA and blood variables

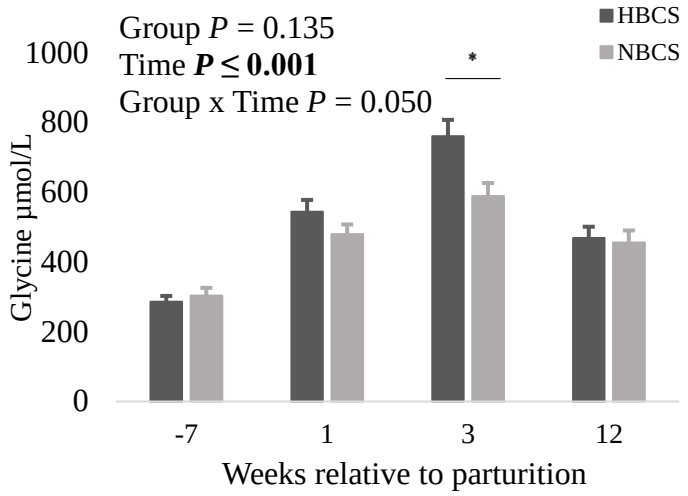
At wk 1 p.p., a weak correlation was observed between TMCA(a+b) and NEFA (r = 0.465; $P = 0.006$). At wk 12 p.p., NEFA correlated negatively with TMCA(a+b) (r = -0.410; $P = 0.016$). At wk 3 p.p., BHB was associated with TMCA(a+b) (r = 0.556; $P = 0.001$), GCDCA

(r = 0.358; *P* = 0.041), MCA(b) (r = 0.493; *P* = 0.007), TCDCA (r = 0.468, *P* = 0.006) and TUDCA (r = 0.481; *P* = 0.005).

Serum glycine concentrations in HBCS and NBCS cows during the study period are shown in Figure 4A. Glycine was lowest before calving and increased 1.73-, 2.30-, and 1.57-fold (*P* < 0.001) at wk 1, 3, and 12 p.p., respectively, when compared to a.p. values. At wk 3 p.p., glycine concentrations were 1.30-fold greater (*P* = 0.005) in HBCS cows compared to NBCS cows. At wk 7 a.p., serum glycine concentrations were positively correlated with CA (r = 0.475; *P* = 0.003) and negatively correlated with TCDCA (r = -0.398, *P* = 0.015), TLCA (r = -0.369, *P* = 0.025), and TUDCA (r = -0.366, *P* = 0.026). At wk 3 p.p., glycine concentrations were positively correlated with GCDCA (r = 0.335, *P* = 0.043), GDCA (r = 0.450, *P* = 0.008), and GUDCA (r = 0.427, *P* = 0.015) as well as with all glycine-conjugated BA (r = 0.338, *P* = 0.050) at wk 12 p.p.

The serum taurine concentrations in HBCS and NBCS cows during the study period are shown in Figure 4B, which were previously published by Ghaffari et al. (2019b). Taurine concentrations were greatest at wk 12 p.p. and were 1.36- (*P* < 0.001), 1.37- (*P* < 0.001), and 1.15-fold (*P* = 0.04) greater than at wk 7 a.p. and wk 1 and 3 p.p., respectively. At wk 7 a.p., taurine concentrations were negatively correlated with GLCA (r = -0.418, *P* = 0.01) and at wk 1 p.p. with CA, GCA, GCDCA, GDCA and GLCA (r = -0.731, *P* = 0.031; r = -0.451, *P* = 0.007; r = -0.481, *P* = 0.004; r = 0.445, P = 0.008; r = -0.457, *P* = 0.007, respectively). At wk 12 p.p., taurine concentrations were negatively correlated with GLCA (r = -0.488, *P* = 0.003) and positively correlated with TCA, TCDCA, TMCA (a+b) and TUDCA (r = 0.524, *P* = 0.001; r = 0.351, *P* = 0.042, r = 0.417, *P* = 0.014, r = 0.412, *P* = 0.015, respectively). Total taurine-conjugated BA were positively correlated (r = 0.471, *P* = 0.005) with taurine concentrations at wk 12 p.p.

[A]



[B]

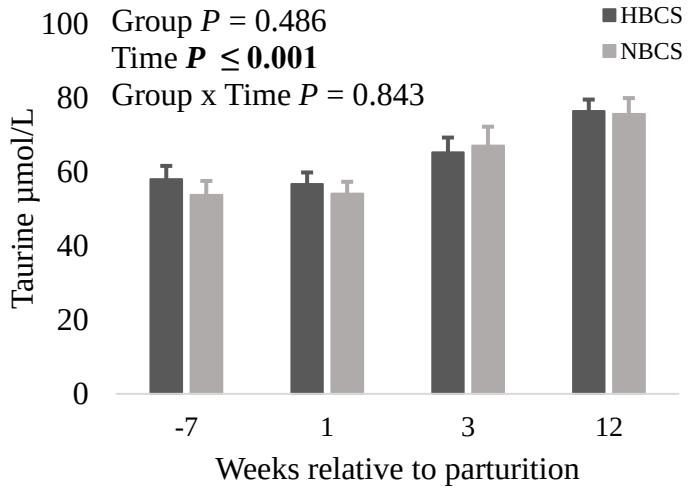


Figure 4. Concentrations of [A] glycine and [B] taurine (µmoL/L) in serum from cows with normal (NBCS) versus high body condition score (HBCS) at wk 7 ante partum (a.p.) and wk 1, 3, and 12 post partum (p.p.). Values are given as means ± SEM. Asterisks indicate significance * $P < 0.05$. The serum taurine concentrations in HBCS and NBCS cows have been published previously by Ghaffari et al. (2019b).

DISCUSSION

In the present study, we analyzed BA profiles in the liver of periparturient dairy cows with different body conditions. Besides lactation-induced changes in the BA liver profiles, we observed varying mRNA expression of BA synthesizing enzymes in the liver. A large proportion of BA passes the enterohepatic circulation and returns to the liver, where they can subsequently be recycled (Hofmann and Hagey, 2008; Chávez-Talavera et al., 2019).

In ruminants, the hepatic BA were predominantly conjugated by the amino acid glycine (Reiter et al., 2021). The increase in serum glycine concentrations in the present study after

parturition may indicate an increased mobilization of muscle protein to meet the high nutritional requirements in early lactating cows, as previously suggested (Klein et al., 2013). Besides glycine, taurine is the second amino acid, playing a role in BA conjugation (Guo et al., 2018; Reiter et al., 2021). Serum glycine concentration was lowest before parturition and increased p.p. Therefore, the greater glycine concentrations in HBCS cows compared to NBCS cows at wk 3 p.p. could indicate an increased mobilization of body reserves from muscle due to the high nutritional requirements in early lactation, as previously shown (Meijer et al., 1995). Whether BA are conjugated with glycine or taurine depends on the availability of amino acids in the liver (Vessey, 1978). Both taurine and glycine can be synthesized endogenously to a certain extent (Ueki and Stipanuk, 2009; Alves et al., 2019). Taurine is involved in many physiological processes, including the defense against oxidative stress during inflammation (Marcinkiewicz and Kontny, 2014). Therefore, the increase in concentrations of taurine-conjugated BA such as TCDCA, TMCA(a+b), and TUDCA in the first wk after calving may be due to physiological changes during the transition from gestation to lactation. The increase of taurine-conjugated BA was associated with increasing taurine availability in the cows studied here, which suffered from the metabolic challenges of early lactation (Ghaffari et al., 2019b). In addition, increasing serum taurine concentrations after calving were positively correlated with taurine-conjugated BA in the liver. However, Ghaffari et al. (2024a) investigated BA in serum and serum taurine concentrations in dairy cows from wk 8 before calving to wk 16 of lactation and found more taurine-conjugated BA in the dry period than during lactation.

In the bovine liver, GCA was the most abundant BA, whereas CA and GCA are most abundant in other bovine matrices, i.e. serum (Washizu et al., 1991, Dicks et al., 2024), follicular fluid (Blaschka et al., 2019) and adipose tissue (Dicks et al., 2024). In general, it is known that BA in the liver and gallbladder are mainly conjugated, while serum contains both conjugated and unconjugated BA (Chiang and Ferrell, 2020b). Regardless of sampling time, GCA accounted for over 50% of total liver BA, while its precursor molecule CA accounted for 1% of total BA. Thus, either the *de novo* synthesis of CA in the liver appears to be very low, and/or CA coming from the portal vein is immediately conjugated in the liver. Furthermore, the low concentrations of CDCA in the present study suggest that BA can either be synthesized to a very low extent in the liver or is immediately conjugated with glycine or taurine when it enters the hepatocytes, as previously postulated (Hofmann, 2009).

In lactating rats, increases in BA and BA-forming enzymes have been associated with increased energy requirements and food intake (Athippozhy et al., 2011; Zhu et al., 2013). Therefore, we suggest that increasing concentrations of CA and GCA after parturition may be

due to increased dry matter intake (DMI) in early lactating dairy cows (Schuh et al., 2019; Supplemental Figure S3). Higher DMI in NBCS cows compared to HBCS cows was associated with higher liver BA concentrations such as GCA, DCA, and CA in the present study. In particular, the increasing concentrations of the primary BA CA could indicate an increased *de novo* BA synthesis in the liver at the beginning of lactation.

Cows with excessive postpartum lipolysis had higher fecal excretion of secondary BA and thus lower concentrations of secondary BA (DCA, LCA) in the blood (Gu et al., 2023). In dairy cows and humans, DCA is recycled in the intestine and conjugated to either GDCA or TDCA or both and reintroduced into the circulating BA pool (Ridlon and Hylemon, 2006; Hofmann et al., 2018). Lower DCA concentrations in the liver of HBCS cows could indicate altered synthesis of secondary BA as well as changes in the microbial composition in the gut of dairy cows due to excessive lipolysis (Gu et al., 2023). In the present study, the lower liver concentrations of taurine-conjugated LCA at wk 1 after calving in HBCS cows may be due to decreased synthesis of secondary BA in the intestine following increased lipolysis (Gu et al., 2023). In addition, greater GUDCA concentrations in HBCS cows than in NBCS cows at wk 3 p.p. could indicate altered microbial characteristics already detected in the gut of dairy cows (Lin et al., 2023). In humans, UDCA, a precursor of GUDCA, has been administered orally to treat liver diseases such as cholestatic liver disorders (Trauner and Graziadei, 1999). In addition, feeding obese mice with UDCA reduced body weight and the lipogenic pathway in the liver, suggesting that UDCA is an important regulator of lipid metabolism (Chen et al., 2019).

BA can be synthesized via either the classical or the alternative synthesis pathway, stored in the gallbladder, and released into the intestine to facilitate digestion (Hofmann, 2009). The BA synthesized via the classical pathway appears to be more effective in forming mixed micelles in the intestine to emulsify fats and fat-soluble vitamins than BA synthesized via the alternative pathway (Wang et al., 2003). Consequently, the synthesis of BA via the alternative pathway could result in reduced intestinal lipid absorption (Jia et al., 2021). The BA synthesized via the alternative pathway, such as UDCA and MCA, have higher hydrophilic properties than BA of the classical pathway, resulting in less effective absorption of cholesterol and fat in the intestine (Wang et al., 2003). In humans, the alternative pathway of BA synthesis was found to be more important during hepatic diseases (Crosignani et al., 2007). In dairy cows, NEB in early lactation leading to fatty liver syndrome is characterized by elevated BHB and NEFA concentrations (Andrews et al., 1991; Van Den Top et al., 1995). In the current study, the alternative synthesis pathway appeared to be favored in HBCS cows, and the positive relationship

between BHB and conjugated BA may thus indicate a preference for the alternative pathway during periods of metabolic challenge.

The enzyme CYP7A1 catalyzes the rate-limiting step of BA synthesis (Chiang, 2009). Therefore, the higher mRNA abundance of *CYP7A1* in bovine liver after calving in the present study could lead to an increased hepatic BA pool, as previously suggested (Schlegel et al., 2012). Increased intestinal BA concentrations could improve energy supply by enhancing the absorption of lipids and fat-soluble nutrients from diets, thereby attenuating NEB after parturition, as shown in rats (Wooton-Kee et al., 2010). In general, negative feedback mechanisms regulate hepatic BA synthesis to prevent BA accumulation in the liver (De Aguiar Vallim et al., 2013). When the BA pool increases, *de novo* BA synthesis can be suppressed by the binding of BA to specific receptors such as the hepatic FXR or by the activation of FGF15 in the intestine, resulting in suppressed *CYP7A1* expression and thus lower BA formation (Goodwin et al., 2000; Lu et al., 2000; Chiang, 2015).

In the alternative pathway, cholesterol is transformed by CYP27A1 in the mitochondria (Björkhem, 2002). Cholesterol is transported into the mitochondria by STAR and TSPO (Li et al., 2014). In rodent hepatocytes, increased expression of *STAR* mRNA led to increased oxysterol levels and subsequently to increased BA synthesis (Pandak et al., 2002). Although *TSPO* mRNA abundance was not affected by treatment, the increased *STAR* mRNA abundance in HBCS at wk 3 p.p. suggests increased transport of cholesterol into the hepatic mitochondria. In addition to the modification of cholesterol by the enzyme CYP27A1, cholesterol can also be degraded via tissue-specific hydroxylation pathways at C24 and C25 (Lund et al., 1998; Russell, 2003). Other enzymatic steps include oxysterol 7α-hydroxylase (CYP7B1; Li et al., 2021), which is considered a marker enzyme of the alternative pathway and mainly produces CDCA (Chiang, 2017). Therefore, the higher mRNA abundance of *CYP7B1* in HBCS cows at wk 3 p.p. suggests greater importance of the alternative pathway.

In mice, CH25H is involved in the alternative metabolic pathway (Pandak and Kakiyama, 2019) and is considered a key enzyme in lipid metabolism that inhibits the sterol regulatory element binding protein (SERBF2; Lund et al., 1998). In the murine liver, increased concentrations of CH25H and 25-hydroxycholesterol activated LXRα, which targets CYP7A1, upregulates the enterohepatic circulation of BA and protects against high-fat diet-induced hepatic steatosis (Dong et al., 2022). The higher mRNA abundance of *CH25H* in NBCS compared to HBCS at wk 1 and 3 p.p. supports the role of the alternative pathway in the bovine liver analyzed here.

The enzyme AKR1D1, which synthesizes CA and CDCA, is involved in both the classical and the alternative pathway for BA synthesis (Monte et al., 2009). Higher mRNA abundance of *AKR1D1* before calving in HBCS cows, accompanied by low concentrations of CA and CDCA, suggests another physiological role of AKR1D1 besides BA synthesis, such as the reduction of steroid hormones, i.e. corticosterone, cortisol androstenedione, progesterone, and 17-hydroxyprogesterone in humans (Palermo et al., 2008; Nikolaou et al., 2019).

Transporters excrete and reabsorb BA after passage through the intestine and portal vein, thereby significantly influencing the enterohepatic circulation of BA (Alrefai and Gill, 2007). As one of the major transport mechanisms for BA uptake from the portal vein into the liver, the NTCP transporter has a higher affinity for taurine- and glycine-conjugated BA than for unconjugated BA (Hata et al., 2003). The majority of BA reabsorbed from the portal vein into the liver are conjugated BA (Kullak-Ublick et al., 2000; Ferrebee and Dawson, 2015). The reabsorption of conjugated BA by NTCP from the portal vein at the basolateral membrane of the liver supports the maintenance of the enterohepatic circulation (Dawson et al., 2009). Higher post-calving mRNA abundance of *NTCP* compared to pre-calving levels was associated with higher p.p. hepatic BA concentrations, suggesting effective BA transport from BA to the liver. In addition to BA, steroid and thyroid hormones can also be transported by NA^+-dependent transport via NTCP (Kullak-Ublick et al., 2000; Da Silva et al., 2013).

In addition to NA^+-dependent import, BA can also enter hepatocytes independently of NA^+ via the organic anion-transporting polypeptides, including OATP1A2, which are mainly responsible for unconjugated BA (Meier, 1995; Trauner and Boyer, 2003; Kullak-Ublick et al., 2004). Thus, the higher mRNA abundance of *OATP1A2* in NBCS cows compared to HBCS cows after parturition could indicate an increased influx of unconjugated BA, i.e. CA and DCA, into the liver.

In hepatocytes, BA are mainly excreted into the biliary canaliculi by the transporter BSEP (Kullak-Ublick et al., 2004; Ferrebee and Dawson, 2015). Therefore, the higher mRNA abundance of *BSEP* in HBCS cows indicates increased BA excretion in over-conditioned cows. In case of accumulation of BA in the liver, BSEP may protect hepatocytes from cytotoxic effects (Eloranta and Kullak-Ublick, 2005). In *ob/ob* mice, injection of leptin increased the mRNA of genes related to BA synthesis and transport, including *BSEP* mRNA (Liang and Tall, 2001). Since over-conditioned cows in the present study showed increased serum leptin concentrations after calving (Schuh et al., 2019), we hypothesize that leptin from adipose tissue may upregulate *BSEP* mRNA abundance.

In addition to the excretion of bilirubin conjugates and other organic substances (Gerk and Vore, 2002), MRP2 transports divalent BA such as TCA and GCA from hepatocytes (Trauner and Boyer, 2003). In rat hepatocytes, mRNA expression of *MRP2* was stable during gestation and lactation; however, MRP2 protein expression decreased during pregnancy compared to lactation (Cao et al., 2001). The decreasing mRNA abundance of *MRP2* after calving in HBCS and NBCS cows suggests that MRP2 plays a minor role in BA export compared to BSEP.

In hepatocytes, the OSTα-OSTβ transport complex facilitates the transport of BA and steroids into the systemic circulation via an alternative pathway of BA efflux (Wang et al., 2001; Trauner and Boyer, 2003). The OSTα-OSTβ complex is upregulated to prevent high hepatic BA concentrations in the liver and to efflux BA into the systemic circulation via an alternative export system (Boyer et al., 2006). In the present study, the mRNA abundance of *OSTβ* tended to be higher in HBCS at 7 wk before calving; however, we were unable to quantify OSTα mRNA in bovine liver due to methodological issues.

Nuclear receptors can regulate BA concentrations at the transcriptional level (Goodwin et al., 2000). Bile acids have been identified as natural ligands for the nuclear receptor FXR (Parks et al., 1999). Since BA concentrations are much higher in the intestine than in the liver, it is hypothesized that feedback regulation of BA metabolism via FXR is mainly controlled from the gut (Chiang and Ferrell, 2020a). The treatment- and time-independent hepatic expression of *FXR* mRNA in the current study suggests the ubiquitous presence of FXR. The mRNA abundance of *TGR5* is expressed in several tissues, such as gallbladder (highest expression), brown adipose tissue, liver, and intestine (Watanabe et al., 2006). In murine hepatocytes, the G protein-coupled receptor TGR5 had a higher affinity for secondary than primary BA, i.e. LCA > DCA > CDCA > CA (Kawamata et al, 2003; Thomas et al, 2008; Chen et al, 2011; Holter et al, 2020). The BCS of cows in the present study did not affect the hepatic mRNA abundance of *TGR5*. However, an increase in *TGR5* mRNA after calving could be related to metabolic changes in the periparturient period of dairy cows, as TGR5 signaling has been identified in the maintenance of glucose homeostasis and insulin sensitivity in mouse models of metabolic disease (Thomas et al., 2009).

Activated either through direct ligand binding or indirectly (Li and Wang, 2010), the nuclear hormone receptor CAR is involved in the regulation of BA synthesizing enzymes and BA transport proteins (Beilke et al., 2009). Although CAR is suggested to be involved in BA signaling, it remains unclear whether BA serves as natural ligand for CAR (Kovács et al., 2019). Given that CAR plays a role in several physiological processes, including energy metabolism

(Konno et al., 2008), triglyceride homeostasis (Maglich et al., 2009), and lipids (Roth et al., 2008), higher mRNA abundance of *CAR* in HBCS cows before calving does not necessarily indicate a role of CAR in bovine BA metabolism.

CONCLUSIONS

This study provides new insights into the dynamics of BA metabolism in periparturient dairy cows and shows the profound influence of lactation and body condition on BA profiles, enzyme activities, and transporter expressions in the bovine liver. The results show a prevalence of glycine-conjugated BA in the liver, with marked differences between cows with different body conditions. Notable upregulation of enzymes after parturition, particularly *CYP7A1*, indicates an increased hepatic BA pool. In addition, increased expression of key BA transporters such as *NTCP* and *MRP2* were observed in the study, indicating an adaptive response of the liver to the physiological changes during this period. The correlations between specific BA and blood parameters such as NEFA and BHB highlight the interplay between BA metabolism and energy balance in early lactation.

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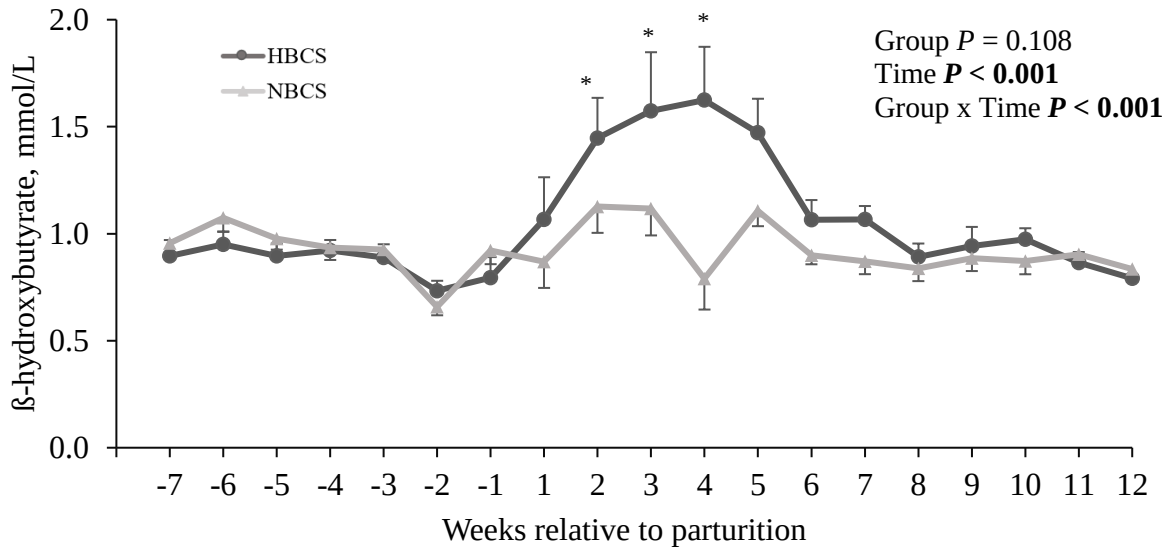
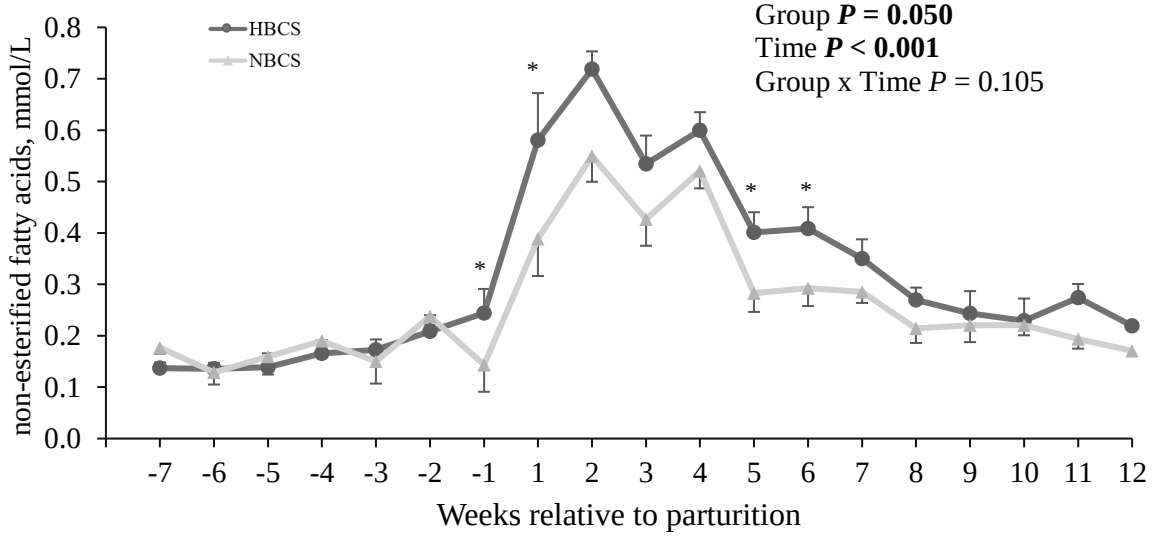
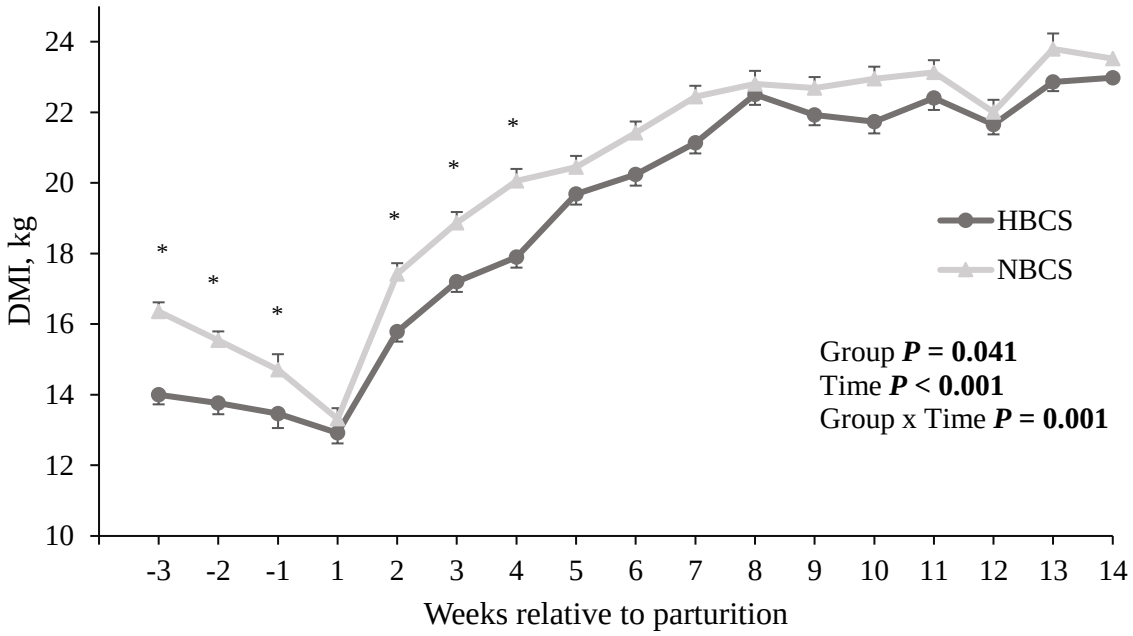
SUPPLEMENTAL FIGURES



Supplemental Figure S1: ß-hydroxybutyrate (BHB) from 7 wk antepartum (a.p.) to 12 wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data for BHB are from Schuh et al. (2019).



Supplemental Figure S2: Non-esterified fatty acids (NEFA) from 7 wk antepartum (a.p.) to 12 wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data for NEFA are from Schuh et al. (2019).



Supplemental Figure S3: Dry matter intake (DMI) from 3 wk antepartum (a.p.) to 14 wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data for DMI are from Schuh et al. (2019).

4 Manuscript II

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Bile acid profiles and mRNA abundance of bile acid-related genes in adipose tissue of dairy cows with high versus normal body condition

Lena Dicks[1], Katharina Schuh-von Graevenitz[2], Cornelia Prehn[3], Hassan Sadri[4†], Eduard Murani[5], Morteza Hosseini Ghaffari[1], Susanne Häussler[1*]

[1]Institute of Animal Science, Physiology Unit, University of Bonn, 53115 Bonn, Germany

[2]Department of Life Sciences and Engineering, Animal Nutrition and Hygiene Unit, University of Applied Sciences Bingen, 55411 Bingen am Rhein, Germany

[3]Helmholtz Zentrum München, German Research Center for Environmental Health, Metabolomics and Proteomics Core, 85764 Neuherberg, Germany

[4]Department of Clinical Science, Faculty of Veterinary Medicine, University of Tabriz, 516616471 Tabriz, Iran

[5]Research Institute for Farm Animal Biology (FBN), Institute for Genome Biology, Wilhelm Stahl Allee 2, 18196 Dummerstorf, Germany

*Corresponding author: susanne.haeussler@uni-bonn.de

†H. Sadri was a visiting scientist at the Institute of Animal Science, Physiology Unit, University of Bonn, 53115 Bonn, Germany, at the time the research was done.

ABSTRACT

Besides their lipid-digestive role, bile acids (BA) influence overall energy homeostasis, such as glucose and lipid metabolism. We hypothesized that BA along with their receptors, regulatory enzymes, and transporters are present in subcutaneous adipose tissue (scAT). In addition, we hypothesized that their mRNA abundance varies with the body condition of dairy cows around calving. Therefore, we analyzed BA in serum and scAT as well as the mRNA abundance of BA -related enzymes, transporters, and receptors in scAT during the transition period in cows with different body conditions around calving. In a previously established animal model, 38 German Holstein cows were divided into either a high (HBCS; n = 19) or normal BCS (NBCS; n = 19) group based on their body condition score (BCS) and back fat thickness (BFT). Cows were fed different diets to achieve the targeted differences in BCS and BFT (NBCS: BCS < 3.5, BFT < 1.2 cm; HBCS: BCS > 3.75, BFT > 1.4 cm) until dry-off at 7 wk ante partum. During the dry period and subsequent lactation, both groups were fed the same diets regarding their demands. Using a targeted metabolomics approach via LC-ESI-MS /MS, BA were analyzed in serum and scAT at wk -7, 1, 3, and 12 relative to parturition. In serum, 15 BA (cholic acid (CA), chenodeoxycholic acid (CDCA), glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), deoxycholic acid (DCA), lithocholic acid (LCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), β-muricholic acid (MCA(b)), tauromuricholic acid (sum of alpha and beta) (TMCA (a+b)), glycoursodeoxycholic acid (GUDCA)) were observed, whereas in scAT 7 BA (CA, GCA, TCA, GCDCA, TCDCA, GDCA, TDCA) were detected. In serum and scAT samples, the primary BA CA and its conjugate GCA were predominantly detected. Increasing serum concentrations of CA, CDCA, TCA, GCA, GCDCA, DCA, and MCA(b) with the onset of lactation might be related to the increasing DMI after parturition. Furthermore, serum concentrations of CA, CDCA, GCA, DCA, GCDCA, TCA, LCA, and GDCA were lower in HBCS cows compared with NBCS cows, concomitant with increased lipolysis in HBCS cows. The correlation between CA in serum and scAT may point to the transport of CA across cell membranes. Overall, the findings of the present study suggest a potential role of BA in lipid metabolism depending on the body condition of periparturient dairy cows.

Key words: adipose tissue, bile acids, body condition, periparturient period

INTRODUCTION

Bile acids (BA) are formed in the liver from cholesterol and are synthesized by various enzymatic processes within the classical/neutral or alternative/acidic synthetic pathway (Russell, 2003; Ma and Patti, 2014; Shapiro et al., 2018). Although BA classically support the digestion and absorption of nutrients such as lipids and lipophilic vitamins from the intestine, they also act as signaling molecules and can influence glucose and lipid metabolism (Ferrebee and Dawson, 2015; Shapiro et al., 2018).

The primary BA cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized in the liver and conjugated by the amino acids glycine or taurine to form the conjugated primary BA (Armstrong and Carey, 1982). After feed intake, the entero-hormone cholecystokinin (CCK) stimulates gallbladder contraction, and the BA are released into the intestine where they are deconjugated and 7α-dehydroxylated by intestinal bacteria. Finally, the resulting secondary BA (Di Ciaula et al., 2017) can be conjugated in the intestine by either glycine or taurine (Chiang, 2004). The BA biosynthetic pathway is shown in Figure 1.

In humans, the majority of BA (90-95% in total) return to the liver at the terminal ilium via the portal vein; however, BA that are not reabsorbed are excreted via feces (Yang et al., 2003; Chiang, 2004; Chen et al., 2019a; Chen et al., 2019b). Moreover, a small portion of BA enters the systemic circulation (Hofmann, 2009). In addition to enterohepatic circulation, BA have also been detected in tissues such as the kidney and heart in rats (Swann et al., 2011), bovine follicular fluid (Blaschka et al., 2019), and human adipose tissue (AT; Jäntti et al., 2014). Furthermore, a variety of different BA transporters have been described to control BA flux, either by absorption and enterohepatic circulation or by excretion and removal from the body (Dawson et al., 2009).

In mice and humans, BA activate both, nuclear and membrane receptors (Ticho et al., 2019). The farnesoid X receptor (FXR) is considered to be a metabolic feedback sensor for the formation of BA by inhibiting the transcription of BA forming enzymes (Chiang, 2009). Moreover, the Takeda G protein-coupled receptor 5 (TGR5) is expressed in many murine tissues, such as placenta, gallbladder, liver, intestine, and brown AT (Maruyama et al., 2002; Maruyama et al., 2006). Via TGR5, BA stimulate energy expenditure in brown AT and skeletal muscle of mice (Watanabe et al., 2006). Furthermore, several membrane and nuclear receptors such as the nuclear receptors vitamin D receptor (VDR), pregnane X receptor (PXR, NR1I2), and constitutive androstane receptor (CAR, NR1I3), G protein-coupled sphingosine-1-phosphates receptor 2 (S1PR2), can be activated by BA and indirectly affect BA homeostasis (Kliewer et al., 1998; Timsit and Negishi, 2007; Wan and Sheng, 2018; Studer et al., 2012).

In the periparturient period, lipid mobilization, mainly from AT, can affect the metabolism of dairy cows (Drackley et al., 2005). Cows with higher pre-calving body condition mobilize more body reserves after parturition and are therefore more prone to metabolic disorders compared to thinner cows (Bernabucci et al., 2005). Increased lipid mobilization from AT, which is associated with an increase in free fatty acids (FFA) in the blood, may increase the risk of metabolic diseases such as ketosis and fatty liver (Adewuyi et al., 2005). Dairy cows suffering from the fatty liver syndrome had increased plasma concentrations of bile components, i.e., bilirubin (West, 1990) and BA (Rehage et al., 1999), indicating a decrease in bile flow. In dairy cows, plasma BA profiles were affected after excessive lipolysis around calving (Gu et al., 2023); however, BA profiles in bovine scAT have not yet been investigated so far. In the present study, we aimed to investigate BA in serum and scAT by metabolomics approach, as well as the mRNA abundance of BA -related enzymes, receptors, and transporters by quantitative reverse transcription real-time PCR (RT -qPCR) in scAT from dairy cows with different body condition. We hypothesized that (1) cows with different pre-calving body condition and thus different levels of lactation-induced lipolysis would differ in their BA profiles and (2) BA as well as the mRNA abundances of BA receptors, transporters, and regulatory enzymes are present in subcutaneous AT (scAT) of dairy cows. By investigating variables involved in BA metabolism within bovine scAT, we aimed to further elucidate lipid metabolism in the periparturient period of dairy cows.

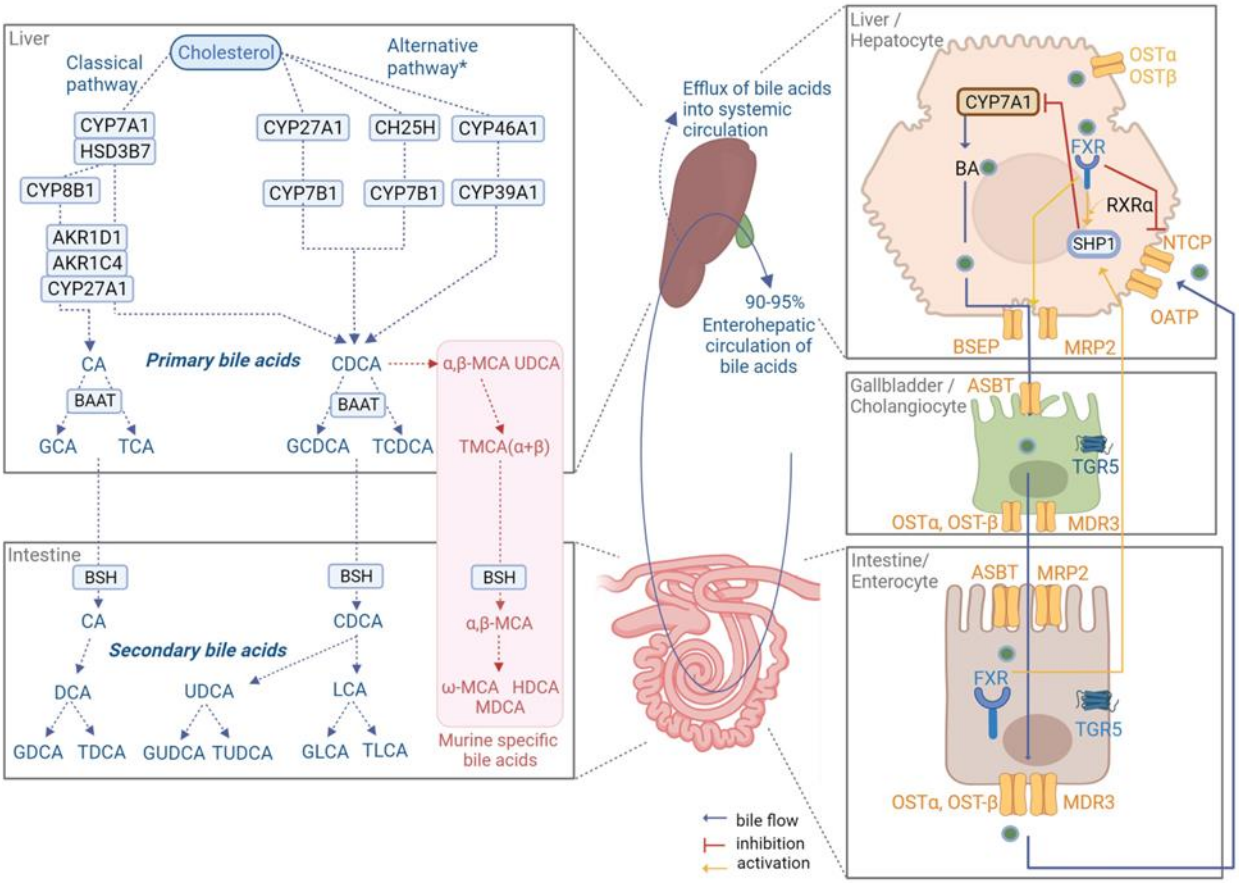

Figure 5: Bile acid synthesis pathway in liver and intestine. *: BA-related enzymes are ubiquitously expressed and not limited to the hepatic alternative pathway. Involved enzymes: CYP7A1: Cholesterol 7alpha-Hydroxylase; HSD3B7: 3 Beta-Hydroxysteroid Dehydrogenase Type 7; CYP8B1: Sterol 12-Alpha-Hydroxylase; AKR1D1: Aldo-Keto Reductase Family 1 Member D1; AKR1C4: Aldo-Keto Reductase Family 1 Member C4; CYP27A1: Sterol 27-Hydroxylase; CYP7B1: Oxysterol 7-Alpha-Hydroxylase; CH25H: Cholesterol 25-Hydroxylase; CYP46A1: Cholesterol 24-Hydroxylase; CYP39A1: Cytochrome P450 Family 39 Subfamily A Member 1; BAAT: Bile Acid Coenzyme A: Amino Acid N-Acyltransferase; BSH: Bile Salt Hydrolase Transporters: NTCP, SLC10A1: Na+-taurocholate cotransporting polypeptide; SLC10A2, ASBT: Apical Sodium-Dependent Bile Acid Transporter; OSTα, SLC51A1: solute carrier family 51 subunit alpha; OSTβ, SLC51B: solute carrier family 51 subunit beta; MRP2, ABCC2: Multidrug Resistance-Associated Protein; BSEP, ABCB11: Bile Salt Export Pump; MDR3, ABCB4: Multiple Drug Resistance 3; OATP: Solute Carrier Organic Anion Transporter. Receptors: FXR, NR1H4: Farnesoid X Receptor; TGR5, GPBAR1: Takeda G protein–coupled receptor 5; RXRα, NR2B1: Retinoid X Receptor Alpha; SHP: Small Heterodimer Partner. Bile acids: cholic acid (CA). chenodeoxycholic acid (CDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA) taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), taurolithocholic acid (TLCA), glycolitocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), α-muricholic acid (αMCA), β-muricholic acid (βMCA), tauromuricholic acid (sum of alpha and beta) (α, β-TMCA), ω-muricholic acid (ωMCA), hyodeoxycholic acid (HDCA), murideoxycholic acid (MDCA). The Figure was created using BioRender.com (SL25XUBGY1).

MATERIALS AND METHODS

Basic trial

The animal experiment was performed at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a.d. Alsenz, Germany. The trial was conducted following European regulations for the protection of experimental animals and was approved by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany [G 14-20-071]). The experiment was described previously in detail (Schuh et al., 2019). In brief, the experimental period started fifteen wk before calving and lasted until fourteen wk after calving. In total, 38 multiparous German Holstein cows were preselected based on their body condition score (BCS) and backfat thickness (BFT) of the previous lactation and divided into two groups (HBCS; n = 19 and NBCS; n = 19). They received different feeding regimens from 15 wk ante partum (a.p.) to 7 wk a.p. (HBCS: 7.2 NEL MJ/kg of dry matter (DM)); NBCS: 6.8 NEL MJ/kg of DM) to reach the targeted BCS and BFT at dry-off (HBCS: BCS > 3.75 and BFT > 1.4 cm; NBCS: BCS < 3.5 and BFT < 1.2 cm). During the dry period and subsequent lactation, both groups received identical diets. Performance data (BCS, BFT, EB, and DMI) were reported earlier (Schuh et al., 2019) and are presented herein as Supplemental Figures S1- S4.

Sampling and BA analyses

Blood and tissue samples were collected at wk 7 a.p., as well as wk 1, 3, and 12 postpartum (p.p.). Blood samples were collected from the coccygeal vein after morning milking and before the new presentation of fresh feed. The scAT taken from the tail head region were rinsed with 0.9% NaCl solution and immediately frozen in liquid nitrogen. Samples were stored at -80°C until analysis.

Bile acids in serum and scAT have been quantified using the AbsoluteIDQTM Bile Acids kit (biocrates life sciences ag, Innsbruck, Austria). This standardized assay includes sample preparation and LC-ESI-MS/MS measurements. The assay allows simultaneous quantification of 20 BA, including CA, CDCA, deoxycholic acid (DCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), hyodeoxycholic acid (HDCA), lithocholic acid (LCA), alpha-muricholic acid (MCA(a)), beta-muricholic acid (MCA(b)), omega-muricholic acid (MCA(o)), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), tauromuricholic acid (sum of alpha and beta) (TMCA(a+b)), tauroursodeoxycholic acid (TUDCA), and ursodeoxycholic acid

(UDCA). Identification and quantification of the compounds were based on scheduled multiple reaction measurements (sMRM). The method of the Bile Acids kit has been proven to be in conformance with the EMEA "Guideline on bioanalytical method validation" (EMEA, 2011), which implies proof of reproducibility within a given error range. The complete assay procedures of the Bile Acid kit for the analysis of plasma or serum and the results of an inter-laboratory ring-trial have been described in detail previously (Pham et al., 2016; McCreight et al., 2018).

Serum samples were applied directly to the assay, whereas scAT samples were applied as homogenate supernatant. The homogenization and extraction protocol have been developed specifically for this application. Samples from scAT have been prepared as follows: frozen bovine AT samples were weighted into homogenization tubes with ceramic beads (1.4 mm). To ensure comparable extraction efficiency and to provide stable pH values, 12 µL of a cooled (4 °C) mixture of ethanol/phosphate buffer (70/30 v/v) was added per 1 mg frozen AT. These tissue/buffer samples were homogenized using a Precellys24 homogenizer (PEQLAB Biotechnology GmbH, Germany) 4 × for 20 sec at 5,500 rpm and 10-15 °C, with 30 sec pause intervals to ensure constant temperature. After sample centrifugation at 10,000 × g for 5 min, supernatants were used for metabolite quantification. Internal standards were included in the Bile Acid kit and were added after homogenization of scAT. To prepare the assay, 10 µL of the internal standard solution in methanol was pipetted onto the filter inserts of the 96-well sandwich plate. After drying the filters for 5 min at RT in a stream of nitrogen, 10 µL of blank, calibration standards, quality control samples, or plasma samples, or 40 µL of the freshly prepared tissue homogenate were pipetted into the respective wells and the filters were dried again for 5 min. The tissue homogenates (40 µL) were applied in two steps of 20 µL each, with a separate drying step in between to avoid sample leakage from the filter insert. For extraction of metabolites and internal standards, 100 µL of methanol was added and the plate was shaken at 650 rpm for 20 min. The metabolite extracts were eluted into the lower deep-well plate by a centrifugation step (5 min at 500 × g at RT). The upper filter plate was removed, the extracts were diluted with 60 µL ultrapure water, and the plate was shaken at 450 rpm for 5 min and placed in the cooled auto-sampler (10 °C) for LC-MS /MS measurements.

The LC -separation was performed using 10 mM ammonium acetate in a mixture of ultrapure water/formic acid v/v 99.85/0.15 as mobile phase A and 10 mM ammonium acetate in a mixture of methanol/acetonitrile/ ultrapure water/formic acid v/v/v/v 30/65/4.85/0.15 as mobile phase B. The BA were separated on the UHPLC column for the Bile Acids kit (Product No. 91220052120868) combined with the precolumn SecurityGuard ULTRA Cartridge

C18/XB-C18 (for 2.1 mm ID column, Phenomenex Cat. No. AJ0-8782). All solvents used for sample preparation and measurement were of HPLC grade. Samples were processed using a Hamilton Microlab STARTM robot (Hamilton Bonaduz AG, Bonaduz, Switzerland), an Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, U.K.), and standard laboratory equipment. Mass spectrometric analyzes were performed using an API 4000 triple quadrupole system (SCIEX Deutschland GmbH, Darmstadt, Germany) equipped with a 1260 series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and an HTC-xc PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by Analyst 1.6.2 software. Data evaluation for quantification of metabolite concentrations and quality assessment were performed using MultiQuant 3.0.1 (Sciex) software and the MetIDQ™ software package, which is an integral part of the Bile Acids kit. Metabolite concentrations were calculated using internal standards and reported in µM.

Primer Design and Quantitative real-time PCR

Bovine-specific primer pairs were designed using the National Center for Biotechnology Information (NCBI) primer blast. In addition, eight reference genes (low-density lipoprotein receptor-related protein 10 (*LRP10*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), RNA polymerase II (*POLR2A*), eukaryotic translation initiation factor 3, subunit K (EIF3K), marvel domain containing 1 (*MARVELD1*), hippocalcin-like 1 (*HPCAL1*), emerin (*EMD*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*)), previously shown to be stable in bovine AT (Saremi et al., 2012), were measured. The primer pairs used in this study are presented in Table 1. Specific primers were selected based on an optimal melting temperature of 59°C. Using a Bio-Rad CFX cycler each primer pair was tested by reverse transcription quantitative real-time PCR (RT-qPCR) in pooled cDNA samples from scAT. Primers were tested according to the following RT-qPCR protocol: 3 min at 90°C initial denaturation and 40 cycles of amplification (95°C for 30 sec, 59°C for 60 sec, and 72°C for 60 sec).

Table 1: Characteristics of primers and real-time qPCR conditions

Gene	Target	Sequence 5'- 3'*	Primer	Ass.No	(bp)
Enzymes					
CYP7A1		F	CTACCCAGACCCGTTGACTT	NM_001205677	270
		R	GGTAAAATGCCCAAGCCTGC		
HSD3B7		F	CCCAGGAGACACAGAAGACC	NM_001034696.1	74
		R	CGGCCATACCTGGCTGC		
CYP8B1		F	GGGAAGGCTTGGAGGAGC	NM_001076139.2	142
		R	GGAGGTGATGAGGAGCCAGA		
AKR1D1		F	ACTCGGAACCTAAATCGACTCC	NM_001192358.1	103
		R	TTCTGGTAGAGGTAGGCCCC		
CYP27A1		F	GGCTGGAGTAGACACGACAT	NM_001083413.2	201
		R	GGGACCACAGGATAGAGACG		
CYP7B1		F	ACAATTGGACAGCCTGGTCT	XM_025001826.1	220
		R	ACTGGAAAATAGCAGCCCATCT		
CH25H		F	ACGCTTGAGGTGGACTTGAG	NM_001075243.1	375
		R	AATCTGAGTCACTGCCCAGC		
CYP46A1		F	TTTCCTTCTAGGGCACCTCC	NM_001076810.1	96
		R	CCGTACTTCTTAGCCCAATCC		
BAAT		F	ACCTGCCTTTCAGAGTGGAG	XM_015472664.1	90
		R	CTGGCCCAAGGACCTTAGTAT		
Transporter					
SLC10A1	*NTCP*	F	GCTATGTCACCAAGGGAGGG	NM_001046339.1	272
		R	GGGGAAGGTCACATTGAGGA		
SLC10A2	*ASBT*	F	TTTCCTTCCAGCGTCAGCAT	XM_019971692.1	566
		R	TATACCACGTACACTGCCAGG		
SLC51A1	*OSTα*	F	CCCAGCTTTTGAGAGCCATC	NM_001025333.2	676
		R	GGTGAACAAGCAATCTGCCC		
SLC51B	*OSTβ*	F	AGCAGACCAGACGAGTCCT	NM_001077867.2	261
		R	TTCCAAGGAGTTGCGTCCTC		
ABCC2	*MRP2*	F	GATGAGGCCACAGTCAATGAG	XM_024985942.1	81
		R	CACGTCCTCTGGGATTTCCT		
ABCB1	*MDR1*	F	GCGGCTCTTCAAGACTCAGTG	XM_024991021.1	137
		R	AGATCCATCGCGACCTCGG		
ABCB11	*BSEP*	F	GCACTGAGTAAGGTTCAGCA	NM_001192703.3	241
		R	TCTCAAGTAAGGCATCTTCGG		
ABCB4	*MDR3*	F	TGGGGCCGGACACTCT	XM_024991318.1	395
		R	TTAGCTTGGCTGCTGCTGA		

Table 1: Continued

Gene	Target	Sequence 5'- 3'*	Primer	Ass.No	(bp)
Receptors					
NR1H4	*FXR*	F	AAGCCCGCTAAAGGTGTACT	NM_001034708.2	298
		R	TGATTCTCCCTGCTGATGCT		
GPBAR1	*TGR5*	F	GACCTCAACGGTCAGGACAC	NM_175049.3	126
		R	GGCATGCATGACTGTAGGTG		
NR1I2	*PXR*	F	GCGGCATGAGAAAAGAGATGAT	NM_001103226.1	998
		R	AGCCAGTCAGCCATTTGTG		
S1PR2	*S1PR2*	F	GATCGGCCTAGCCAGCATCA	NM_001081541.1	650
		R	AAGATGGTCACCACGCAGAG		
VDR	*VDR*	F	CACCCGCAGGACCAGAGTC	NM_001167932.2	701
		R	GAGAAGCTGGTTGGCTCCAT		
CHRM2	*CHRM2*	F	ACCTCCAGACCGTCAACAAT	NM_001080733.1	139
		R	CAAAGGTCACACACCACAGG		
NR2B1	*RXRα*	F	CCATTTTCGACAGGGTGCTG	NM_001304343.1	171
		R	CCAGGGACGCATAGACCTTC		
SHP1	*SHP1*	F	TCCTCTTCAACCCTGACGTG	XM_002685759.5	173
		R	GCTGGGTGGAATGGACTTGA		
NR1I3	*CAR*	F	GAACAACGGAGGCTACACAC	NM_001079768.2	197
		R	TGTTGACTGTTCGCCTGAAG		
Reference genes					
YWHAZ		F	CCACCTACTCCGGACACAG	NM_174814.2	464
		R	GACTGGTCCACAATCCCTTTC		
EIF3K		F	CCAGGCCCACCAAGAAGAA	NM_001034489	125
		R	TTATACCTTCCAGGAGGTCCATGT		
HPCAL1		F	GCCGGCTTCCTTTTGTCTTT	NM_001098964	216
		R	CTAGACCATGCCCTGCTCC		
POLR2A		F	CTATCGCAGAACCCACTCACC	NM_001206313.2	91
		R	CACAGCGGGAAGGATGTCTG		
GAPDH		F	GAAGGTCGGAGTGAACGGATTC	NM_001034034.2	153
		R	TTGCCGTGGGTGGAATCATA		
MARVELD1		F	TCGGTGCTTTGATGTCTTGC	NM_001101262.1	71
		R	CAATCCACGGGCACTTCCTA		
LRP10		F	TTTTCCCGAATCCTGCCTGT	NM_001100371.1	73
		R	ACAGGCCTCTGTAAGGTGC		
EMD		F	GCCAGTACAACATCCCACAC	NM_203361.1	155
		R	CGCCGAATCTAAGTCCGAGA		

CYP7A1: Cholesterol 7alpha-Hydroxylase; *HSD3B7*: 3 Beta-Hydroxysteroid Dehydrogenase Type 7; *CYP8B1*: Sterol 12-Alpha-Hydroxylase; *AKR1D1*: Aldo-Keto Reductase Family 1 Member D1; *CYP27A1*: Sterol 27-Hydroxylase; *CYP7B1*: Oxysterol *CH25H*: Cholesterol 25-Hydroxylase; *CYP46A1*: Cholesterol 24-Hydroxylase; *BAAT*: Bile Acid Coenzyme A:Amino Acid N-Acyltransferase; *NTCP, SLC10A1*: Na+-taurocholate cotransporting polypeptide; *SLC10A2, ASBT*: Apical Sodium-Dependent Bile Acid Transporter;

OSTα, SLC51A1: solute carrier family 51 subunit alpha; *OSTβ, SLC51B*: solute carrier family 51 subunit beta; *MRP2, ABCC2*: Multidrug Resistance-Associated Protein; *MDR1, ABCB1*: Multidrug Resistance Protein 1; *BSEP, ABCB11*: Bile Salt Export Pump; *MDR3, ABCB4*: Multiple Drug Resistance 3; *FXR, NR1H4*: Farnesoid X Receptor; *TGR5, GPBAR1*: Takeda G protein–coupled receptor 5*; NR1I2, PXR*: Nuclear Receptor Subfamily 1, Group I, Member 2; *S1PR2*: Sphingosine-1-Phosphate Receptor 2; *VDR*: Vitamin D Receptor; *CHRM2*: Cholinergic Receptor Muscarinic 2; *RXRα, NR2B1*: Retinoid X Receptor Alpha; *SHP1*: Small Heterodimer Partner; *CAR*: Constitutive Androstane Receptor; *YWHAZ*: Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta; *EIF3K*: Eukaryotic Translation Initiation Factor 3 Subunit K; *HPCAL1*: Hippocalcin Like 1; P*OLR2A*: RNA Polymerase II Subunit A; *GAPDH*: Glyceraldehyde-3-Phosphate Dehydrogenase; *MARVELD1*: MARVEL Domain Containing 1; *LRP10*: LDL Receptor Related Protein 10; *EMD*: Emerin.
*F = forward; R = reverse.

RT-qPCR Measurements

The mRNA abundance of 26 target genes and 8 reference genes in scAT from 38 cows at four time points was measured by RT -qPCR using the Biomark HD 96.96 system (Fluidigm Co., San Francisco, CA, USA), as described in detail previously (Alaedin et al., 2021). In brief, samples were measured in triplicates using the Biomark HD RT-qPCR system and 96.96 integrated fluidic circuits (IFCs). Preparation of the IFCs was performed according to the protocol "Fast Gene Expression Analysis Using EvaGreen on Biomark HD or Biomark" from Fluidigm. To compensate for variations between IFCs, three inter-run calibrators were added to each IFC. For subsequent gene expression (GE) analysis, the Biomark HD real-time PCR reader was used with the protocol "GE Fast 96 × 96 PCR + Meltv2".

Quality control of the melting and amplification curves was performed using Fluidigm real-time PCR Analysis Software (V4.5.2). Inter-run calibration was performed using qBASEplus software (V3.3, Biogazelle, Ghent, Belgium). Differences in the quantification cycle between the inter-run calibrators of the runs were compared, and corrections or calibration factors were determined to compensate for the differences between runs. Three reference genes were determined by GeNormPlus (included in qBASEplus) to serve as optimal numbers for normalization, i.e., *EIF3K, LRP10*, and *POLR2A*). Reference genes were determined as described in detail by Alaedin et al. (2021). The normalized values were used for statistical analysis of the mRNA data.

Statistical Analyses

Statistical analyses of BA concentrations in blood and scAT as well as mRNA abundance of BA-associated enzymes, receptors, and transporters were performed using a linear mixed model with repeated measures (IBM SPSS version 28). The model consisted of group, time, and interaction of group and time as fixed effects and cow as the random effect. Time was classified as repeated measures. Different variance-covariance structures were tested to determine the most appropriate variance-covariance structure. An autoregressive type 1 covariance structure and an identity covariance structure (scaled identity matrix) were selected as the best fit based on the Akaike and Bayesian information criteria. Bonferroni´ correction was used to perform multiple comparisons. All residuals were tested for normality using the Kolmogorov-Smirnov test. Data that did not meet the assumptions of normality of residuals had to be log-transformed (base 10). Data was back-transformed for the Figures and Tables (mean ± SEM). Relationships between BA in serum and scAT were calculated by Spearman correlation using non-transformed data and represented by a heat map generated using JASP 0.17.1 (JASP Team, 2019). Correlations between mRNA abundance of BA -related enzymes, transporters, and receptors were calculated only for the data analyzed under the mixed model. Correlation coefficients were considered as very strong ($1.0 \geq r \geq 0.9$), strong ($0.9 > r \geq 0.7$), moderate ($0.7 > r \geq 0.5$), weak ($0.5 > r \geq 0.3$), very weak to zero correlation ($r < 0.3$). The threshold of significance was set at $P \leq 0.05$; trends were declared at $0.05 < P \leq 0.10$.

RESULTS

BA concentrations in Serum

A total of 6 primary and 9 secondary BA, including their conjugates, were evaluated in serum. The mean percentage of each BA relative to the total BA in serum is shown in Figure 2A. In serum, CA and its conjugated form GCA account for the largest proportion of the total BA (approximately 65%). The concentrations of BA in serum from wk 7 a.p. to wk 12 p.p. are shown in Figure 3. The concentration of serum BA changed over time, except for GLCA. For CA, CDCA, TCA, GCA, GCDCA, DCA and MCA(b) concentrations were greater after calving than a.p.. Regardless of time, concentrations of CA, CDCA, GCA, DCA, GCDCA, TCA, LCA, and GDCA were greater ($P \leq 0.05$) in NBCS cows than in HBCS cows.

BA concentrations in scAT

A total of 5 primary and 2 secondary BA were detected in scAT. The average percentages of each BA relative to the total BA in scAT are shown in Figure 2B. In scAT, CA and its conjugated form GCA had the highest proportion of the total BA. The concentrations of BA in scAT from wk 7 a.p. to wk 12 p.p. are presented in Figure 4. The concentrations of CA, GDCA and GCA were lower ($P \leq 0.001$) before calving and at wk 1 p.p. compared to wk 3 and 12 p.p. In addition, GCDCA concentrations a.p. were lower compared to p.p. concentrations ($P \leq 0.001$). Across all time points, higher concentrations of GCA (1.46-fold, $P \leq 0.001$), GCDCA (1.40-fold, $P \leq 0.001$), GDCA (1.63-fold, $P \leq 0.001$), TDCA (1.19-fold, $P = 0.02$), and TCDCA (1.35-fold, $P = 0.01$) were measured in NBCS cows compared with HBCS cows. At wk 3 p.p., an interaction ($P = 0.01$) between group and time was observed in CA concentrations, with NBCS cows showing 2.4 times higher ($P \leq 0.001$) CA levels than HBCS cows, indicating a time-specific differential response between the groups.

[A] [B]

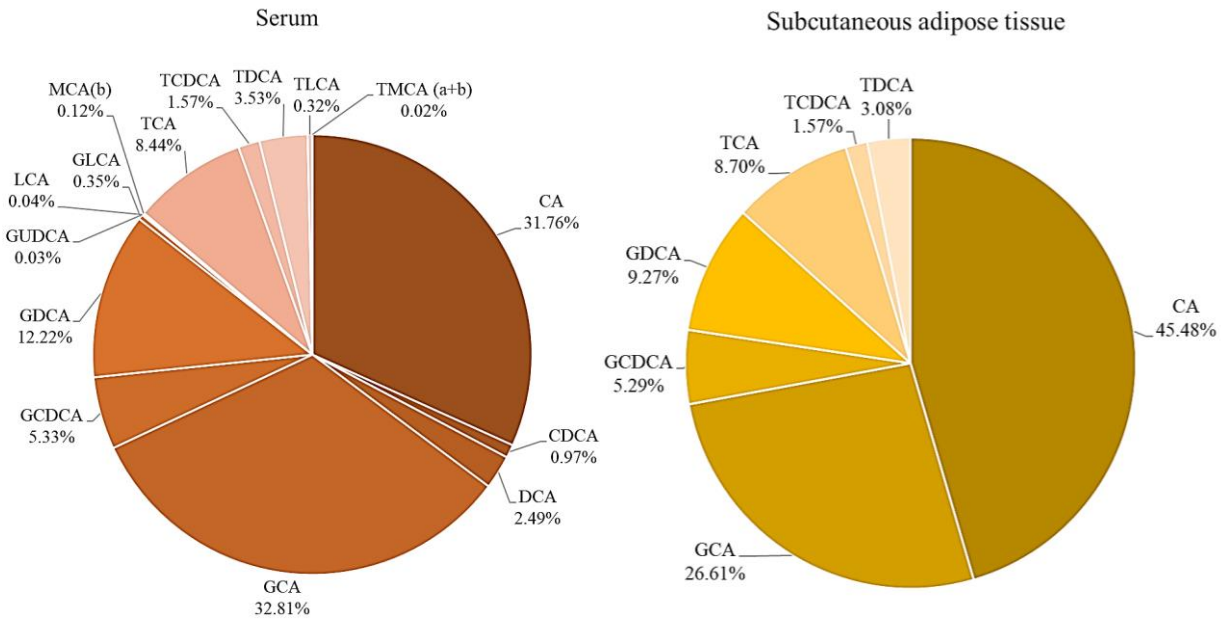

Figure 2: Mean percentage (%) of total bile acids in serum [A] and subcutaneous adipose tissue [B]. Bile acids: cholic acid (CA). chenodeoxycholic acid (CDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), deoxycholic acid (DCA), lithocholic acid (LCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), taurolithocholic acid (TLCA), glycolitocholic acid (GLCA), glycoursodeoxycholic acid (GUDCA), tauroursodeoxycholic acid (TUDCA), muricholic acid b (MCA beta).

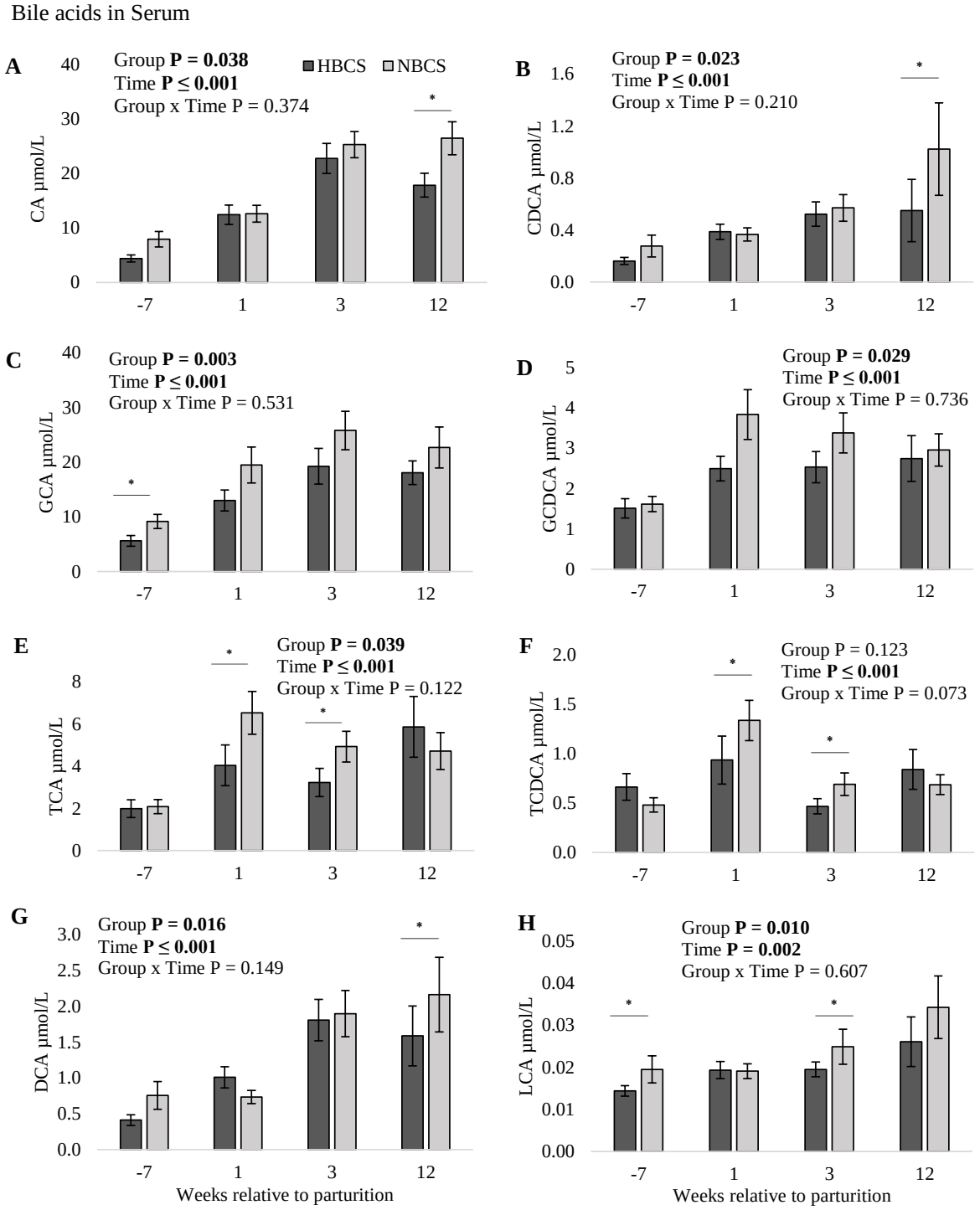
Bile acids in Serum
A
Group P = 0.038
Time P ≤ 0.001
Group x Time P = 0.374
HBCS
NBCS
CA µmol/L
B
Group P = 0.023
Time P ≤ 0.001
Group x Time P = 0.210
CDCA µmol/L
C
Group P = 0.003
Time P ≤ 0.001
Group x Time P = 0.531
GCA µmol/L
D
Group P = 0.029
Time P ≤ 0.001
Group x Time P = 0.736
GCDCA µmol/L
E
Group P = 0.039
Time P ≤ 0.001
Group x Time P = 0.122
TCA µmol/L
F
Group P = 0.123
Time P ≤ 0.001
Group x Time P = 0.073
TCDCA µmol/L
G
Group P = 0.016
Time P ≤ 0.001
Group x Time P = 0.149
DCA µmol/L
H
Group P = 0.010
Time P = 0.002
Group x Time P = 0.607
LCA µmol/L
Weeks relative to parturition
Weeks relative to parturition

Figure 3

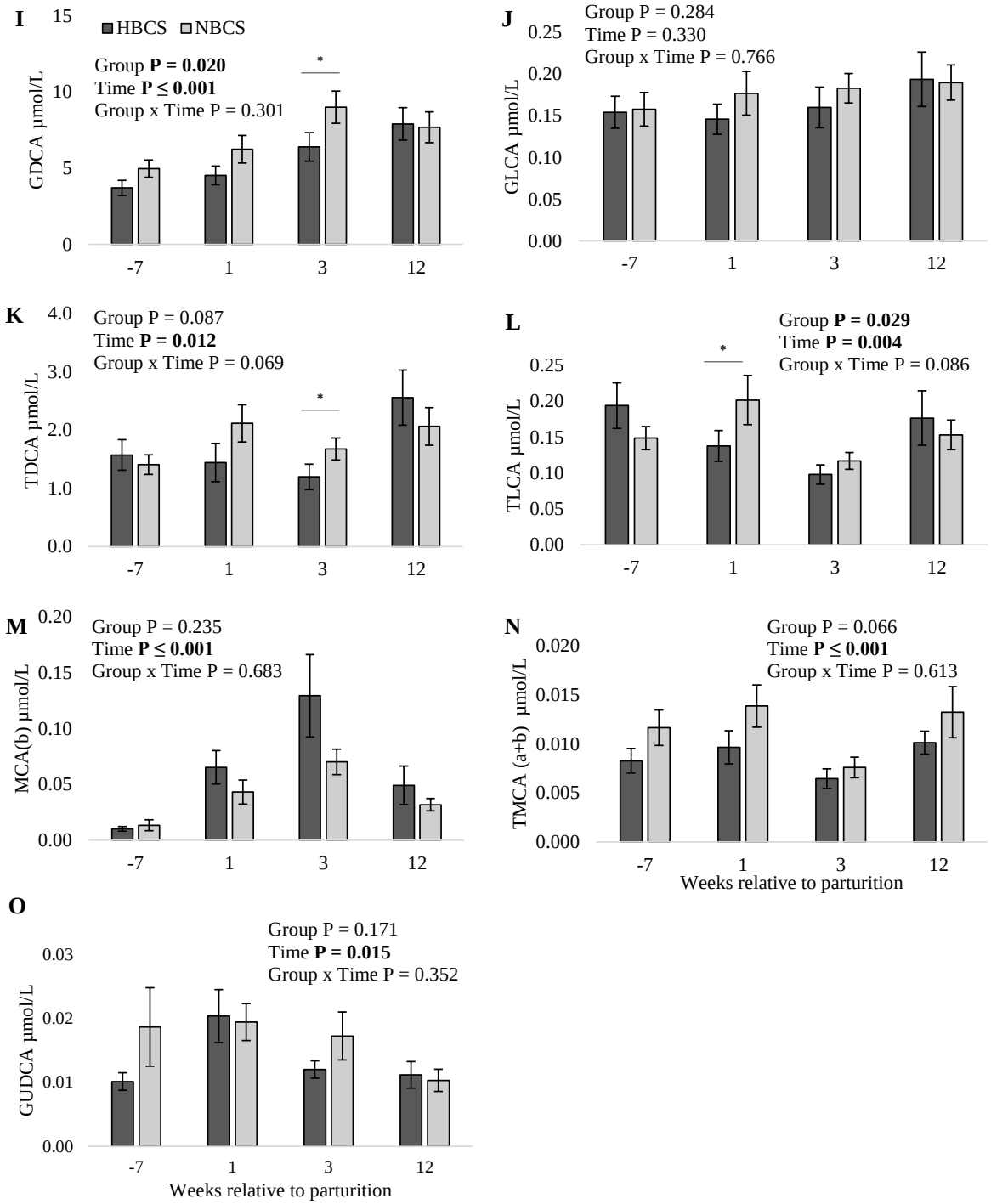

Figure 3 continued: Concentration of bile acids (µmol/L) in serum from cows with normal (NBCS) versus high body condition score (HBCS) at wk -7 ante partum (a.p.) and wk 1, 3, and 12 postpartum (p.p.). Values are given as means ± SEM. Significant differences ($P \leq 0.05$) between the groups are indicated by asterisks.: cholic acid (CA), chenodeoxycholic acid (CDCA), glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA), deoxycholic acid (DCA), lithocholic acid (LCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), β-muricholic acid (MCA(b)), tauromuricholic acid (sum of alpha and beta) (TMCA (a+b)), glycoursodeoxycholic acid (GUDCA).

Bile acids in subcutaneous adipose tissue

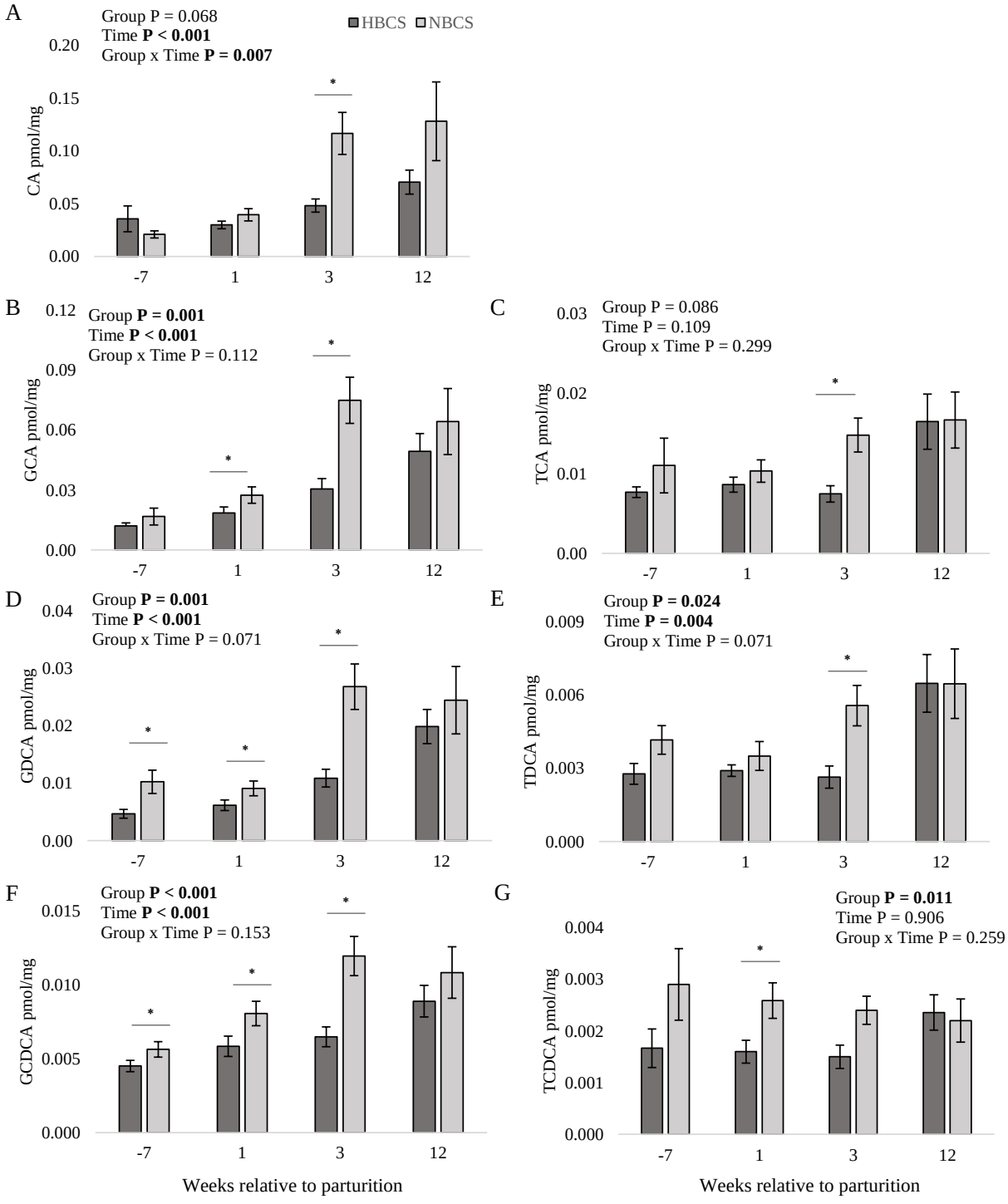

Figure 4: Concentration of bile acids (pmol/ng) in scAT from cows with normal (NBCS) versus high body condition score (HBCS) at wk -7 ante partum (a.p.) and wk 1, 3 and 12 postpartum (p.p.). Values are given as means ± SEM. Significant differences ($P \leq 0.05$) between the groups are indicated by asterisks. Bile acids: cholic acid (CA), glycocholic acid (GCA), taurocholic acid (TCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA).

Relationship between BA in Serum and scAT

The correlations between BA in serum and scAT are shown in Figure 5. Glycine-conjugated BA in serum and scAT were weakly correlated, with correlation coefficients ranging from r = 0.447 to 0.498. Significant positive correlations between taurine-conjugated BA in serum and scAT ranged from r = 0.276 to 0.356. Serum CA was positively associated with CA as well as with glycine-conjugated BA (i.e., GCA, GCDCA, and GDCA; $P \leq 0.001$) in scAT.

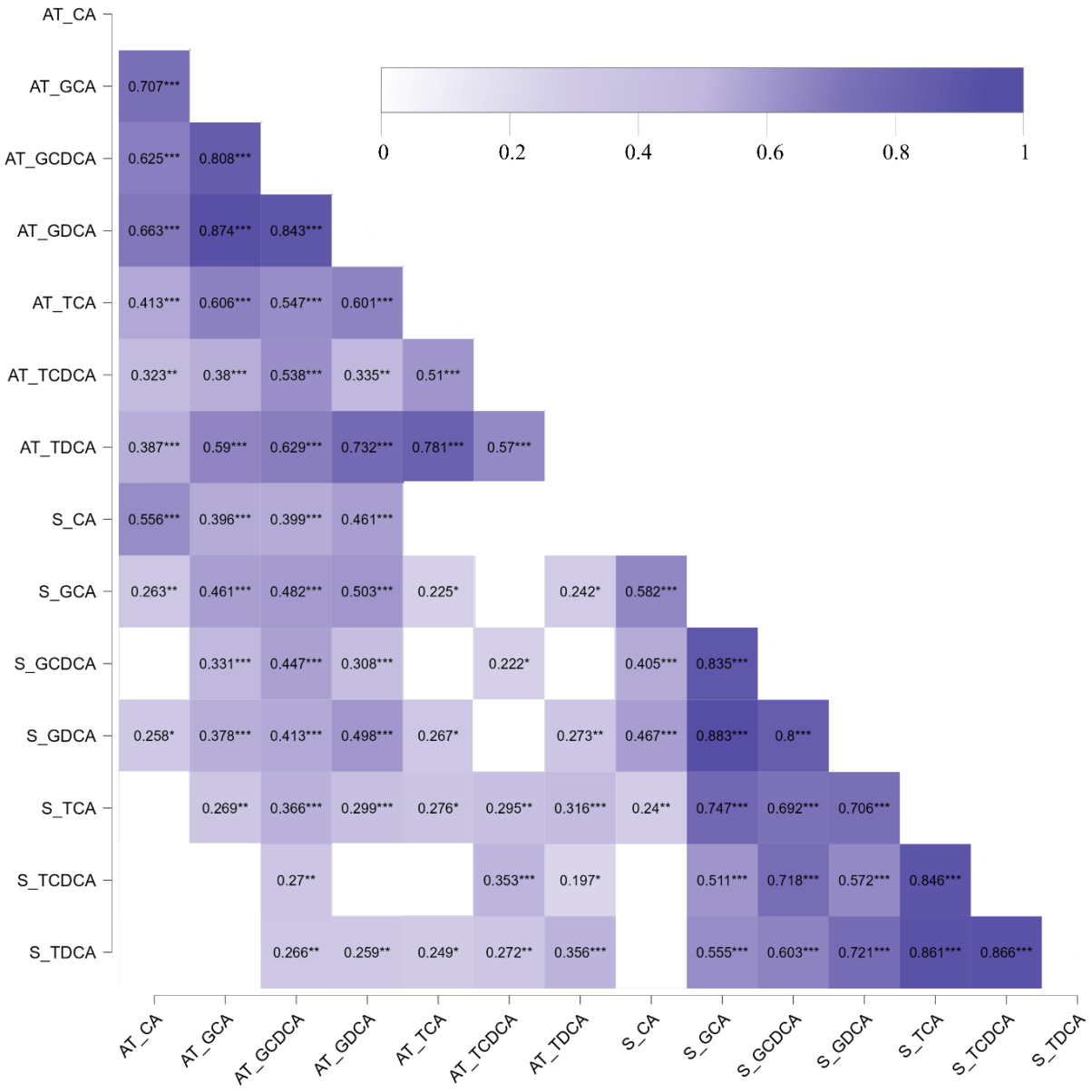

Figure 5: Correlations between serum (S) BA and scAT (AT) BA independent of group and time. Asterisks indicate significant differences: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. Bile acids: cholic acid (CA), glycocholic acid (GCA), taurocholic acid (TCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), taurochenodeoxycholic acid (TCDCA).

mRNA abundance of BA-related Enzymes in scAT

The mRNA abundance of enzymes related to the BA metabolism in scAT are shown in Table 2. In HBCS cows, the mRNA abundance of 3 beta-hydroxysteroid dehydrogenase type 7 (*HSD3B7*) was 1.53-fold higher at 3 wk p.p. ($P \leq 0.001$) and 1.41-fold higher at 12 wk p.p. ($P = 0.02$) compared to NBCS cows. An interaction between group and time was observed for the mRNA abundance of cholesterol-24S-hydroxylase (CYP46A1; $P = 0.04$), with HBCS cows having a 2.16-fold higher ($P = 0.01$) mRNA abundance than in NBCS cows at wk 7 a.p.. Furthermore, in HBCS cows, the mRNA abundance of CYP46A1 was higher before calving compared to wk 3 p.p. (3.76-fold; $P \leq 0.001$).

Table 2: mRNA abundance of enzymes related to bile acid metabolism in scAT from cows with normal (NBCS) and high body condition score (HBCS) at wk 7 a.p., as well as wk 1, 3 and 12 p.p.. Statistically significant P-values are given in bolt ($P \leq 0.05$).

		Weeks relative to parturition								***P*-value**		
		-7		**1**		**3**		**12**		**Group**	**Time**	**Group x Time**
		HBCS	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**			
	mean	0.002	0.001	0.000	0.001	0.002	0.009	-	0.001	-	-	-
CYP7A1	SEM	0.001	0.000				0.007	-				
	N	3	4	1	1	1	2	-	1			
	mean	1.034	1.212	1.384	0.906	1.440	1.463	1.951	1.384	**0.008**	**0.009**	0.067
HSD3B7	SEM	0.092	0.268	0.161	0.107	0.191	0.216	0.273	0.181			
	N	19	17	18	19	17	16	16	11			
	mean	0.159	0.269	0.545	0.483	0.262	0.434	0.555	0.423	0.250	< **0.001**	0.268
CYP8B1	SEM	0.036	0.067	0.125	0.133	0.046	0.079	0.178	0.055			
	N	11	13	12	10	12	8	10	7			
	mean	0.010	0.004	0.002	0.008	0.002	0.012	0.001	-	-	-	-
AKR1D1	SEM	0.004	0.001	0.000	0.004	0.000	0.010	0.000	-			
	N	8	13	2	8	6	6	2	-			
	mean	0.376	0.341	0.335	0.340	0.342	0.318	0.336	0.392	0.832	0.714	0.643
CYP27A1	SEM	0.035	0.024	0.032	0.029	0.027	0.029	0.026	0.050			
	N	19	17	19	19	17	16	17	11			
	mean	0.388	0.287	0.589	0.471	0.795	0.684	0.943	0.648	0.309	< **0.001**	0.897
CYP7B1	SEM	0.089	0.043	0.108	0.067	0.182	0.078	0.224	0.142			
	N	12	12	11	10	8	4	7	4			
	mean	0.785	0.894	1.742	3.359	1.570	2.324	2.250	2.668	**0.038**	< **0.001**	0.982
CH25H	SEM	0.194	0.153	0.409	1.472	0.447	0.483	0.423	0.563			
	N	18	16	15	17	16	15	17	11			
	mean	4.282	1.986	1.878	2.194	1.138	1.301	1.473	1.163	0.363	< **0.001**	**0.039**
CYP46A1	SEM	0.653	0.287	0.425	0.354	0.216	0.205	0.207	0.238			
	N	19	17	17	18	15	11	14	10			
	mean	0.737	0.541	0.672	0.772	0.711	0.566	0.488	0.719	0.903	0.779	0.095
BAAT	SEM	0.112	0.070	0.115	0.119	0.090	0.081	0.082	0.114			
	N	14	15	15	17	13	11	12	10			

CYP7A1: Cholesterol 7alpha-Hydroxylase; *CYP27A1*: Sterol 27-Hydroxylase; *HSD3B7*: 3 Beta-Hydroxysteroid Dehydrogenase Type 7; *CYP8B1*: Sterol 12-Alpha-Hydroxylase; *AKR1D1*: Aldo-Keto Reductase Family 1 Member D1; *CH25H*: Cholesterol 25-Hydroxylase; *CYP46A1*: Cholesterol 24-Hydroxylase; *BAAT*: Bile Acid Coenzyme A: Amino Acid N-Acyltransferase; *CYP7B1*: Oxysterol 7-Alpha-Hydroxylase.

Relationship between BA and the mRNA abundance of BA-related Enzymes in scAT

Before parturition, the mRNA abundance of sterol 27-hydroxylase (*CYP27A1*) was negatively correlated to GDCA (r = -0.34, $P \leq 0.05$). Moreover, negative correlations were observed between the mRNA abundance of *CYP46A1* and GDCA at wk 7 a.p. (r = -0.34, $P \leq 0.05$), as well as TCDCA (r = -0.42, $P \leq 0.05$) and TDCA (r = -0.43, $P \leq 0.05$), both at wk 1 p.p.. At wk 1 p.p., the mRNA abundance of cholesterol 25-hydroxylase (*CH25H*) was positively associated with GCDCA (r = 0.37, $P \leq 0.05$), TCDCA (r = 0.59, $P \leq 0.01$), TDCA (r = 0.49, $P \leq 0.05$) and between the mRNA abundance of *CH25H* and GCDCA at wk 12 p.p. (r = 0.42, $P \leq 0.05$).

BA Transporters in scAT

The mRNA abundance of BA transporters in scAT is shown in Table 3. The mRNA abundance of the apical sodium-dependent BA transporter (*ASBT/ SLC10A2*) and the organic solute transporters (*OST-α/ SLC51A1*) were not detectable in scAT. Irrespective of grouping, the mRNA abundances of the Na+-taurocholate co-transporting polypeptide (*NTCP/ SLC10A1*) were higher a.p. when compared to wk 1, 3, and 12 p.p. (3.71-, 4.81- and 3.82-fold, respectively; all $P \leq 0.001$). An interaction between group and time was observed for the mRNA abundance of *NTCP*, with 2.52-fold higher mRNA abundance in HBCS compared to NBCS cows before calving ($P \leq 0.001$).

Table 3: mRNA abundance of transporters related to bile acid metabolism in scAT from cows with normal (NBCS) and high body condition score (HBCS) at wk 7 ante partum, as well as wk 1, 3 and 12 p.p.. Statistically significant P-values are given in bolt ($P \leq 0.05$).

		Weeks relative to parturition								***P*-value**		
		-7		**1**		**3**		**12**		**Group**	**Time**	**Group x Time**
		HBCS	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**			
NTCP	mean	0.136	0.054	0.016	0.033	0.011	0.034	0.018	0.034	0.297	**< 0.001**	**< 0.001**
(*Gene:*	SEM	0.019	0.015	0.004	0.007	0.001	0.021	0.003	0.012			
***SLC10A1*)**	N	18	17	11	15	13	8	12	10			
OST-β	mean	0.005	-	0.003	0.024	-	0.014	-	0.009	-	-	-
(*Gene:*	SEM	0.003	-		0.017	-		-	0.001			
***SLC51B*)**	N	3	-	1	3	-	1	-	3			
MRP2	mean	0.008	0.005	0.006	0.010	0.003	0.015	0.006	0.005	-	-	-
(*Gene:*	SEM	0.003	0.002	0.002	0.005	0.000	0.011	0.001	0.004			
***ABCC2*)**	N	8	10	6	6	6	5	5	2			
MDR1	mean	1.637	1.997	2.097	2.171	2.419	2.045	1.658	1.830	0.838	**0.001**	0.135
(*Gene:*	SEM	0.153	0.289	0.198	0.202	0.239	0.227	0.255	0.207			
***ABCB1*)**	N	19	17	19	19	17	16	16	11			
BSEP	mean	0.012	0.009	0.013	0.014	0.012	0.021	0.017	0.013	0.930	0.229	0.104
(*Gene:*	SEM	0.003	0.001	0.003	0.003	0.004	0.007	0.005	0.001			
***ABCB11*)**	N	9	14	6	12	10	7	5	4			
MDR3	mean	1.399	0.869	1.615	1.419	-	1.612	1.523	2.614	-	-	-
(*Gene:*	SEM	0.690	0.464	1.161	1.204	-	-	1.378	1.262			
***ABCB4*)**	N	3	3	3	3	-	1	3	2			

NTCP, SLC10A1: Na+-taurocholate cotransporting polypeptide; *OSTβ, SLC51B*: solute carrier family 51 subunit beta; *MRP2, ABCC2*: Multidrug Resistance-Associated Protein; *MDR1, ABCB1*: Multidrug Resistance Protein 1; *BSEP, ABCB11*: Bile Salt Export Pump; *MDR3, ABCB4*: Multiple Drug Resistance 3.

Relationship between BA and the mRNA abundance of BA-related Transporters in scAT

The mRNA abundance of *NTCP* and GDCA were negatively correlated at wk 7 a.p. (r = - 0.36; $P \leq 0.05$). Regarding wk 1 p.p., the mRNA abundance of the bile salt export pump (*BSEP*) was negatively correlated to all scAT BA except CA [GCA (r = - 0.62; $P \leq 0.05$), GCDCA (r = - 0.61; $P \leq 0.01$), GDCA (r = - 0.55; $P \leq 0.05$), TCA (r = - 0.80; $P \leq 0.01$), TCDCA (r = - 0.78; $P \leq 0.01$) and TDCA (r = -0.67; $P \leq 0.01$)]. Furthermore, the mRNA abundance of the multidrug resistance protein 1 (*MDR1*) was negatively correlated to the glycine-conjugated BA GCA (r = - 0.34; $P \leq 0.05$) and GCDCA (r = - 0.46; $P \leq 0.01$) at wk 1 p.p.. In addition, the mRNA abundance of *MDR1* was negatively correlated with CA (r = - 0.54; $P \leq 0.01$), GCA (r = - 0.52; $P \leq 0.01$), GDCA (r = - 0.55; $P \leq 0.001$) and TDCA (r = - 0.50; $P \leq 0.001$) at wk 3 p.p., whereas at wk 12 p.p., the mRNA abundance of *MDR1* was negatively correlated to CA (r = - 0.51; $P \leq 0.01$), GCA (r = - 0.67; $P \leq 0.001$), GCDCA (r = - 0.58; $P \leq 0.01$), GDCA (r = - 0.63; $P \leq 0.001$), TCA (r = - 0.52; $P \leq 0.01$), TCDCA (r = - 0.49; $P \leq 0.05$), TDCA (r = - 0.48; $P \leq 0.05$) in scAT.

BA Receptors in scAT

The mRNA abundance of BA receptors in scAT are shown in Table 4. The mRNA abundance of TGR5 and cholinergic receptor muscarinic 2 (*CHRM2*) were up to 3.70- and 4.13-fold higher ($P \leq 0.001$) at wk 3 p.p. compared to a.p.. Moreover, the mRNA abundance of the retinoid X receptor alpha (*RXRα; NR2B1*) was highest at wk 12 p.p. compared to all other time-points ($P \leq 0.001$). Regarding group differences, the mRNA abundance of *S1PR2* was 2.12-fold ($P = 0.04$) higher in NBCS cows than in HBCS cows.

Table 4: mRNA abundance of receptors related to bile acid metabolism in scAT from cows with normal (NBCS) and high body condition score (HBCS) at wk 7 ante partum, as well as wk 1, 3 and 12 p.p.. Statistically significant *P*-values are given in bolt ($P \leq 0.05$).

		Weeks relative to parturition								***P*-value**		
		-7		**1**		**3**		**12**		**Group**	**Time**	**Group x Time**
		HBCS	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**	**HBCS**	**NBCS**			
FXR	mean	0.001	0.006	-	0.004	0.0059	-	-	-	-	-	-
(*Gene:*	SEM	0.000	0.003	-	-	-	-	-	-			
***NR1H4*)**	N	3	3	-	1	1	-	-	-			
TGR5	mean	0.102	0.113	0.312	0.285	0.374	0.419	0.273	0.340	0.462	**< 0.001**	0.908
(*Gene:*	SEM	0.032	0.026	0.090	0.051	0.089	0.114	0.060	0.165			
***GPBAR1*)**	N	11	10	11	12	9	10	9	3			
S1PR2	mean	1.038	2.580	0.954	1.462	0.778	1.277	1.008	0.683	**0.039**	0.276	0.121
(*Gene:*	SEM	0.338	1.085	0.254	0.492	0.114	0.170	0.246	0.051			
***S1PR2*)**	N	6	5	7	7	5	10	5	4			
VDR	mean	1.636	0.931	2.372	1.977	0.818	0.995	1.074	1.572	-	-	-
(*Gene:*	SEM	0.459	0.256	1.449	0.455	0.176	0.995	0.251	1.249			
***VDR*)**	N	5	8	4	6	5	4	6	2			
CHRM2	mean	0.486	0.484	0.825	1.596	1.916	2.101	1.781	1.598	0.103	**< 0.001**	0.713
(*Gene:*	SEM	0.123	0.119	0.112	0.617	0.975	0.619	0.413	0.516			
***CHRM2*)**	N	16	13	16	15	15	14	16	11			
RXR α	mean	0.584	0.599	0.583	0.550	0.498	0.563	0.751	0.741	0.664	**< 0.001**	0.621
(*Gene:*	SEM	0.047	0.038	0.028	0.026	0.030	0.049	0.032	0.042			
***NR2B1*)**	N	19	17	19	19	17	16	16	11			
CAR	mean	0.007	0.003	0.005	0.006	0.002	0.017	-	-	-	-	-
(*Gene:*	SEM	0.003	0.001	0.002	0.001		0.011	-	-			
***NR1I3*)**	N	7	9	3	4	1	3	-	-			

FXR, NR1H4: Farnesoid X Receptor; *TGR5, GPBAR1*: Takeda G protein–coupled receptor 5; *VDR*: Vitamin D Receptor; *S1PR2*: Sphingosine-1-Phosphate Receptor 2; *CHRM2*: Cholinergic Receptor Muscarinic 2; *RXRα, NR2B1*: Retinoid X Receptor Alpha; *CAR:* Constitutive Androstane Receptor.

DISCUSSION

Synthesized from cholesterol, BA are known to affect metabolic processes such as lipid and glucose metabolism as well as general energy homeostasis (Shapiro et al., 2018). In the periparturient period, the metabolism of high-yielding dairy cows is challenged by calving and the onset of lactation. Over-conditioned cows, mobilizing more body reserves, are more susceptible to metabolic disorders compared to thinner cows (Bernabucci et al., 2005). In the present study, increased mobilization of AT in HBCS cows was indicated by higher NEFA concentrations as well as the loss of BFT and BCS in HBCS cows compared to NBCS cows after parturition (Schuh et al., 2019, see Supplemental Figures S1, S2, S5). Excessive lipolysis in over-conditioned dairy cows, knowingly affected plasma BA and activated secondary BA biosynthesis in the gut microbiome (Gu et al., 2023). In our study, cows with different body condition around calving had different serum and scAT BA profiles, with increasing serum BA concentrations at the onset of lactation. In the current study, CA and GCA were the dominant BA in serum and scAT, as reported in ruminants (Sheriha et al., 1968; Washizu et al., 1991; Reiter et al., 2021). Postprandial stimuli are known to affect BA synthesis in the liver, BA circulation in enterohepatic tissues, and serum (LaRusso et al., 1978; Hofmann, 1999). Herein we assumed, that higher p.p. BA concentrations in serum might be related to increasing DMI after parturition. However, the relationship between serum BA and DMI (an increase in DMI was previously reported by Schuh et al. (2019), see Supplemental Figure S3) was limited to a few BA at wk 1 p.p. (i.e., TCA, TCDCA, TMCA, GDCA, and TDCA; data not shown). In dairy cows, most serum levels of BA change during the dry period and lactation (Ghaffari et al., 2023). The increasing metabolic demand for milk synthesis associated with dietary changes resulted in increased BA synthesis to facilitate digestion and absorption of dietary lipids (Ghaffari et al., 2023). In humans, the body mass index (BMI) was positively correlated to BA concentrations in the fasting period (Prinz et al., 2015) and negatively correlated with postprandial BA concentrations (Brufau et al., 2010). Moreover, obesity suppressed the normal postprandial increase in circulating BA (Ahmad et al., 2013; Haeusler et al., 2016). Given that excessive lipolysis seven days after calving altered the gut microbiota in transition cows, leading to changes in the composition of secondary BA (Gu et al., 2023), it may suggest that the lower serum BA concentrations in HBCS cows compared to NBCS cows might be due to higher fecal BA excretion.

In the present study, we observed 7 BA in scAT using a targeted metabolomics approach via LC-ESI-MS /MS that allowed detection of 20 BA. Since both primary and secondary BA as well as their conjugates were present in scAT, we assume that BA can be taken up from circulation into scAT. The trend toward lower concentrations of BA in scAT before calving and increasing concentrations after the onset of lactation were consistent with higher circulating BA concentrations after parturition. However, the weak to moderate correlations between BA in serum and in scAT were not adequate to indicate clear bioactive mechanisms. In bovine estrus, very strong relationships between serum and follicular fluid CA (up to r = 0.97; $P \leq 0.001$) indicated predominant diffusion of circulating CA across the follicular membrane (Blaschka et al., 2019). The relationship was stronger for glycine-conjugated BA than for taurine-conjugated BA. The moderate correlation between CA in serum and scAT may indicate the ability of CA to cross cell membranes by passive diffusion, whereas transport of conjugated BA into cells depends on specific transporters (Hofmann, 1999). Since secondary BA are synthesized exclusively by the gut microbiome (Chiang, 2015), de novo synthesis in scAT seems unlikely. Furthermore, the tissue-specific conjugation patterns of BA as well as the specific expression of BA transporters suggest selective uptake of conjugated BA in peripheral tissues such as serum, kidney, and heart (Swann et al., 2011). The mRNA abundance of BA transporters, i.e., the mRNA abundance of *NTCP* and *BSEP*, being mainly responsible for the import and export of BA within the liver (Trauner and Boyer, 2003), were detectable in scAT from dairy cows in the present study. In mouse adipocyte cell culture, expression of *BSEP* mRNA and export of BA from cells to the circulation via BSEP appeared to be essential for preventing cytotoxic accumulation of BA within cells (Schmid et al., 2019). Whether this also applies for AT from dairy cows has not been investigated so far. However, the negative moderate correlations at wk 1 p.p. with all conjugated BA, led to the assumption, that BA might be eliminated from scAT through BSEP. As an adaptive regulation of BA entering into hepatocytes, *NTCP* gene expression is associated with the total hepatic BA concentration, thyroid and steroid hormones, cytokines, or injury in the liver (Geier et al., 2007; Alrefai and Gill, 2007; Dawson et al., 2009). Furthermore, BA indirectly regulate the expression of *NTCP* and *BSEP*, through the activation of signaling cascades via FXR, small heterodimer partner (SHP), and RXRα in humans and rodents (Anwer, 2004). As known in rats, FXR can bind BA, inducing the expression of *SHP*, and thus activating RXRα, which finally initiates NTCP (Jung et al., 2004). In the present study, the *FXR* mRNA abundance was below the limit of detection (LOD); however, the absence of mRNA does not definitely rule out FXR activity in general. Previous research, employing proteomic methods, has demonstrated substantial FXR pathway activity in AT in late-pregnant dairy cows (Zachut

et al., 2017). Since MDR1 does not exclusively transport BA (Klaassen and Aleksunes, 2010), the negative correlation between *MDR1* mRNA and BA after calving should be considered cautiously. MDR1 is responsible for the excretion of BA (Ayewoh and Swaan, 2022), organic cations, phospholipids (Anwer, 2004) and cholesterol from the liver into the bile (Honig et al., 2003). Therefore, transporters may depend on factors other than BA concentration in scAT.

The mRNA of key enzymes such as cholesterol 7α-hydroxylase (*CYP7A1*; Chiang and Ferrell, 2020) and aldo-keto reductase family 1 member D1 (*AKR1D1*; Chiang, 2004), being relevant for the *de novo* synthesis within the classical pathway, could only be detected in few samples below the LOD. However, oxysterol-7α -hydroxylase (*CYP7B1*), which is a marker enzyme of the alternative BA synthesis pathway (Chiang, 2017), was detected in scAT. Moreover, the weak to moderate negative correlations between the mRNA abundance of enzymes involved in the alternative pathway (*CYP27A1*, *CH25H*, and *CYP46A1*) and conjugated BA, point to a predominance of the alternative pathway. However, CDCA, the main BA of the alternative pathway, was detected below the LOD in scAT. Therefore, increasing mRNA abundance of *CYP7B1* throughout the experimental period, might rather control cellular oxysterol concentrations as recently reported in murine liver (Pandak and Kakiyama, 2019). Also, the cholesterol hydroxylase enzymes mRNA, *CH25H*, *CYP27A1*, and *CYP46A1*, generating oxysterols (Björkhem et al., 2002), have been detected in bovine scAT in this study. Oxysterols are precursors of BA (Russell, 2000), influencing lipid metabolism through activating the liver X receptor (LXR), which increases lipid synthesis by an induced expression of genes, such as sterol element binding protein-1c (*SREBP-1c*), FA synthase (*FAS*), stearoyl-CoA desaturase 1 (*SCD-1*), and acetyl-CoA carboxylase 1 (*ACC-1*) (Joseph et al., 2002; Talukdar and Hillgartner, 2006). In 3T3-L1 preadipocytes, oxysterol-forming enzymes (CYP27A1, CYP7B1) as well as oxysterols themselves, were synthesized (Li et al., 2014). Therefore, oxysterols might serve as an alternative way to metabolize cholesterol and thus protect adipocytes against cholesterol overload (Li et al., 2014). Catalysis of cholesterol to the oxysterol 25-hydroxycholesterol, CH25H, has been previously studied in obese humans, where weight reduction downregulated CH25H mRNA in the visceral AT (Dankel et al., 2010). In our study, HBCS cows that exhibited greater postpartum BCS loss than NBCS cows (Schuh et al., 2019) had lower mRNA abundance of *CH25H* than NBCS cows, suggesting a specific role for CH25H in lipid metabolism at least during periods of lipid mobilization. The higher mRNA abundance of CYP46A1 in scAT of HBCS cows 7 wk before calving may indicate higher cholesterol degradation as described in human embryonic kidney 293 cells (Mast et al., 2003). The consistent abundance of *CYP27A1* mRNA across all time-points irrespective of body condition, suggests that this enzyme is of

permanent importance in scAT. In addition to the formation of BA, CYP27A1 is also involved in the formation of oxysterol 27-hydroxycholesterol, an oxysterol, is formed de novo in adipocytes to protect against cholesterol overload (Li et al., 2014). Recently, the formation of oxysterols via the enzyme CYP27A1 was discussed in context with steroid biosynthesis in scAT of cows from the same study (Schuh et al., 2022).

Within the classical and alternative pathway of BA synthesis, HSD3B7 is involved in the production of CA and CDCA (Chiang, 2013; Li and Dawson, 2019); however, HSD3B7 serves as an important enzyme for the synthesis of oxysterols (Griffiths and Wang, 2019). Due to the lack of correlations between *HSD3B7* and BA, we assumed that HSD3B7 may be involved in oxysterol rather than BA synthesis in scAT. In addition, the higher mRNA abundance of *HSD3B7* in HBCS animals may point to the formation of oxysterols, which affects lipid metabolism (Russell, 2000). In addition, the present study detected the mRNA abundance of *BAAT* in scAT, the enzyme that conjugates BA in the liver (Falany et al., 1994). As postulated earlier, conjugation could protect adipocytes from cytotoxic BA overload (Monte et al., 2009). In the present study, mRNA from both transmembrane (i.e., *TGR5, CHRM2*, and *S1PR2*) and nuclear BA receptors (*RXRα*) were present in scAT. The TGR5 is activated by BA concentrations (LCA, TLCA, CA, DCA, and CDCA) in the nanomolar range (Prawitt and Staels, 2010). In this study, CA could serve as the major ligand for TGR5 in scAT. Since ligand activation in AT and liver induced lipolysis and energy expenditure in mice and humans (Chávez-Talavera et al., 2017; Velazquez-Villegas et al., 2018), the upregulation of mRNA abundance of *TGR5* with the onset of lactation could point to similar effects in dairy cows. Furthermore, although the mRNA abundance of *CHRM2* was detected in scAT, the concentrations of secondary BA DCA and LCA binding to CHRM2 (Evangelakos et al., 2021) in the present study were below the LOD. Moreover, albeit GCDCA, GDCA, and TCDCA are not considered as potential agonists for CHRM2, the positive correlation between *CHRM2* and these BA may suggest a role as ligand precursor molecules (Xie et al., 2021). The *S1PR2*, a ubiquitously expressed G protein-coupled receptor (Adada et al., 2013) that serves as a receptor for sphingosine-1-phosphate and conjugated BA in liver (Wan and Sheng, 2018), was detected herein in scAT. As a ligand for S1PR2, TCA could regulate glucose and lipid metabolism as suggested in rodent hepatocytes (Studer et al., 2012). The nuclear receptor *RXRα*, being present in scAT, forms a heterodimer with FXR in the liver, which is activated via BA and subsequently prevents BA synthesis via inhibiting CYP7A1 (Lu et al., 2000). BA are not direct ligands of the RXRα but bind to FXR (Jenkins and Hardie, 2008). Given that *FXR* mRNA was occasionally present in this study

(with values below the LOD), the importance of the heterodimer formation (FXR and RXRα) is questionable.

CONCLUSION

In conclusion, our study detects BA in serum and scAT of cows, as well as the mRNA abundance of BA -related enzymes, receptors, and transporters, suggesting a potential role of BA in lipid metabolism. Higher concentrations of BA in both serum and scAT, after parturition, may be associated with increasing DMI. Increasing lipid mobilization in over-conditioned cows after parturition was accompanied by lower circulating BA concentrations. Conjugated BA may be actively transported from the circulation to the scAT via NTCP and exported via BSEP as well as metabolized by BA -related enzymes. Finally, the presence of specific BA receptors in scAT supports the potential role of BA in lipid metabolism during the periparturient period of dairy cows.

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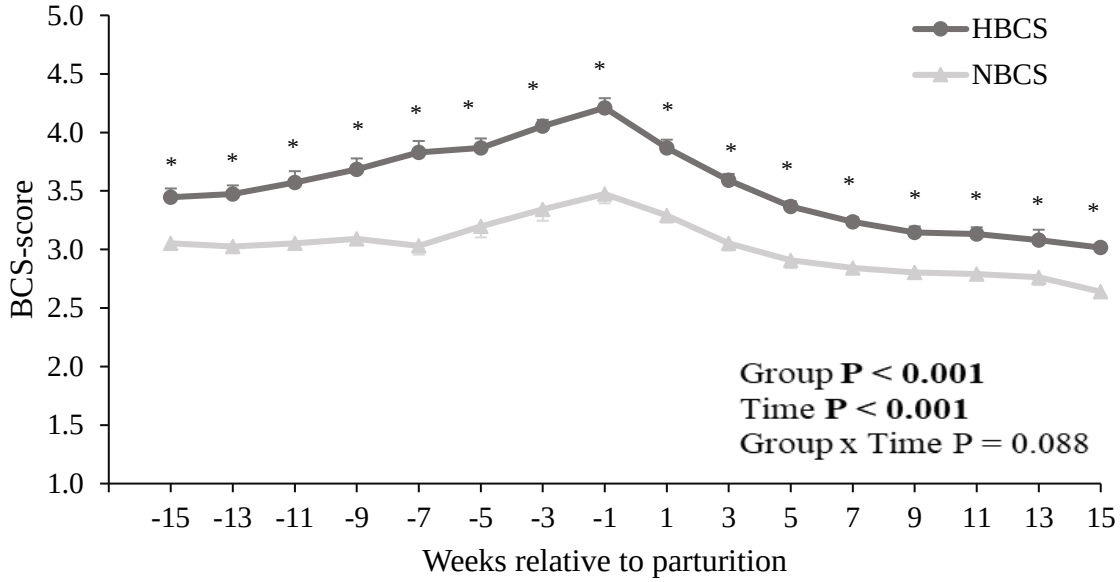
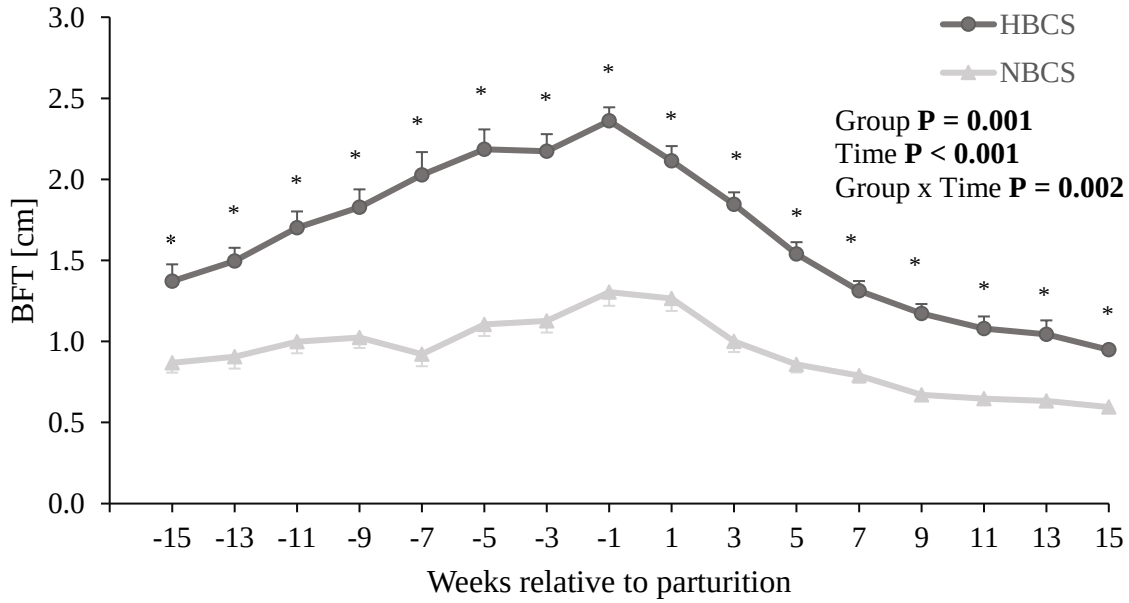
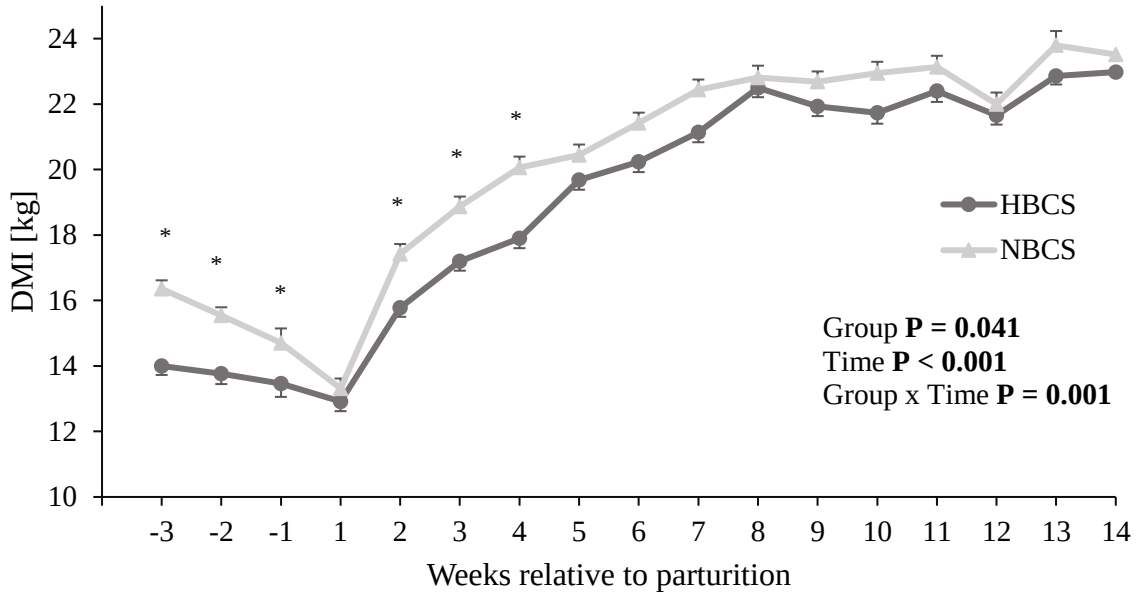
SUPPLEMENTAL FIGURES



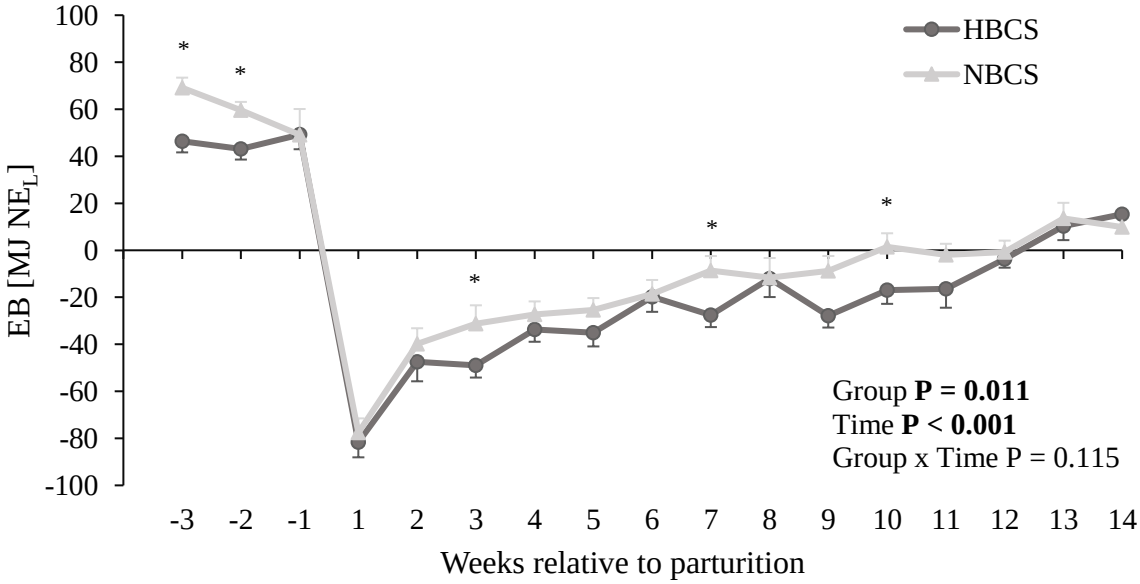
Supplemental Figure S1: Changes of body condition score (BCS) from 15 wk antepartum (a.p.) to 15 wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data were already published by Schuh et al. (2019).



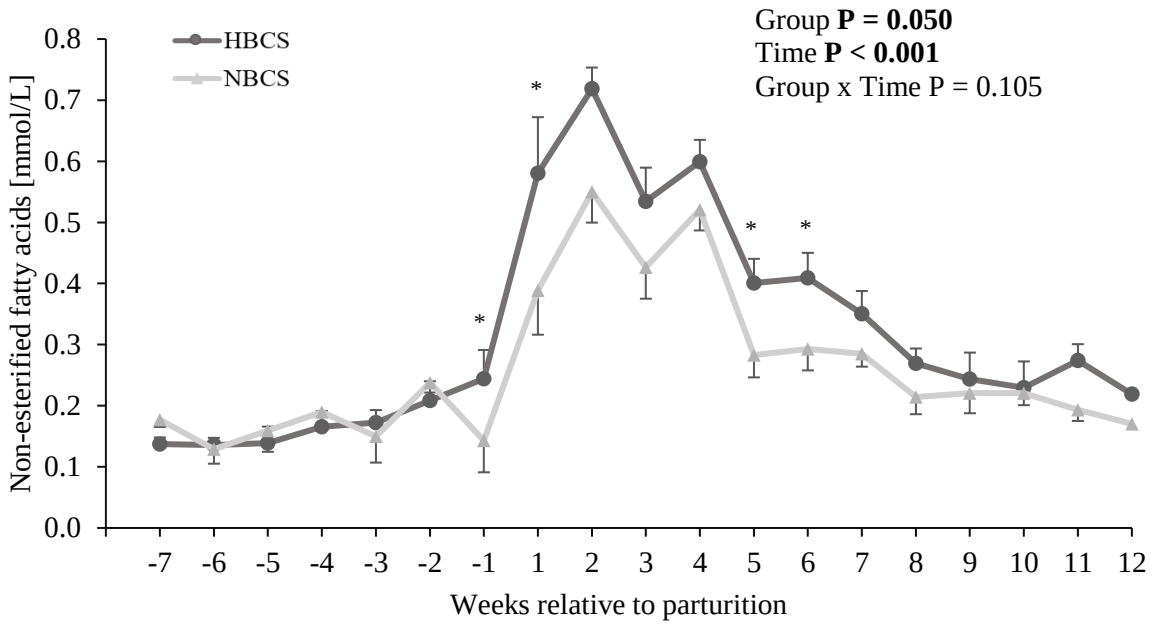
Supplemental Figure S2: Changes of back fat thickness (BFT) from 15 wk antepartum (a.p.) to 15 wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data were already published by Schuh et al. (2019).



Supplemental Figure S3: Dry matter intake (DMI) from 3 wk antepartum (a.p.) to 14 wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data were already published by Schuh et al. (2019).



Supplemental Figure S4: Energy balance (EB) from 3 wk antepartum (a.p.) to 14 wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data were already published by Schuh et al. (2019).



Supplemental Figure S5: Non-esterified fatty acids (NEFA) from 7 wk antepartum (a.p.) to 12wk postpartum (p.p.) in high BCS (HBSC) and normal BCS (NBCS) cows. Results are given as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$. Data were already published by Schuh et al. (2019).

5 General Discussion and Perspectives

This thesis aimed to investigate the BA metabolism in dairy cows with HBCS compared to those with NBCS in the period around calving. We hypothesized that cows with different body condition before calving would differ in their BA metabolism. To test this hypothesis, we compared the BA concentrations in serum and liver between HBCS and NBCS cows. Furthermore, we analyzed the mRNA abundance of BA synthesizing enzymes, transporters, and receptors in the liver of HBCS and NBCS cows to determine potential differences at the transcriptional level. In scAT, we hypothesized that BA as well as the mRNA of BA receptors, transporters, and enzymes are present and differ between cows with different body condition around calving.

5.1 Bile Acids in Liver and Serum of Dairy Cows

The BA contribute to optimal feed digestion by supporting the emulsification of dietary fats and facilitating nutrient digestion and absorption in the intestine (Chiang, 2013). Under the influence of postprandial stimuli, BA are released into the intestine and pass through the enterohepatic circulation several times a day (Hofmann, 1999).

The bovine liver contains mainly conjugated BA with a predominance for glycine conjugation and the main BA being GCA (Reiter et al., 2021). Our results go in line with GCA being predominant, accounting for 53%. In total, we were able to quantify 14 BA within the liver with a total of 81% being primary or primary conjugated BA. Secondary BA were present at 19% with a predominance for GDCA. In contrast, intestinal formed DCA returns to the liver being rehydroxylated to its precursor CA in rodents (Yamashita et al., 1989); in humans and cattle, hepatic biotransformation of secondary BA is limited to the conjugation (Hofmann, 1984; Hofmann et al., 2018), which may explain the high concentrations of TDCA and GDCA within this study. The BA are synthesized *de novo* to the amount of BA excreted via feces (Dawson et al., 2010), and are represented by the small amounts of the primary BA CA (1%) and CDCA, the latter being below the limit of detection in liver in this study. After the synthesis, BA undergo enterohepatic circulation, passing the gallbladder, intestinal epithelium, intestinal veins and then reach the liver via the portal vein (Hofmann, 1999). Small amounts spill over into the systemic circulation (Chiang, 2013).

Being the organ of the BA synthesis (Hofmann, 1999), the liver had higher concentrations (pmol/g tissue) of total BA, compared to serum (pmol/mL) in dairy cows within this study. By comparing 1 mL of serum and 1g of liver tissue, serum contained 4.3-fold higher amounts of CA, which could be due to intestinal modifications like deconjugation of GCA through BSH (Chiang, 2004; Di Ciaula et al., 2017). Furthermore, the circulating BA pool in bovines mainly

contains CA and GCA to equal amounts (Washizu et al., 1991), as we also could demonstrate within this study. Differences in composition are probably due to modifications in the intestine and absorption via active transport or passive diffusion at the intestinal epithelial cells (Poland and Flynn, 2021). Also, the capacity of the liver to re-absorb BA and to pass them via bile into entero-hepatic circulation, rather than forwarding them into the systemic circulation, impacts the serum BA concentrations (LaRusso et al., 1978). The origin of the BA present in the systemic circulation is related to the composition of the intestinal microbiome, as well as to the extraction rate of the liver and the re-absorption of the BA into the enterohepatic circulation (Lin et al., 2023; LaRusso et al., 1978). Therefore, perspectives for further investigation might be extending the study to the individual processes of BA metabolism in dairy cows, including BA concentrations in the intestine, the excretion via the feces, and also the BA transported in the portal vein.

5.2 Bile Acids in subcutaneous Adipose Tissue

The BA that enter the circulation can accumulate in tissues outside the enterohepatic circulation, and have already been detected in bovine follicular fluid, urine (Sanchez et al., 2014; Blaschka et al., 2019), udder, muscle, heart, bone, tongue, esophagus, omasum, abomasum, lung, spleen, and kidney (Reiter et al., 2021). To the best of our knowledge, BA have not been investigated in bovine scAT. Being a metabolically active and also an endocrine organ, AT produces cytokines and hormones secreting them into the circulation (Bélanger et al., 2002; Kershaw and Flier, 2004). It is also capable of storing lipophilic steroid hormones (Schuh et al., 2022), which have a structural similarity to BA (Amaral et al., 2009). Steroid hormones as well as steroidogenic enzymes have already been detected in bovine scAT within the animals used for this study (Schuh et al., 2022). Sharing the same precursor molecule, i.e., cholesterol, their structural similarity enables them to undergo similar biochemical processes and utilize common enzymes and metabolic pathways, in particular concerning cholesterol synthesis and conversion (Li et al., 2014). With this background, we aimed to investigate BA in scAT within this study to elucidate their abundance and potential mechanisms.

In 2014, 17 BA were quantified for the first time in human AT via ultra-high performance liquid chromatography tandem mass spectrometry (**UHPLC-MS/MS**) with a Kit targeting 61 BA. Comparatively, we were able to quantify 7 BA in bovine scAT, including primary and secondary BA by using the Biocrates BA kit, by which 20 BA can be detected by liquid chromatography electrospray ionization tandem mass spectrometry (**LC ESI MS/MS**). The lower number of BA in our study as compared to the results of Jäntti et al. (2014) may be

explained by the lesser coverage of the method we used. Besides BA in AT, Jäntti et al. also measured BA concentrations in blood. The authors attempted to explain BA concentrations in AT by assuming 1% blood volume being present in AT. Showing that the amount of explainable BA concentrations varied between 0 and 80% (depending on the different BA), led to the assumption that BA are released from AT into the circulation (Jäntti et al., 2014).

We aimed to investigate the absolute BA concentrations and the ratio between serum and AT by calculating the BA in total body fat (**TBF**) and in the total blood volume (**TBV**). As HBCS and NBCS differed in body fat and blood volume we calculated values separately. Based on BFT equivalents [BFT, HBCS: 2.04 cm; NBCS: 0.95 cm], with the conversion factor indicating that 10 mm BFT is equivalent to 50 kg of body fat (Schröder and Staufenbiel, 2006) the mean TBF was determined to be 102 kg and 48 kg for HBCS and NBCS cows, respectively, at 7 weeks a.p.. Calculations were done without considering the heterogeneous nature of different AT depots. Additionally, TBV was considered, amounting to 44 L and 42 L for HBCS and NBCS cows, respectively, relying on data from Turner and Herman (1931), who specified 61 mL/kg BW for mature non-lactating cows [BW, HBCS: 727 kg; NBCS: 693 kg]. Assuming that one liter of blood is equal to one kilogram, we adopted the following methodology:

BA in TBF (pmol) = BA in scAT [pmol/g tissue] * 1.000 * TBF (kg)
BA in TBV (µmol) = BA in serum [µmol/mL] * 1.000 * TBV (L)

After converting the BA concentration in serum from µmol to pmol, BA concentrations in TBF were about 1.2 to 6.5-fold higher compared to BA in TBV in both groups. These results may indicate local uptake and accumulation of BA in bovine scAT, as BA diffusion from blood into a peripheral tissue would assume BA concentrations to be nearly identical (Blaschka et al., 2019). Also, the detection of secondary BA, which are exclusively synthesized by the gut microbiome (Chiang, 2015), points to an accumulation in scAT, rather than generation of BA from scAT. All BA which we detected in scAT, were also detected in serum, however, weak to moderate correlation coefficients between BA in scAT and in serum could not entirely clarify the underlying bioactive mechanisms. The moderate correlation between CA in serum and scAT may indicate the ability of CA to cross cell membranes by passive diffusion, whereas conjugated BA could be actively transported into cells (Hofmann, 1999) and accumulate. However, these calculations are very speculative as this assumption is based on equalizing all fat depots within the cow. The comparison of scAT in different body locations in dairy cows (sternum, tail-head, withers) revealed that the location contributed to its metabolic activeness (Singh et

al., 2014), which could also be indicative for the lack of clear bioactive mechanisms between scAT and serum BA within this study. With respect to the anatomical location of the AT depots, visceral AT in dairy cows may be metabolically more active (Klein et al., 2004; Kelley 2004), which could respond to physiological changes caused by lactation-induced and condition-dependent lipolysis differently.

To further evaluate the origin of the BA located in scAT, we investigated the mRNA abundance of various BA transporters. By detecting the mRNA expression of the main BA transporters *BSEP* and *NTCP*, being responsible for the export and import of BA (Trauner and Boyer, 2003), we were able to show the potential for transport of conjugated BA in scAT. In human and murine adipocyte cell cultures, *BSEP* mRNA was detected earlier, already indicating that adipocytes may contain active transport mechanisms for BA (Schmid et al., 2019). By analyzing the mRNA abundance of BA synthesizing enzymes, we further evaluated the possibility of *de novo* BA synthesis in scAT. In the present study, we could detect the mRNA abundance of key enzymes involved in the classical BA synthesis pathway only in a few samples, therefore, we assume that the *de novo* synthesis of BA is lacking or of minor importance in scAT. Nevertheless, the mRNA abundance of enzymes being involved in the alternative pathway, point to a predominant role of the alternative pathway in scAT. Enzymes of the alternative pathway like the cholesterol hydroxylase enzymes, CH25H, CYP27A1, and CYP46A1, are also involved in synthesis of oxysterols (Björkhem, 2002), being precursor molecules of BA (Russell, 2000). Oxysterols and oxysterol-forming enzymes have previously been detected in 3T3-L1 preadipocytes, pointing to an alternative way to metabolize cholesterol in adipocytes (Li et al., 2014). Therefore, this metabolic pathway could also be pronounced in bovine scAT rather than the *de novo* synthesis of BA. Furthermore, the detection of secondary BA, which are exclusively formed by the gut microbiota (Chiang, 2015), *de novo* synthesis in AT seems unlikely. Subsequently, investigations concerning cellular cholesterol as well as oxysterol concentrations in scAT might be beneficial to evaluate clear mechanisms of cholesterol degradation. Since conjugated BA are unable to diffuse through cell membranes, their presence in scAT may also point to a local conjugation of BA with glycine and taurine in AT (Jäntti et al., 2014). Our work supports this assumption, as we were able to detect the mRNA of the enzyme BAAT, being responsible for the conjugation of BA. However, enzymatic activity is not only determined by transcriptional regulation and does not necessarily correspond to mRNA levels (Rodríguez-Antona et al., 2001).

In human medical research, BA are increasingly recognized as hormone-like signaling molecules (Ferrebee and Dawson, 2015; Shapiro et al., 2018), and BA receptors being present

in human adipocytes provided evidence for functional signaling pathways that might represent a hormonal network, being able to regulate adipocyte physiology including lipolysis (Schmid et al., 2019). By detecting the mRNA of both, transmembrane and nuclear BA receptors in bovine scAT, BA could probably serve as signaling molecules in cattle. The presence of BA and the mRNA of related enzymes, transporters, and receptors in scAT opens up new perspectives for the understanding of AT physiology and interactions with bioactive molecules such as BA. Potential avenues for future research include the detection of transport proteins and enzyme activity. In summary, the presence of BA and of the mRNA for BA-forming enzymes, transporters, and receptors provides new insights into these molecules and their involvement in whole-body physiology.

5.3 Bile Acids and Body Condition

Body condition in transition dairy cows is knowingly impacting metabolic processes, including liver health but research on the BA metabolism in dairy cows is scarce. Excessive body fat mobilization after parturition due to over-conditioning before calving, reinforces triglyceride accumulation in the liver, which in turn interferes with the normal hepatic function (Heuer et al., 2001). We hypothesized that differences in body fat mobilization would affect BA metabolism, as the liver is the main organ for BA synthesis (Hofmann, 2009).

Cows experiencing excessive lipolysis tended to excrete higher levels of total secondary BA compared to cows with normal lactation-induced lipolysis, suggesting that the lipolysis status affects the composition and function of fecal microbiota, which in turn affects the synthesis of secondary BA (Gu et al., 2023). By demonstrating different hepatic BA concentrations, and the expression of BA associated enzymes and transporters in HBCS and NBCS in this study, lipolysis could already affect the BA metabolism in the liver, affecting the substrate for the microbiome in the gut.

Within this study, NBCS cows had higher BA concentrations of several BA in liver, serum, and scAT than HBCS cows, indicating that different extents of body fat mobilization affect the BA concentrations. The liver function of transition cows underlies dramatic changes and challenges which are even more pronounced with excessive lipolysis (Roche et al., 2009). The liver is the limiting step for the enterohepatic circulation of BA (Fuchs, 2003; Kullak-Ublick et al., 2004) and impairments of liver function due to liver diseases lead to a dysregulation of the BA metabolism (Zhang and Deng, 2019). Liver disorders such as NAFLD in humans and rodents altered the BA pool size and the BA pool composition (Zhang and Deng, 2019). In

NASH patients, hepatic CA and GDCA concentrations decreased by 69% and 91%, respectively, whereas TCA, TDCA, and GCDCA increased compared to healthy control subjects (Lake et al., 2013). Lower concentrations of GCA, DCA, and a trend for CA could be due to an impaired liver function of the HBCS cows, as indicated by greater ketogenesis (Schuh et al., 2019). Changes in liver BA could indicate that HBCS cows have impaired liver function and that adaptation to metabolic changes cannot be regulated as in the NBCS animals. Furthermore, HBCS had a lower DMI, compared to the NBCS animals (Schuh et al., 2019), which could cause lower BA concentrations in HBCS as well.

Transcription factors, which respond to environmental, autocrine, or paracrine signals, are the primary regulators of hepatic gene expression (Costa et al., 2003). As there is evidence that the alternative BA synthesis pathway is elevated during liver diseases in humans (Crosignani et al., 2011; Lake et al., 2013), we investigated the mRNA abundance of BA synthesizing enzymes within the liver of HBCS and NBCS cows. Within mitochondria, CYP27A1 catalyzes the hydroxylation of cholesterol, being the first step of the alternative pathway of BA synthesis (Crosignani et al., 2011). The hepatic mRNA abundance of CYP27A1 tended to be higher in HBCS cows, which may indicate a pronounced use of the alternative pathway. Also, the mRNA abundance of *CYP7B1*, the rate-limiting enzyme of the alternative pathway (Chiang, 2017) was upregulated in HBCS compared to NBCS cows, which may further indicate a predominance of this pathway.

The concomitant analysis of the mRNA abundance of the BA transporters showed that HBCS cows had a higher mRNA expression of hepatic *OSTβ*, and a trend for higher values of *BSEP*-mRNA, both being responsible for hepatic BA export (Trauner and Boyer, 2003, Kullak-Ublick et al., 2004; Ferrebee and Dawson, 2015), indicating a higher BA excretion into the bile canaliculi and systemic circulation (Arab et al., 2017). Even though there is evidence for reduced re-uptake of BA in cows with fatty liver (Mohamed et al., 2004), the *NTCP* mRNA abundance was not affected by treatment. However, the capacity of the liver to re-absorb BA is also reflected in the amount of BA within the circulation (Mohamed et al., 2002). Serum BA correlated with the degree of clinical illness in hepatic lipidosis in cattle and may represent an indicator for hepatic diseases (West, 1991). Within our study, serum BA concentrations of CA, CDCA, GCA, DCA, GCDCA, TCA, LCA, and GDCA were higher in NBCS cows compared to HBCS cows, which does not go in line with the previous hypothesis. Since we did not measure the degree of fatty degeneration of the liver within our study, we can only rely on the higher BHBA concentrations in the serum of the HBCS animals, indicating a higher degree of fatty infiltration and ketogenesis compared to NBCS.

A recent study compared cows with excessive and normal lactation-induced lipolysis, presenting results which are in line with our study. Cows with excessive lipolysis had higher serum concentrations of GLCA, TLCA, and, as a trend, of GCDCA in cows with normal lipolysis (Gu et al., 2023). The authors showed that the lipolysis status of dairy cows is linked to the microbial composition of the gut, being related to the BA composition in serum (Gu et al., 2023). Thus, lower BA concentrations in HBCS cows could also be due to a higher fecal BA excretion in cows with higher body fat mobilization as reported by Gu et al. (2023). In scAT, GCA, GCDCA, GDCA, TDCA, and TCDCA concentrations were higher in NBCS, compared to HBCS cows. Higher concentrations of glycine conjugates (GCA, GCDCA, GDCA) could be due to their higher concentrations in serum of NBCS compared to HBCS animals. However, calculating correlations between serum and scAT BA did not indicate a direct BA diffusion from serum to scAT, as correlations were weak to moderate.

In summary, the HBCS animals had lower concentrations of numerous BA, including the main bovine BA CA, GCA, and DCA in both, the tissues and serum, indicating that body condition around parturition affects the BA metabolism of high yielding dairy cows. Previous studies showed that body condition in the period around calving has an impact on related metabolic processes such as steroid hormone metabolism (Schuh et al., 2022). Metabolic changes associated with excessive lipolysis and the increased accumulation of triglycerides in the liver alter liver function and the metabolic processes located in the liver (Wankhade et al., 2017). The synthesis of primary BA as well as the hepatic excretion of BA could be altered in HBCS animals which in turn affects the BA concentrations in the liver as well as those within the circulation (Mohamed et al., 2004). In addition, according to Gu et al., the intestinal microbiome appears to change during excessive lipolysis, which in turn influences the synthesis of secondary BA, the excretion of BA, and serum BA (Gu et al., 2023). Assuming that the BA metabolism in NBCS cows is less affected by other metabolic challenges compared to HBCS cows, the higher BA concentrations in NBCS cows support the notion of superior adaptability of these cows to the conditions of pregnancy and lactation.

5.4 Bile Acids in the Period around Calving

The time around calving is a challenging period for dairy cows, as the metabolism needs to adapt to the conditions of late pregnancy and then the onset of lactation (Drackley, 1999). Transition from pregnancy to lactation physiologically affects multiple organ and tissue systems including the liver and AT (Wankhade et al., 2017). The BA synthesis, mainly taking

place within the liver, is also affected by the metabolic changes occurring in this phase in dairy cows (Ghaffari et al., 2023).

Within the liver, CA and GCA, the predominant bovine BA (Reiter et al., 2021), had higher concentrations after calving than before calving, indicating an increase in BA synthesis at the onset of lactation (Zhu et al., 2013). Increasing feed intake (DMI was reported by Schuh et al., 2019) after parturition could induce a higher production of BA. Elevated BA concentrations and mRNA abundance of BA forming enzymes have already been reported in the liver of lactating rats and suggest an effect of the increased energy demand and food intake (Athippozhy et al., 2011; Zhu et al., 2013). Increasing CA concentrations within the liver could also indicate a higher *de novo* synthesis of BA, induced by lactation (Klassen and Strom, 1978; Kilpatrick et al., 1980). In rats, the mRNA abundance of *CYP8B1*, *CYP27A1*, *CYP7B1*, *NTCP*, and *BSEP* was higher during lactation compared to gestation, indicating stimulated BA formation and uptake into the circulation (Zhu et al., 2013). In addition, we also observed a higher hepatic mRNA abundance of *CYP7A1* after parturition. Improvements of lipid and nutrient absorption in the intestine mediated by increased BA concentrations could improve the energy intake from feed in times of the prevailing NEB (Wooton-Kee et al., 2010). Furthermore, in lactating rats, prolactin may reinforce hepatic CYP7A1 and HMG-CoA reductase, being the rate-limiting enzymes for the BA and cholesterol synthesis (Bolt et al., 1984). We further observed a higher mRNA abundance of hepatic *NTCP* after parturition, going along with higher hepatic BA concentrations. NTCP is mainly responsible for the re-uptake of BA from the portal blood into the liver (Trauner and Boyer, 2003). Within this study, serum and liver BA concentrations were elevated after parturition, assuming a greater circulating BA pool size and a higher reabsorption through NTCP in line with the higher hepatic expression of *NTCP*.

Higher serum concentrations of CA, CDCA, TCA, GCA, GCDCA, DCA, and MCA(b) after parturition, compared to the pre-calving values, could be due to the higher hepatic BA synthesis related to milk production and an increased DMI after calving as reported by Schuh et al. (2019). In scAT, the concentrations of CA, GDCA, GCDCA, and GCA increased, a finding that is consistent with increasing hepatic and circulating BA levels after parturition. However, comparisons between BA concentration in tissues versus serum should be made with caution. Comparisons of different biological matrices must be done with caution due to their different biological and biochemical properties, in particular tissue samples may have different distributions and regulations of metabolite concentrations that may influence results. Furthermore, underlying the control of the enterohepatic circulation, BA metabolism is subject to multifactorial influences, such as diurnal and individual variations (Abdelkader and Ropstad,

1989), the diet (Liu et al., 2020), and health status (Kalaitzakis et al., 2007; Gu et al., 2023; West, 1990) in dairy cows.

The results of the studies included in this doctoral thesis provide evidence that the BA metabolism changes with the onset of lactation and that body condition before calving affects BA the metabolism in liver and serum, as well as in scAT of dairy cows. A holistic approach and the inclusion of all organs and fluids involved in the enterohepatic circulation would provide an even better overview for future studies. Additionally, it is not yet clear from this study how the alterations in BA metabolism may affect the overall metabolism of dairy cows and, potentially, the ability of the cow to absorb lipids. Furthermore, we showed that BA are present in scAT from the tailhead region, but no clear relationships with serum BA have been identified. Given that BA act as signaling molecules, further research may help to elucidate potential relationships to energy and lipid metabolism in the periparturient period of dairy cows.

6 Summary

The periparturient period is characterized by significant metabolic, physiological, and hormonal changes in dairy cows. Early lactation milk production increases, while feed intake does not increase proportionally, resulting in a negative energy balance (NEB). Energy reserves are mobilized primarily from adipose tissue to meet energy demands and maintain milk synthesis. Adipose tissue (AT) triglycerides are hydrolyzed to glycerol and free fatty acids, the latter are either oxidized or re-esterified in the liver. Excessive AT mobilization can exceed the oxidative capacity and triglyceride export from the liver, promoting both ketogenesis and triglyceride accumulation. Over-conditioned cows are more susceptible to excessive body fat mobilization, increasing their risk for metabolic diseases such as ketosis and fatty liver. Triglyceride accumulation in the liver can impact numerous metabolic processes, including bile acid (BA) synthesis. The liver synthesizes BA from cholesterol through both the classical and alternative pathways. After synthesis, BA are conjugated with either glycine or taurine, and stored in the gallbladder as conjugated BA. Postprandially, BA are released into the intestine, where they aid in the emulsification of dietary fats and fat-soluble vitamins. The intestinal microbiota deconjugate BA, converting them to both secondary and secondary conjugated BA. The majority of BA are absorbed by intestinal epithelial cells and are returned to the liver via the portal vein. Maintaining BA homeostasis, the BA pool circulates several times a day, with only a small fraction being excreted in the feces and a small fraction not being re-absorbed by the liver, entering the circulation. BA have been detected in body fluids and tissues outside the enterohepatic circulation, although their origin and function in these tissues are controversially discussed and require further research. Furthermore, BA act as signaling molecules by binding to receptors, influencing various metabolic processes, including glucose, lipid, and energy metabolism. The presence of BA receptors in human adipocytes suggests that BA might have functional signaling pathways in adipocytes being able to impact metabolic processes such as lipolysis. Therefore, the aim of this study was to investigate the impact of the body condition and the extend of body fat mobilization on BA metabolism in dairy cows.

The animal experiment included 38 multiparous German Holstein cows, which were divided into either a high-body condition score (HBCS; N=19) or a normal-body condition score group (NBCS; N=19) 15 weeks before the calculated calving date. In order to achieve the target values for body condition score (BCS) and backfat thickness (BFT) at dry off (NBCS: BCS < 3.5, BFT < 1.2 cm; HBCS: BCS > 3.75, BFT > 1.4 cm), the groups received different diets (HBCS: 7.2 NEL MJ/kg dry matter (DM)); NBCS: 6.8 NEL MJ/kg DM) from 15 to 7

weeks before calving. Both in the dry period (6.8 MJ NEL/kg DM) and in the following lactation (7.2 MJ NEL/kg DM), the animal groups received identical diets for ad libitum intake. Liver and subcutaneous AT (scAT) biopsies as well as serum samples were taken 7 weeks ante partum (a.p.), 1-, 3-, and 12-weeks postpartum (p.p.) and were analyzed for 20 BA using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in a targeted metabolomics kit format. The mRNA abundance of the target genes in the liver and scAT was analyzed by an automated, high-performance polymerase chain reaction (PCR)/quantitative PCR (qPCR) system that uses microfluidics technology to process samples at nanoliter-scale volumes.

In total, 14 BA could be quantified in the liver, the majority being primary or primary conjugated with a predominance for glycocholic acid (GCA). In serum, 15 BA were detected with a predominance for GCA and cholic acid (CA) in almost equal concentrations. Liver and serum BA differed in their composition, which is probably related to intestinal microbial biotransformation, fecal BA loss or reabsorption conditions at the liver.

Tissues and body fluids outside the enterohepatic circulation could contain BA passed from the liver vein into the systemic circulation. However, there is evidence that BA may be synthesized *de novo* in extrahepatic tissues. Also, the detection of BA specific receptors in extra-enterohepatic tissues pointed to a role of BA acting as signaling molecules besides the liver and gut. Focusing on scAT, a metabolically active endocrine organ, that is highly involved in energy metabolism during the transition from pregnancy to lactation, we were able to quantify 7 BA. The BA were primary and secondary, as well as conjugated. The mRNA of key enzymes for BA synthesis was not detectable in scAT, therefore a *de novo* synthesis of BA in scAT seemed unlikely. However, the expression of BA synthesizing enzymes of the alternative pathway may indicate the formation of oxysterols for the degradation of cholesterol. The detection of the mRNA of specific BA transporters in scAT suggests that BA are actively transported into scAT. Along with the presence of mRNA of specific BA receptors, we hypothesized that BA might act as signaling molecules in scAT.

Considering the effect of body condition on the BA concentrations, we showed that animals with increased body condition before calving, had lower concentrations of BA than animals with normal body condition. The liver, being highly involved in energy and lipid metabolism, is the key organ for BA synthesis. Lower BA concentrations in HBCS compared to NBCS cows could be due to an impaired liver metabolism in the HBCS. In the liver, the mRNA of key enzymes of the alternative synthesis pathway were upregulated in the HBCS animals, being consistent with the results from human research, indicating impairment of liver function.

Lower BA concentrations were also detected in serum and scAT in the HBCS animals compared to the NBCS animals. This might be interpreted as a reduced circulating BA pool, which in turn could affect the circulating BA. Furthermore, the intestinal microbiome might have been altered in the HBCS cows which in turn would affect BA metabolism and excretion via the feces, as already shown in a comparable study. With the onset of lactation, the concentrations of several BA in the liver, serum and scAT increased. Along with the increase in the concentration of BA, an increase in the mRNA abundance of the key enzyme *CYP7A1* in the liver was observed. We assume that the BA synthesis is upregulated during lactation in response to the increasing energy requirements for milk synthesis.

Our results clearly showed that lactation-induced and condition-dependent lipolysis has an effect on the BA metabolism in dairy cows. However, in order to characterize the pathophysiological effects of prepartum over-conditioning on BA metabolism, research should focus on all compartments of the enterohepatic circulation. The BA metabolism is subjected to complex interactions between the intestinal microbiome, receptor-controlled or concentration-based altered synthesis, which in turn can be influenced by a variety of factors. The results presented within this doctoral thesis help to further characterize the role of BA in peripheral tissues and provide important insights into the involvement of BA outside the enterohepatic circulation.

7 Zusammenfassung

Die Zeit rund um die Kalbung der Milchkuh ist geprägt durch enorme metabolische, physiologische und hormonelle Veränderungen. In der Frühlaktation kann die schnell ansteigende Milchleistung mit einer nicht äquivalent ansteigenden Futteraufnahme zu einer negative Energiebilanz (NEB) führen. Zur Deckung des Energiebedarfes und zur Aufrechterhaltung der Milchsynthese werden Energiereserven hauptsächlich aus dem Fettgewebe mobilisiert. Triglyceride im Fettgewebe werden zu Glycerol und freien Fettsäuren gespalten; letztere erreichen über die Zirkulation die Leber wo sie entweder oxidiert oder re-verestert werden. Übermäßige Fettgewebsmobilisation führt dazu, dass die Oxidationskapazität und der Triglycerid-export aus der Leber überschritten wird und sowohl die Ketogenese als auch die Akkumulation von Triglyceriden gefördert wird. Überkonditionierte Tiere vor der Kalbung mobilisieren mehr Körperfettreserven und sind folglich einem erhöhten Risiko für Stoffwechselerkrankungen, wie Ketose und Fettleber ausgesetzt. Die Belastung der Leber durch Triglyceridakkumulation beeinflusst eine Vielzahl metabolischer Prozesse. Studien zufolge wirken sich Stoffwechsel- und Lebererkrankungen wie das nicht-alkoholische Fettlebersyndrom (NAFLD) sowohl auf die *de novo* Synthese, den Syntheseweg als auch auf die Resorption der Gallensäuren im Darm und an der Leber aus.

Primäre Gallensäuren werden in der Leber aus Cholesterin sowohl über den klassischen als auch alternativen Syntheseweg gebildet. Die Gallensäuren werden nach der Synthese mit den Aminosäuren Glycin oder Taurin konjugiert und als konjugierte Gallensäuren in der Gallenblase gespeichert. Durch enterohormonelle Reize werden Gallensäuren postprandial in den Darm abgegeben, wo sie die Emulgierung von Nahrungsfetten und fettlöslichen Vitaminen unterstützen. Im Darm werden die Gallensäuren durch das dortige Mikrobiom de-konjugiert, und sowohl zu sekundären als auch zu sekundär konjugierten Gallensäuren umgewandelt. Ein Großteil wird anschließend über die Darmschleimhaut resorbiert und über die Pfortader zurück in die Leber geführt. Der Gallensäurepool unterliegt einer Homöostase und zirkuliert zwischen diesen Kompartimenten mehrmals täglich, wobei nur geringe Anteile über den Kot ausgeschieden werden und ein kleiner Teil von der Leber nicht resorbiert wird und in den systemischen Kreislauf gelangt. So konnten Gallensäuren auch in Körperflüssigkeiten und Geweben außerhalb des enterohepatischen Kreislaufes nachgewiesen werden, wobei ihre Herkunft und Funktion in diesen Geweben kaum erforscht und auch umstritten ist. Forschungsergebnisse aus dem Humanbereich haben zudem gezeigt, dass Gallensäuren als Signalmoleküle fungieren und sich über die Bindung an Rezeptoren auf verschiedenste Stoffwechselprozesse, inclusive dem Glu-

cose-, Lipid- und Energiestoffwechsel auswirken können. Die Detektion von Gallensäurerezeptoren in humanen Adipozyten lieferte erste Hinweise darauf, dass im Fettgewebe funktionelle Gallensäure-Signalwege vorliegen, die in der Lage sind, die Physiologie der Adipozyten einschließlich der Lipolyse zu regulieren.

Im Rahmen dieser Arbeit sollte der Gallensäurestoffwechsel der Milchkuh in der Zeit rund um die Kalbung in Abhängigkeit von der Körperkondition untersucht werden. Die Leber und das subkutane Fettgewebe (scAT) als Schlüsselorgane des Energiestoffwechsels der Milchkuh wurden hinsichtlich der Gallensäurekonzentrationen und der mRNA-Menge von Gallensäurebildenden Enzymen, sowie von Rezeptoren und Transportern untersucht. Zudem wurden Gallensäuren im Serum aus dem systemischen Kreislauf untersucht, um Rückschlüsse über die Herkunft im peripheren Gewebe zu schließen.

Der Tierversuch beinhaltete die Untersuchung von 38 Multiparen deutschen Holstein Kühen, welche 15 Wochen vor dem errechnetem Kalbetermin entweder in eine High Body Condition Score Gruppe (HBCS; n=19) oder in eine Normal-BCS-Gruppe (NBCS; n = 19) eingeteilt wurden. Um die Zielgrößen in der Körperkondition (BCS) und Rückenfettdicke (BFT) (NBCS: BCS < 3.5, BFT < 1.2 cm; HBCS: BCS > 3.75, BFT > 1.4 cm) zum Zeitpunkt des Trockenstellens zu erreichen, wurden die Gruppen von der 15. bis zur 7. Woche vor der Kalbung mit Rationen unterschiedlicher Energiedichte gefüttert (HBCS: 7.2 NE_L MJ/kg Trockenmasse (DM)); NBCS: 6.8 NE_L MJ/kg DM). Sowohl in der Trockenstehzeit (6.8 MJ NE_L/kg DM), als auch in der darauffolgenden Laktation (7.2 MJ NE_L/kg DM) erhielten die Tiergruppen jeweils identische Rationen zur *ad libitum* Aufnahme.

Leber-, Fettgewebsbiopsien und Serumproben wurden 7 Wochen ante partum (a.p.), 1, 3, und 12 Wochen post partum (p.p.) entnommen und mittels Flüssigchromatographie Elektrospray-Ionisierung Tandem-Massenspektrometrie (LC-ESI-MS/MS) auf 20 verschiedene Gallensäuren in einem gezielten Metabolom-Kit-Format getestet. Die mRNA-Menge der Zielgene wurde mittels eines automatisierten Hochleistungs-qPCR-Systems, das die Mikrofluidik-Technologie nutzt, um Proben im Nanoliterbereich zu verarbeiten (Fluidigm Technik) untersucht.

Insgesamt konnten in der Leber 14 Gallensäuren quantifiziert werden, wobei der Großteil in Form von primären oder primären konjugierten Gallensäuren vorlag und überwiegend Glycocholsäure (GCA) nachgewiesen wurde. Im Serum konnten 15 Gallensäuren nachgewiesen werden, wobei GCA und Cholsäure (CA) zu annähernd gleichen Teilen dominierten. Die Gallensäurekonzentrationen in der Leber und im Serum aus dem systemischen Kreislauf weisen Unterschiede in ihrer Zusammensetzung auf, was vermutlich durch Modifikationen der Gallensäuren durch intestinale mikrobielle Biotransformationen hervorgerufen wurde.

Gallensäuren in Geweben und Körperflüssigkeiten außerhalb des enterohepatischen Kreislaufes könnten aus dem Blut aus dem systemischen Kreislauf angereichert werden, wobei es Hinweise auf eine mögliche *de novo* Synthese in extrahepatischen Geweben gibt. Die Detektion von gallensäureaffinen Rezeptoren in extra-enterohepatischen Geweben lässt ebenfalls darauf schließen, dass Gallensäuren dort in ihrer Funktion als Signalmoleküle Wirkungsmechanismen beeinflussen könnten. Mit der Fokussierung auf das scAT und seiner Eigenschaft als metabolisch aktives, endokrines Organ, haben wir im Zuge dieser Arbeit die Rolle der Gallensäuren in diesem Gewebe untersucht. Wir konnten insgesamt 7 primäre und sekundäre Gallensäuren im scAT nachweisen. Einhergehend mit der Untersuchung der mRNA-Menge gallensäurebildender Enzyme halten wir die *de novo* Gallensäuresynthese im scAT für unwahrscheinlich, wobei Teilreaktionen auf die Bildung von Oxysterolen zum Abbau des Cholesterins hindeuten könnten. Der Nachweis der mRNA spezifischer Gallensäuretransporter im scAT lässt jedoch vermuten, dass die Gallensäuren aktiv in das scAT transportiert werden. Mit dem Nachweis der mRNA spezifischer Gallensäurerezeptoren stellten wir die Hypothese auf, dass Gallensäuren im scAT als Signalmoleküle fungieren könnten.

Im Hinblick auf die Untersuchung des Einflusses der Körperkondition auf den Gallensäurestoffwechsel der Milchkuh konnten wir zeigen, dass Tiere mit einer erhöhten Körperkondition vor der Kalbung, einhergehend mit einer erhöhten postpartalen Fettgewebsmobilisation, eine Vielzahl an Gallensäuren in geringerer Konzentration aufwiesen als die Tiere mit normaler Körperkondition. Die Leber, welche maßgeblich in den Energie und Lipidstoffwechsel involviert ist, ist zudem das Schlüsselorgan der Gallensäuresynthese. Geringere Gallensäurekonzentrationen könnten darauf hindeuten, dass die Lebern der HBCS-Tiere durch vermehrte Triglyceridakkumulation in ihrer normalen Stoffwechselleistung beeinträchtigt waren. In der Leber konnten wir ebenfalls feststellen, dass die mRNA von Schlüsselenzymen des alternativen Syntheseweges bei den HBCS-Tieren hochreguliert waren, was im Hinblick auf die mögliche Beeinträchtigung der Leberfunktion mit den Ergebnissen aus der humanmedizinischen Forschung übereinstimmt. Im Serum und scAT konnten ebenfalls geringere Gallensäurekonzentrationen bei den HBCS-Tieren im Vergleich zu den NBCS-Tieren nachgewiesen werden. Wir stellten die Hypothese auf, dass die HBCS-Tiere einen verminderten zirkulierenden Gallensäurepool aufweisen könnten, was sich wiederum auf die zirkulierenden Gallensäuren in der Peripherie auswirken könnte. Zudem stellten wir die Hypothese auf, dass die HBCS-Tiere eine veränderte intestinale mikrobielle Gallensäuremodifikation aufweisen und eine erhöhte Gallensäureausscheidung über den Kot aufweisen könnten, wie es bereits in einer vergleichbaren Studie gezeigt wurde.

Mit dem Einsetzen der Laktation stiegen die Konzentrationen bei einer Vielzahl von Gallensäuren in der Leber, dem Serum und im scAT an. Einhergehend mit dem Konzentrationsanstieg der Gallensäuren konnten wir eine Erhöhung in der mRNA-Expression des Schlüsselenzyms *CYP7A1* in der Leber nachweisen, was darauf hindeuten könnte, dass sich der Gallensäurestoffwechsel in der Laktation an den steigenden Energiebedarf für die Milchleistung anpasst. Unsere Ergebnisse zeigen deutlich, dass die laktations-induzierte und konditionsabhängige Lipolyse einen Einfluss auf den Gallensäurestoffwechsel der Milchkuh hat.

Der Gallensäurestoffwechsel unterliegt komplexer Wechselwirkungen zwischen dem Darmmikrobiom, rezeptor-gesteuerten oder auf Konzentrationsbasis veränderten Syntheseraten, die wiederum über eine Vielzahl von Faktoren beeinflusst werden können. Eine ganzheitliche Betrachtung des Gallensäurestoffwechsels in allen Organen des enterohepatischen Kreislaufes könnte dazu beitragen, klare Rückschlüsse über Wirkungsketten zu fassen. Die in diesem Forschungsprojekt erlangten Ergebnisse zum Vorliegen der Gallensäuren im scAT tragen dazu bei, die Rolle der Gallensäuren in peripheren Geweben weiter zu charakterisieren und liefern wichtige Erkenntnisse in Bezug auf potenzielle Wirkungsmechanismen der Gallensäuren außerhalb des enterohepatischen Kreislaufes.

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Publications and proceedings derived from this doctorate thesis

Publications

Dicks L., K. Schuh-von Graevenitz, C. Prehn, H. Sadri, E. Murani, M. H. Ghaffari, S. Häussler (2024): Bile acid profiles and mRNA abundance of bile acid-related genes in adipose tissue of dairy cows with high versus normal body condition. Journal of Dairy Science. https://doi.org/10.3168/jds.2024-24346.

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