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Bile acid profiles and messenger RNA 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] and 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

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 (HBCS; n = 19) or normal body condition score (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 liquid chromatography electrospray ionization 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 with HBCS cows was associated with higher liver BA concentrations such as GCA, deoxycholic acid, and cholic acid. 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 from adipose tissue, leading to an accumulation of lipids and an increase in

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

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

the production of ketone bodies, particularly 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, 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 bile acids (**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. Although 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 such as 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, 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% to 95% of these BA are reabsorbed and returned to the liver via the portal vein, largely through passive diffusion or transporters, such as the Na^+-taurocholate cotransporting polypeptide (NTCP; Yang et al., 2003; Chiang, 2004). Intriguingly, in patients with nonalcoholic fatty liver disease, BA uptake in the liver is diminished compared with 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 2 treatment groups based on their BCS and backfat thickness (**BFT**) from the previous lactation. Cows were assigned to 2 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 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), whereas 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

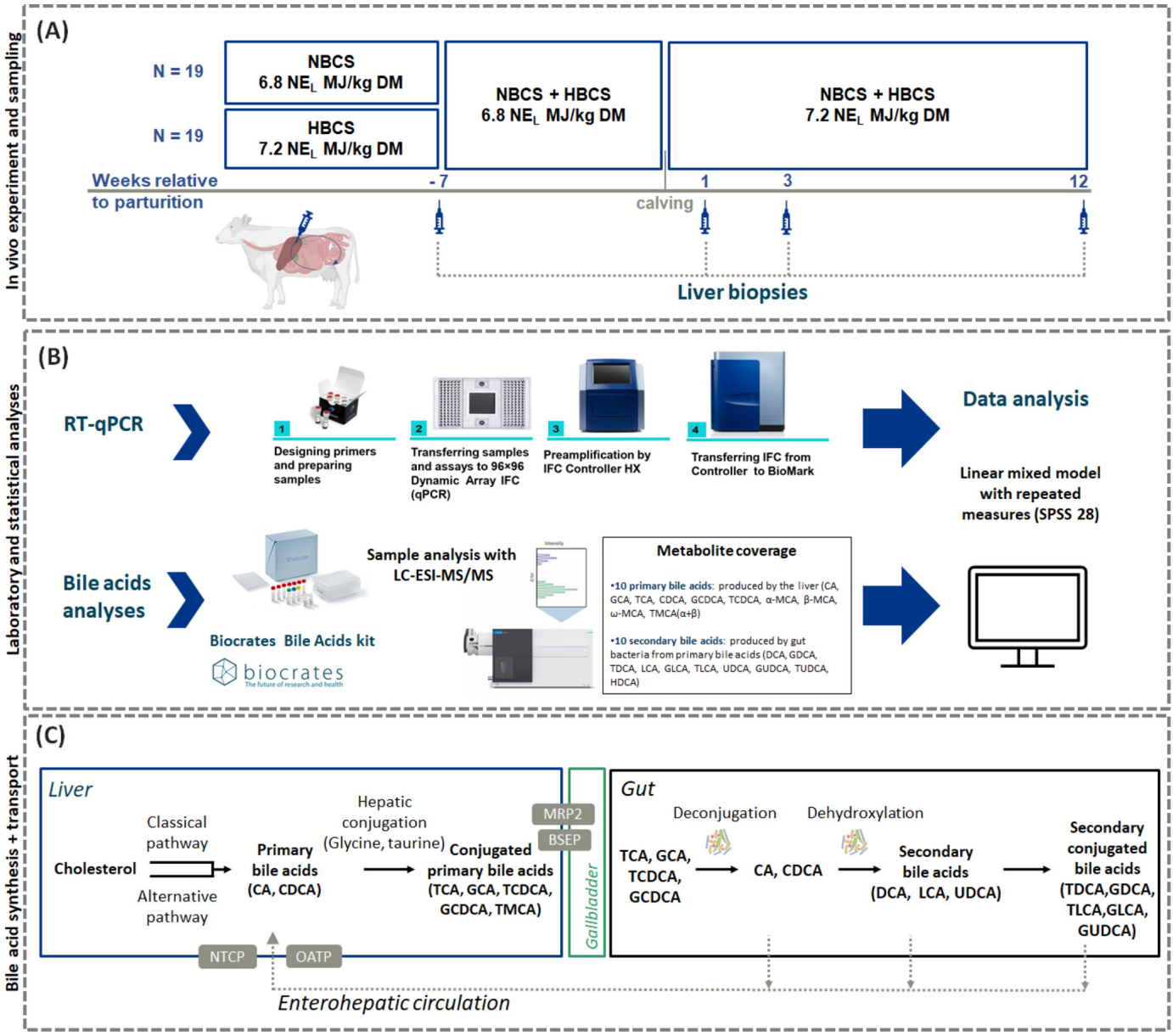

Figure 1. Schematic representation of the study and the analytical workflow. Timing of treatment and data collection during the study period from wk 15 before the expected calving date to wk 12 after calving. (A) The baseline experiment consisted of a high BCS (HBCS) group and a normal BCS (NBCS) group 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 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. CA = cholic acid; CDCA = chenodeoxycholic acid; TCA = taurocholic acid; GCA = glycocholic acid; TCDCA = taurochenodeoxycholic acid; GCDCA = glycochenodeoxycholic acid; DCA = deoxycholic acid; LCA = lithocholic acid; UDCA = ursodeoxycholic acid; TDCA = taurodeoxycholic acid; GDCA = glycodeoxycholic acid; TLCA = taurolithocholic acid; GLCA = glycolitocholic acid; GUDCA = glycoursodeoxycholic acid; TUDCA = tauroursodeoxycholic acid; α-MCA = α-muricholic acid; β-MCA = β-muricholic acid; TMCA(α+β) = tauromuricholic acid (sum of α and β); ω-MCA = ω-muricholic acid; HDCA = hyodeoxycholic acid; MDCA = murideoxycholic acid. *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).

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 TMR (7.2 MJ NE_L/kg DM) after calving.

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 Bile Acids Kit (Biocrates Life Sciences AG, Innsbruck, Austria; Figure 1B). This standardized assay includes sample preparation and liquid chromatography (**LC**) electrospray ionization (**ESI**) MS/MS (**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**), α-muricholic acid (**α-MCA**), β-muricholic acid (**β-MCA**), omega-muricholic acid (**ω-MCA**), taurocholic acid (**TCA**), taurochenodeoxycholic acid (**TCDCA**), taurodeoxycholic acid (**TDCA**), taurolithocholic acid (**TLCA**), tauromuricholic acid [sum of α and β; **TMCA(α+β)**], tauroursodeoxycholic acid (**TUDCA**), and ursodeoxycholic acid (**UDCA**). Compound identification and quantification were based on scheduled multiple reaction monitoring measurements. The method of Biocrates Bile Acids Kit has been proven to be in conformance with EMEA (2011), which implies proof of reproducibility within a given error range. The assay procedures of the bile acid kit and the results of an interlaboratory 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 vol/vol; 4°C) per 1 mg of 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 at 15°C. 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 robot (Hamilton Bonaduz AG, Bonaduz, Switzerland). After drying the filters for 5 min at room temperature with an Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK), 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 of methanol was 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 room temperature). 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 autosampler (10°C) for LC-MS/MS measurements.

Liquid chromatography separation was performed using 10 m*M* ammonium acetate in a mixture of ultrapure water/formic acid (99.85/0.15 vol/vol) as mobile phase A and 10 m*M* ammonium acetate in a mixture of methanol/acetonitrile/ultrapure water/formic acid vol/vol/vol/vol 30/65/4.85/0.15 as mobile phase B. The BA were separated on the ultra-HPLC column for the Biocrates Bile Acids kit (Product No. 91220052120868) combined with the precolumn SecurityGuard Ultra Cartridge C18/XB-C18 (for 2.1 mm i.d. 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 nonesterified fatty acids (**NEFA**) in serum, as previously described (Schuh et al., 2019; Supplemental Figures S1 and S2, see Notes). Furthermore, the amino acids glycine and taurine were previously measured in serum by LC-ESI-MS/MS 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 Primer Blast. In addition, 8 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 s, 59°C for 60 s, and 72°C for 60 s. For the subsequent RT-qPCR experiments, only primer sets with PCR efficiencies between 90% and 110% and an $R^2 > 0.985$ were used. The analysis was performed in accordance with the guidelines of the Minimum Information for Publication of Quantitative RT-PCR Experiments (Bustin et al., 2009).

RT-qPCR

The mRNA abundance of 27 genes in liver tissues from 38 cows at 4-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, 3 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, IBM Corp.). 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 (week 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 pri-

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

Gene[1]	Target	Sequence 5′–3′[2]	Primer	Accession number	bp
Enzyme					
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		
Receptor					
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 gene					
YWHAZ		F	CCACCTACTCCGGACACAG	NM_174814.2	464
		R	GACTGGTCCACAATCCCTTTC		
EIF3K		F	CCAGGCCCACCAAGAAGAA	NM_001034489	125
		R	TTATACCTTCCAGGAGGTCCATGT		

Continued

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

Gene[1]	Target	Sequence 5′–3′[2]	Primer	Accession number	bp
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		

[1]*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 α; *OSTβ*, *SLC51B*: solute carrier family 51 subunit β; *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.

[2]F = forward; R = reverse.

mary and secondary BA). The BA CDCA and LCA were detected above the limit of detection; 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 β-MCA 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 with 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(α+β) 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. and 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. and wk 3 and 12 p.p., respectively. Interactions between treatment and time were detected for CA, GUDCA, β-MCA, 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 β-MCA, 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.

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-α-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 with wk 7 a.p. and wk 3 and 12 p.p. Regardless of time, the mRNA abundance of oxysterol 7-α-hydroxylase (*CYP7B1*)

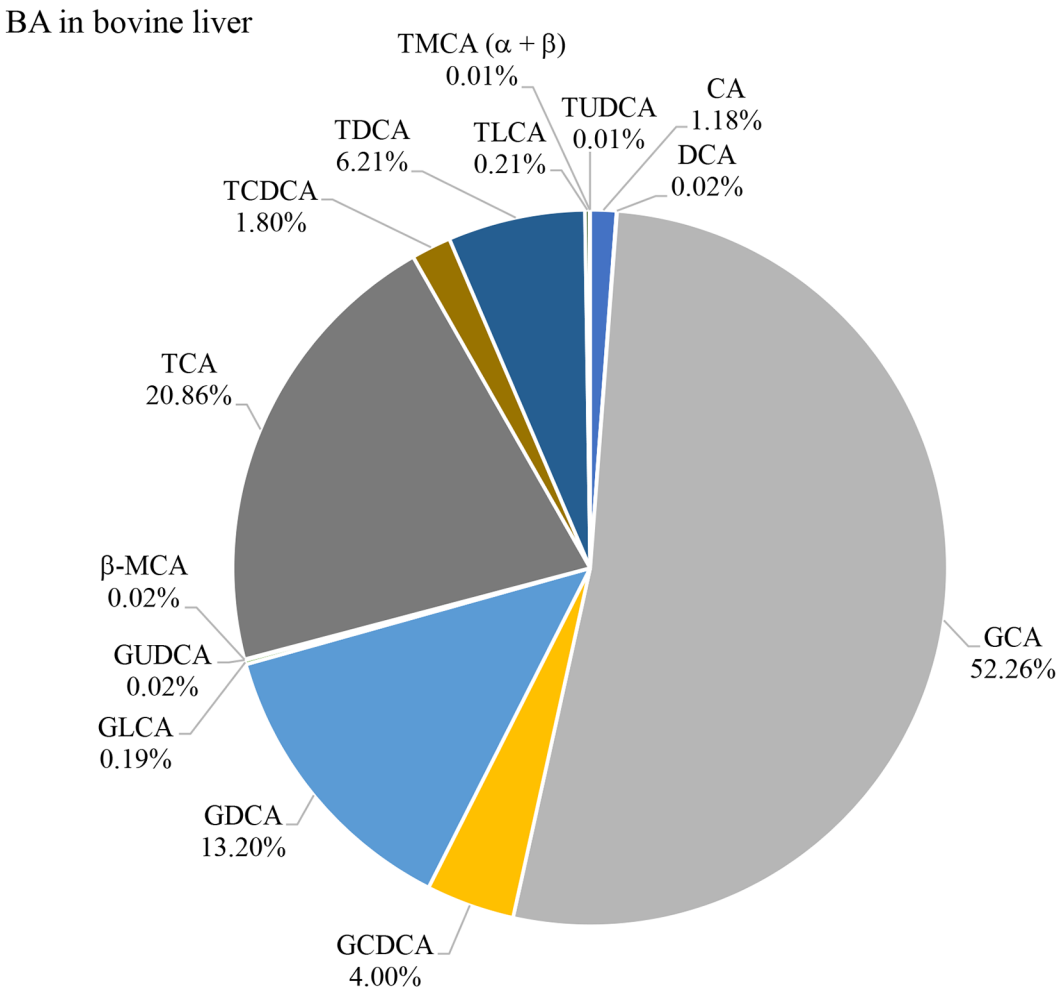

Figure 2. Mean percentage (%) of total bile acids (BA) in liver across all time points. CA = cholic acid; TCA = taurocholic acid; GCA = glycocholic acid; TCDCA = taurochenodeoxycholic acid; GCDCA = glycochenodeoxycholic acid; DCA = deoxycholic acid; TDCA = taurodeoxycholic acid; GDCA = glycodeoxycholic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

was 1.27-fold (P = 0.01) higher in HBCS cows than in NBCS cows, whereas 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.

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 with 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. The 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 with 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 with wk 7 a.p. and wk 1 and 3 p.p., respectively. The mRNA abundance

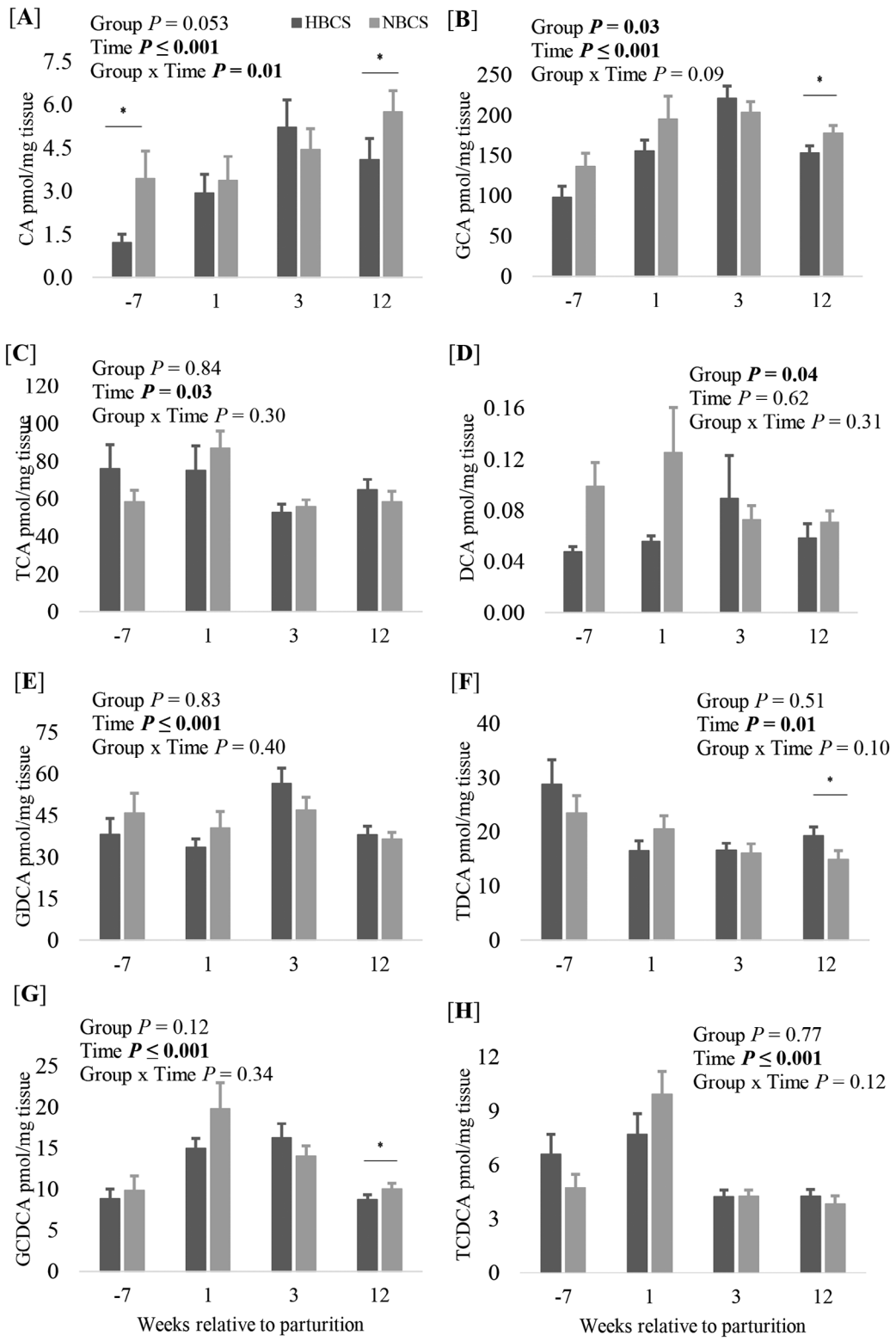

Figure 3. Concentration of bile acids (pmol/mg tissue) in liver from cows with normal (NBCS) versus high BCS (HBCS) at wk 7 antepartum (a.p.) and wk 1, 3, and 12 postpartum (p.p.). Values are given as means ± SEM. Significant differences ($P \leq 0.05$) between the groups are indicated by asterisks.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

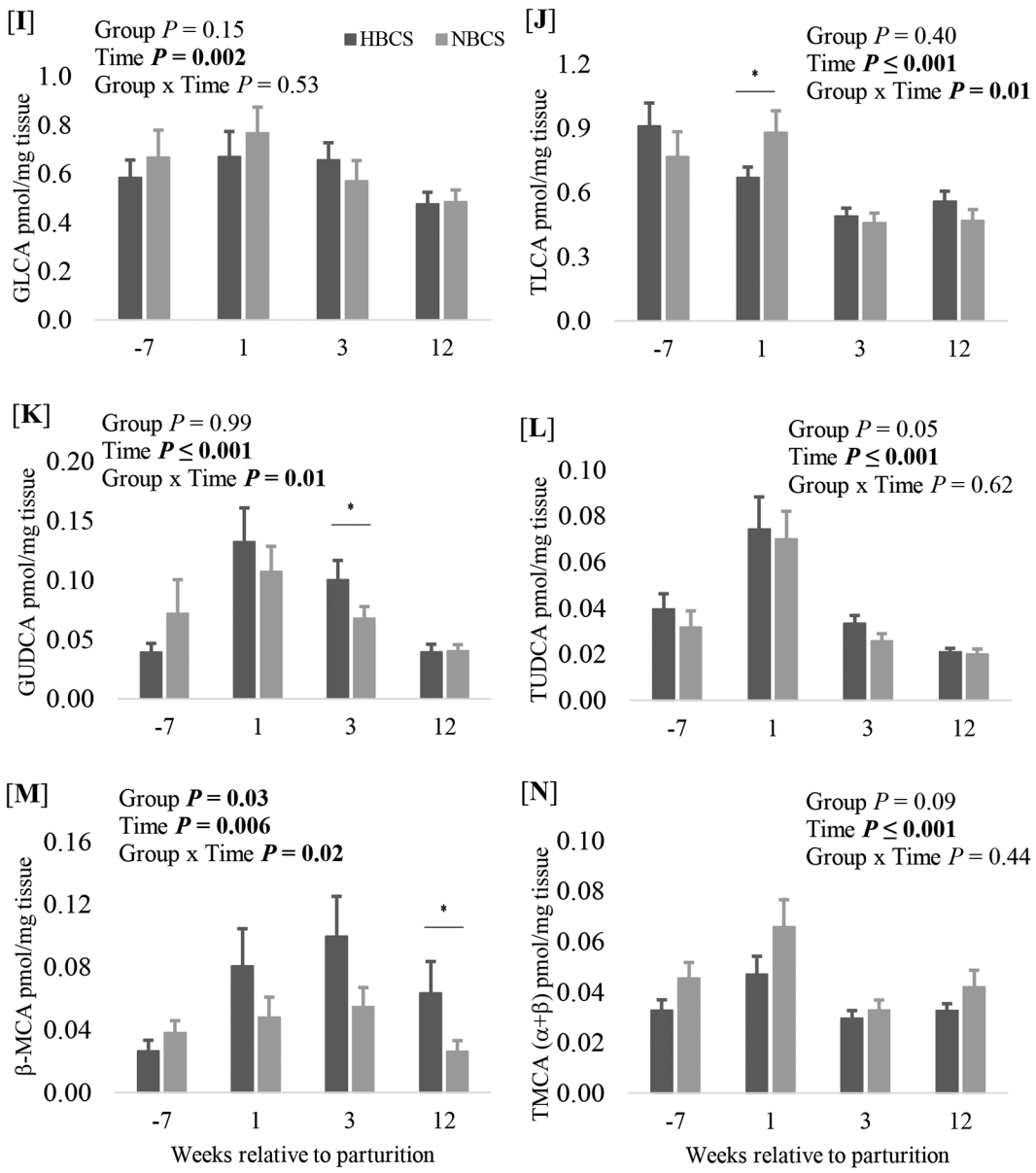

Figure 3 (Continued). Concentration of bile acids (pmol/mg tissue) in liver from cows with normal (NBCS) versus high BCS (HBCS) at wk 7 antepartum (a.p.) and wk 1, 3, and 12 postpartum (p.p.). Values are given as means ± SEM. Significant differences ($P \leq 0.05$) between the groups are indicated by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

of solute carrier family 51 subunit β (*OSTβ*) tended to be 1.12-fold higher ($P = 0.081$) in HBCS compared with NBCS before calving.

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.

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

Table 2. mRNA abundance of enzymes related to bile acid metabolism in the liver from cows with normal (NBCS) and high BCS (HBCS) at wk 7 antepartum, as well as wk 1, 3, and 12 postpartum[1]

		Weeks relative to parturition								*P*-value		
		−7		1		3		12				
Gene[2]	Item	HBCS	NBCS	HBCS	NBCS	HBCS	NBCS	HBCS	NBCS	Group	Time	Group × time
CYP7A1	Mean	1.97	1.45	2.62	2.54	2.12	2.36	3.33	3.02	0.43	<0.001	0.39
	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			
HSD3B7	Mean	0.83	0.90	1.11	1.43	1.25	1.00	0.99	0.70	0.54	<0.001	0.11
	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			
CYP8B1	Mean	6.69	6.15	8.57	6.69	6.92	6.27	8.09	7.63	0.30	0.20	0.89
	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			
AKR1D1	Mean	8.48	8.28	7.20	7.64	7.06	8.64	8.41	8.61	0.12	<0.001	0.005
	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			
CYP27A1	Mean	4.39	3.82	4.49	3.90	3.99	3.98	4.49	4.19	0.07	0.46	0.48
	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			
CYP7B1	Mean	2.69	2.05	2.12	2.01	2.49	1.54	2.52	2.35	0.01	0.25	0.40
	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			
CH25H	Mean	1.24	1.32	0.85	1.46	0.94	1.43	0.92	1.05	0.01	0.48	0.23
	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			
CYP46A1	Mean	0.39	0.37	0.31	0.41	0.33	0.26	0.46	0.33	—	—	—
	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			
BAAT	Mean	2.30	2.09	2.25	2.41	2.28	1.79	1.74	1.63	0.27	0.01	0.19
	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			
TSPO	Mean	0.32	0.42	0.47	0.56	0.41	0.36	0.38	0.35	0.89	0.002	0.17
	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			
STAR	Mean	0.65	1.14	0.97	0.93	1.26	0.48	0.70	1.09	0.60	0.80	0.02
	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			

[1]Data are given as mean ± SEM.

[2]*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.

Table 3. mRNA abundance of transporters related to bile acid metabolism in the liver from cows with normal (NBCS) and high BCS (HBCS) at wk 7 antepartum, as well as wk 1, 3, and 12 postpartum[1]

		Weeks relative to parturition								*P*-value		
		−7		1		3		12				
Gene[2]	Item	HBCS	NBCS	HBCS	NBCS	HBCS	NBCS	HBCS	NBCS	Group	Time	Group × time
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			
OSTβ (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			
OATP1A2 (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			

[1]Data are given as mean ± SEM.

[2]*NTCP*, *SLC10A1*: Na^+-taurocholate cotransporting polypeptide; *SLC10A2*, *ASBT*: apical sodium-dependent bile acid transporter; *OSTα*, *SLC51A1*: solute carrier family 51 subunit α; *OSTβ*, *SLC51B*: solute carrier family 51 subunit β; *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.

Table 4. mRNA abundance of receptors related to bile acid metabolism in the liver from cows with normal (NBCS) and high BCS (HBCS) at wk 7 antepartum, as well as wk 1, 3, and 12 postpartum[1]

		Weeks relative to parturition								*P*-value		
		−7		1		3		12				
Gene[2]	Item	HBCS	NBCS	HBCS	NBCS	HBCS	NBCS	HBCS	NBCS	Group	Time	Group × time
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	0.01	0.01
	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			

[1]Data are given as mean ± SEM.

[2]*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.

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), β-MCA (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 with a.p. values. At wk 3 p.p., glycine concentrations were 1.30-fold greater (P = 0.005) in HBCS cows compared with 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.

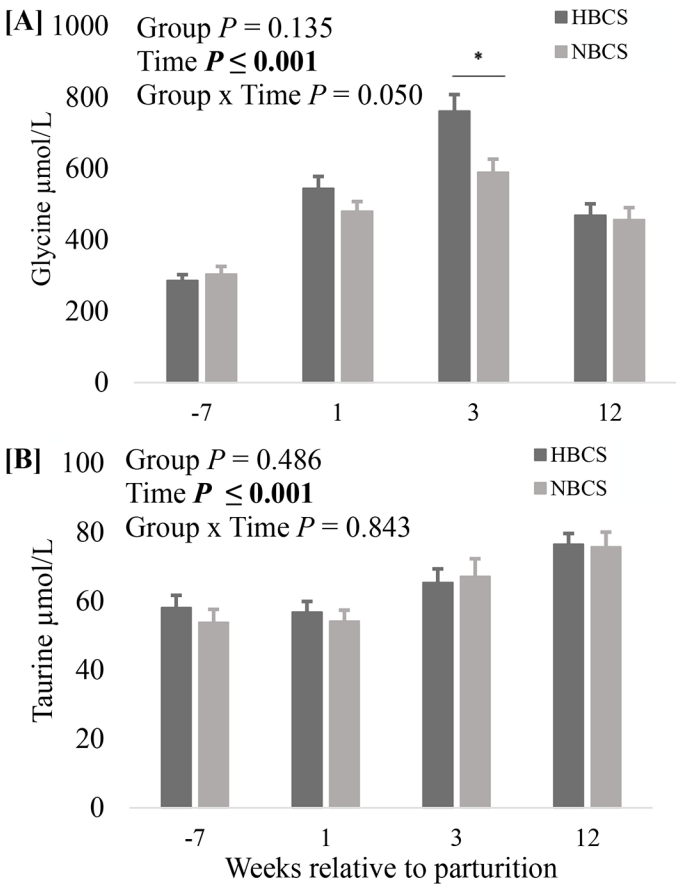

Figure 4. Concentrations of (A) glycine and (B) taurine (µmol/L) in serum from cows with normal (NBCS) versus high BCS (HBCS) at wk 7 antepartum (a.p.) and wk 1, 3, and 12 postpartum (p.p.). Values are given as means ± SEM. Asterisks indicate significance: *P < 0.05. The serum taurine concentrations 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. In addition to 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). In addition to 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 with 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 week 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., 2020) and adipose tissue (Dicks et al., 2024). In general, it is known that BA in the liver and gallbladder are mainly conjugated, whereas serum contains both conjugated and unconjugated BA (Chiang and Ferrell, 2020b). Regardless of sampling time, GCA accounted for over 50% of total liver BA, whereas 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 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 DMI in early lactating dairy cows (Schuh et al., 2019; Supplemental Figure S3, see Notes). Higher DMI in NBCS cows compared with 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 BW and the lipogenic pathway in the liver, suggesting that UDCA is an important regulator of lipid metabolism (Chen et al., 2019).

Bile acids 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 regu-

late 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 Farnesoid X receptor (**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 with 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 a physiological role of AKR1D1 other than 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 postcalving mRNA abundance of *NTCP* compared with precalving 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; Claro 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 with 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). Because 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 with 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 with 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). Because 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.

NOTES

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Nonstandard abbreviations used: a.p. = antepartum; BA = bile acids; BFT = backfat thickness; BSEP = bile salt export pump; CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; ESI = electrospray ionization; FXR = Farnesoid X receptor; GCA = glycocholic acid; GCDCA = glycochenodeoxycholic acid; GDCA = glycodeoxycholic acid; GE = gene expression; GLCA = glycolithocholic acid; GUDCA = glycoursodeoxycholic acid; HBCS = high BCS; HDCA = hyodeoxycholic acid; IFC = integrated fluidic circuits; LC = liquid chromatography; LCA = lithocholic acid; LC-ESI-MS/MS = liquid chromatography-electrospray ionization-MS/MS; α-MCA = α-muricholic acid; β-MCA = β-muricholic acid; ω-MCA = omega-muricholic acid; NBCS = normal BCS; NEB = negative energy balance; NEFA = nonesterified fatty acid; p.p. = postpartum;

RT-qPCR = reverse-transcription quantitative real-time PCR; TCA = taurocholic acid; TCDCA = taurochenodeoxycholic acid; TDCA = taurodeoxycholic acid; TLCA = taurolithocholic acid; TMCA(α+ β) = tauromuricholic acid, sum of α and β; TUDCA = tauroursodeoxycholic acid; UDCA = ursodeoxycholic acid.

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