Hepatic 1-carbon metabolism enzyme activity, intermediate metabolites, and growth in neonatal Holstein dairy calves are altered by maternal supply of methionine during late pregnancy

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ABSTRACT

Maternal supply of methyl donors such as methionine (Met) during late pregnancy can affect offspring growth and development. The objective was to investigate the effect of postruminal Met supply during late pregnancy on 1-carbon, Met cycle, and transsulfuration pathways in the calf liver. During the last 28 d of pregnancy, cows were individually fed a control diet or the control diet plus rumen-protected DL-Met (MET; 0.09% dry matter intake). Liver samples obtained from calves (n = 14/group) at 4, 14, 28, and 50 d of age were used for metabolomics, real-time PCR, and enzyme activity analyses. Genes associated with 1-carbon metabolism, DNA methylation, and the cytidine 5'-diphosphocholine–choline pathway were analyzed via real-time PCR. Activity of betaine homocysteine methyltransferase, cystathionine β -synthase, and 5-methyltetrahydrofolate homocysteine methyltransferase (MTR) was analyzed using ¹⁴C isotopes. Data were analyzed using a mixed model that included the fixed effects of maternal treatment, day, and their interaction, and the random effect was calf within maternal diet. Calves born to dams offered MET tended to have greater birth body weight and had overall greater body weight during the first 9 wk of life. However, no differences were detected for daily feed intake and average daily gain between groups. Concentrations of betaine and choline, reflecting Met cycle activity, at d 14 through 28 were greater in MET calves. Transsulfuration pathway intermediates also were altered in MET calves, with concentrations of cysteine sulfinic acid and hypotaurine (d 4 and 14) and

taurine being greater (d 4, 14, 28, and 50). Despite the lack of differences in daily feed intake, the greater concentrations of the tricarboxylic acid cycle intermediates fumarate and glutamate along with NAD/NADH in MET calves indicated enhanced rates of energy metabolism. Although activity of betaine homocysteine methyltransferase was greater in MET calves at d 14, cystathionine β -synthase was lower and increased at d 14 and 28, where it was greater compared with the control diet. Activity of MTR was lower at d 4 and 50 in MET calves. Among gene targets measured, MET calves had greater overall expression of *MTR*, phosphatidylethanolamine *N*-methyltransferase, and choline kinase α and β . An interaction of maternal diet by time was detected for mRNA abundance of DNA methyltransferase 3 α (involved in de novo methylation) due to greater values at d 4 and 14 in MET calves. Overall, the data indicate that enhanced postruminal supply of Met to cows during late pregnancy may program hepatic metabolism of the calf in the context of maintaining Met homeostasis, phosphatidylcholine and taurine synthesis, DNA methylation, and energy metabolism. These alterations potentially result in better efficiency of nutrient use, hence conferring the calf a physiologic advantage during a period of rapid growth and development. The precise biologic mechanisms remain to be established.

Key words: epigenetics, metabolism, methyl donor, nutritional programming

INTRODUCTION

Methionine (Met) is an EAA and an important methyl donor with crucial roles in 1-carbon metabolism and transsulfuration (Stipanuk, 2004). As a methyl donor, Met may induce programming effects on offspring through the production of S-adenosyl-

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Met (**SAM**), which can methylate DNA (Robertson, 2005). In dairy cattle, maternal supplementation with rumen-protected Met (**RPM**) during late gestation not only altered mRNA abundance of placental AA and glucose transporters (Batistel et al., 2017a) but also was associated with hepatic upregulation of genes associated with fatty acid oxidation, gluconeogenesis, and inflammatory responses in calf liver (Jacometo et al., 2016). Thus, postruminal supply of Met to dairy cows during late gestation seems to elicit biological effects that could affect calf growth during postnatal life.

Enhanced Met supply may promote flux through the transsulfuration pathway, enhancing production of the antioxidants glutathione (**GSH**) and taurine. Increasing antioxidant production may help alleviate oxidative stress and inflammation induced from reactive oxygen metabolite production in the liver (Osorio et al., 2014b; Zhou et al., 2016a; Batistel et al., 2018). Dairy cows experiencing a greater oxidative stress status in late pregnancy delivered calves with lower birth weight and a compromised immune system (Ling et al., 2018). The fact that enhancing postruminal supply of Met during the periparturient period has consistently led to better immunometabolic status underscores the benefits of Met beyond protein synthesis (Osorio et al., 2014b; Zhou et al., 2016a; Batistel et al., 2018).

In the current study, a subset of animals from a previously published study was used to test the hypothesis that increasing maternal Met supply during late pregnancy affects metabolic and epigenetic signatures in calf liver (Batistel et al., 2017b; Alharthi et al., 2018; Xu et al., 2018). Thus, the main objective was to investigate the effects of RPM supplementation during the last 28 d of pregnancy on mRNA abundance, activity of enzymes involved in 1-carbon metabolism and the transsulfuration pathway, and metabolites associated with those pathways in addition to energy metabolism in calf liver.

MATERIALS AND METHODS

All procedures for this study were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Illinois (protocol no. 14270). Calves in the present study were from Holstein cows randomly assigned to receive a basal control (**CON**) close-up diet (from -28 ± 2 d to parturition, $\pm$ SD; $n = 14$; 1.47 Mcal/kg of DM and 15.3% CP) with no added RPM or CON plus ethyl-cellulose RPM (**MET**; $n = 14$; Mepron, Evonik Nutrition and Care GmbH, Essen, Germany). Diet composition is shown in Supplemental Table S1 (<https://doi.org/10.3168/jds.2019-16562>). The Met product

was offered at a rate of 0.09% of diet DM. This supply of Met was based on experiments demonstrating a benefit of achieving a Lys:Met ratio close to 2.8:1 in terms of production performance and health (Osorio et al., 2013; Zhou et al., 2016b).

After parturition, calves were removed from their dams immediately. Cows were milked in the parlor where the volume of colostrum was recorded, and IgG content was estimated based on specific gravity with a bovine colostrometer (Nasco, Fort Atkinson, WI; cat. no. C10978N). Calves were offered 3.8 L of first-milking colostrum from their respective dam within 6 h. If voluntary colostrum intake had not reached the 3.8 L required, calves were force-fed via esophageal tube to ensure that all calves consumed the same amount of colostrum. Calves were housed in individual outdoor hutches bedded with straw and fed twice daily (morning and afternoon) with a milk replacer (Advance Excelerate, Milk Specialties, Carpentersville, IL; 28.5% CP, 15% fat) until 35 d of age. Then, they were switched to once-daily feeding until weaning (42 d of age). From 1 to 10 d of age, calves received 4.54 kg of milk replacer/d (0.59 kg of milk replacer in 3.95 L of water); from 11 to 20 d of age, they received 5.90 kg/d (0.77 kg of milk replacer in 5.13 L of water); and from 21 to 35 d of age, they received 7.26 kg/d (0.94 kg of milk replacer in 6.32 L of water). From 36 to 42 d of age, calves received 3.63 kg/d (0.47 kg of milk replacer in 3.16 L of water) in a single feeding. From 1 until 56 d of life, calves had ad libitum access to a starter grain mix (19.9% CP, 13.5% NDF) at 0800 h. Amino acid profiles of the milk replacer and calf starter are given in Supplemental Table S2 (<https://doi.org/10.3168/jds.2019-16562>). Daily intake of starter and milk replacer was recorded separately. Starter intake was recorded daily for each calf until 56 d of life. Between d 42 and 56, all calves were fed the same starter. Body weight was recorded immediately after birth and once every week until 9 wk of age using a digital scale. Average daily gain was calculated by dividing the difference between initial BW and final BW by number of days. Feed efficiency was calculated by dividing total gain by total feed intake.

Sample Collection

Liver was sampled via puncture biopsy under local anesthesia at approximately 0530 h on d 4, 14, 28, and 50 of age ($n = 14$ calves/group) before the morning feeding. Tissue was frozen immediately in liquid nitrogen and stored at -80°C until further analysis. All samples were analyzed within 6 mo of collection. Calves were monitored after each biopsy for 5 d by checking rectal temperature, respiratory score, fecal score, calf

attitude, incision site, umbilicus, joints, and intake of starter and milk replacer.

Metabolomics

Approximately 100 mg of frozen tissue was extracted using a 2-step protocol described by Wu et al. (2008). Targeted metabolomics (liquid chromatography–MS) was performed to quantify 31 metabolites related to the tricarboxylic acid (TCA) cycle, 1-carbon metabolism, and transsulfuration pathway. Samples were analyzed with the 5500 QTRAP liquid chromatography–tandem MS system (Sciex, Framingham, MA) at the metabolomics core facility of the Roy J. Carver Biotechnology Center, University of Illinois (Urbana). Software Analyst 1.6.2 (Batistel et al., 2019) was used for data acquisition and analysis. Full details of the metabolomics analysis are reported in Supplemental File S1 (<https://doi.org/10.3168/jds.2019-16562>).

Enzyme Activity Assays

Activity of betaine homocysteine methyltransferase (BHMT), 5-methyltetrahydrofolate homocysteine methyltransferase (MTR), and cystathionine β -synthase (CBS) was determined according to protocols detailed by Zhou et al. (2017). Details of the assays are reported in Supplemental File S1 (<https://doi.org/10.3168/jds.2019-16562>).

RNA Extraction, cDNA Synthesis, and Quantitative PCR

All procedures were performed as described previously (Osorio et al., 2014a). Briefly, RNA was extracted from liver samples using the miRNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Samples were treated on-column with DNaseI (Qiagen) to remove genomic DNA. Quantification was accessed using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE), and RNA quality was measured using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA). All samples had an RNA integrity number factor greater than 7. The RNA was diluted to 100 ng with DNase/RNase-free water for cDNA synthesis using real-time PCR, and the cDNA was diluted 1:4 with DNase/RNase-free water. The quantitative PCR (qPCR) performed was SYBER Green based (Quanta Biosciences, Beverly, MA), using a 7-point standard curve obtained from a cDNA pool of all samples. The final data were normalized using the geometric mean of 3 internal control genes: *UXT*, *GAPDH*, and *RPS9*. A total of 24 genes selected related to the Met cycle,

DNA methylation, transsulfuration, GSH, and cytidine 5'-diphosphocholine (CDP)–choline pathway were measured. The qPCR performance and relative mRNA abundance are shown in Supplemental Table S3 (<https://doi.org/10.3168/jds.2019-16562>).

Statistical Analysis

Data were analyzed with the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Fixed effects in the model were maternal treatment, day, and treatment $\times$ day ($T \times D$) interaction. Random effect was calf within maternal diet. The exponential correlation covariance structure SP(POW) for repeated measures was used for the analysis of mRNA abundance, enzyme activity, and metabolites because time intervals were not evenly spaced. The covariance structure AR(1) was used for the analyses of BW and daily starter intake because time was evenly spaced. The qPCR data were $\log_2$ transformed before statistical analysis to obtain a normal distribution. When the interaction was significant ($P \leq 0.05$), least squares means separation between and within time points was performed using the PDIFF statement with Tukey adjustment.

RESULTS

Growth Performance

At birth, calves born to dams offered MET tended to have greater BW ($P = 0.10$; 45.7 vs. 43.0 $\pm$ 1.1 kg; $\pm$ SEM). Calves born to dams offered MET had greater overall BW ($P = 0.04$; 59.8 vs. 55.6 $\pm$ 1.4 kg; $\pm$ SEM) during the first 9 wk of life (Table 1). However, overall daily starter intake, ADG (difference in BW between d 1 and 56), and overall feed efficiency (total gain/total feed intake between d 1 and 56) were not affected by maternal diet ($P > 0.10$). Temporal profiles for BW and daily starter intakes are reported in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-16562>).

Metabolomics

1-Carbon Metabolism. Maternal supplementation with MET led to greater concentrations of adenosine, betaine, choline, glycine, and *N,N*-dimethylglycine ($P \leq 0.05$). There was a $T \times D$ effect ($P \leq 0.05$) for the concentrations of betaine, choline, glycine, and S-5'-adenosyl-homocysteine (Table 2; Figure 1). Calves in the MET group had greater concentrations of betaine ($P < 0.01$) at 14 and 28 d and choline ($P = 0.01$) at 4, 14, and 28 d of age. Concentration of glycine was

Table 1. Weekly BW during the first 9 wk of life, average daily starter intake from 1 to 56 d of age, and ADG and feed efficiency (total gain/total feed intake) in calves born to dams fed a control diet (CON) or CON supplemented with ethyl-cellulose rumen-protected methionine (MET; 0.09% daily diet DMI) during the last 28 d of pregnancy¹

Item	Treatment group			P-value ²		
	CON	MET	SEM	T	D	T × D
BW, kg						
Birth	43.04	45.73	1.11	0.10	—	—
2–9 wk of age				0.04	<0.01	0.92
2	42.8	46.1	1.69			
3	44.9	48.3				
4	49.6	52.8				
5	54.1	57.6				
6	59.2	63.4				
7	63.9	68.9				
8	68.5	74.8				
9	74.9	80.9				
Average of birth to 9 wk	55.6 ^b	59.8 ^a	1.4			
Daily starter intake, ³ kg	0.81	0.86	0.06	0.51	<0.01	0.93
ADG, kg/d	0.57	0.63	0.03	0.19	—	—
Feed efficiency ⁴	0.71	0.73	0.04	0.72	—	—

^{a,b}Means in the same row with different superscripts differ ($P \leq 0.05$).

¹Values are means and pooled SEM; $n = 14$ /treatment group.

²T = maternal treatment; D = day; T × D = maternal treatment × day interaction.

³Daily intake profiles are reported in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2019-16562>).

⁴Calculated as total gain/total feed intake.

consistently greater ($P < 0.01$) in the MET group throughout the first 50 d of age (Figure 1).

Transsulfuration Pathway. Compared with CON calves, the concentrations of cystathionine, cysteine sulfinic acid, hypotaurine, serine, and taurine were greater in MET calves ($P \leq 0.05$; Table 2). Maternal treatment × day effects ($P \leq 0.05$) were detected for cysteine sulfinic acid, hypotaurine, serine, and taurine (Figure 1). Calves in the MET group had greater concentrations of cysteine sulfinic acid ($P < 0.01$) at 4 and 14 d of age and hypotaurine ($P = 0.03$) at 14 and 28 d of age, whereas concentrations of serine ($P = 0.05$) at 4, 14, 28, and 50 d and taurine ($P < 0.01$) at 4, 14, and 28 d of age were greater in the MET calves (Figure 1).

TCA Cycle. Greater concentrations of fumarate, glutamate, NAD, and NADH were detected in MET calves ($P \leq 0.05$; Table 2). Compared with CON calves, the concentrations of NAD ($P < 0.01$) and NADH ($P = 0.01$) were greater in MET calves at 4, 14, and 28 d of age. Concentration of glutamate was also greater ($P < 0.01$) at 4, 14, 28, and 50 d of age in MET calves. Among the metabolites measured in the TCA cycle, only glutamate concentration remained greater in the MET calves at 50 d of age.

Bile Acids. There was a T × D effect ($P < 0.01$) for glycochenodeoxycholic acid, with MET calves having a greater concentration at d 4, 12, and 28. A similar T × D effect was observed for lithocholic acid ($P = 0.03$) and taurocholic acid ($P = 0.06$), where concentrations

were greater in MET calves at d 4 and 14 (Table 2). Greater overall concentrations of glycocholic acid ($P < 0.01$) and taurochenodeoxycholic acid ($P = 0.03$) were detected in MET compared with CON calves (Table 2).

Enzyme Activity

There was a T × D effect ($P < 0.01$) for BHMT, partly due to greater activity in the MET calves at 14 d of age (Figure 2). Similarly, the T × D effect ($P < 0.01$) for CBS was due to lower activity at 4 d and greater activity at 14 and 28 d of age in the MET calves (Figure 2). Unlike BHMT and CBS, the T × D effect ($P < 0.01$) for MTR was due to lower activity at 4 and 50 d of age in the MET calves (Figure 2).

Hepatic mRNA Abundance

Met Cycle. Temporal profiles for all genes are reported in Supplemental Figure S2 (<https://doi.org/10.3168/jds.2019-16562>). Maternal supplementation with MET led to an overall tendency toward greater expression of 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*; $P = 0.06$) and phosphatidylethanolamine *N*-methyltransferase (*PEMT*; $P = 0.07$; Figure 3). A T × D effect was detected in the abundance of betaine-aldehyde dehydrogenase (*BADH*; $P = 0.05$) at 4 d of age due to greater expression in MET calves and a tendency ($P = 0.06$) for greater *BHMT* expression at

Table 2. Liver metabolite concentrations (ng/mg of total protein) in neonatal Holstein calves born to dams fed a control diet (CON) or CON supplemented with ethyl-cellulose rumen-protected methionine (MET; 0.09% daily DMI) during the last 28 d of pregnancy¹

Metabolite, ² ng/mg of total protein	Treatment group			P-value ³		
	CON	MET	SEM	T	D	T × D
1-Carbon metabolism						
Adenosine	4,510 ^b	8,619 ^a	686	<0.01	<0.01	0.26
Methionine	672	760	53	0.23	<0.01	0.16
5-Methyltetrahydrofolic acid	5.01	4.93	0.81	0.95	<0.01	0.06
<i>N,N</i> -Dimethylglycine	26.8 ^b	37.6 ^a	3.7	0.03	<0.01	0.14
Transsulfuration						
Cystathionine	330 ^b	650 ^a	52	<0.01	0.17	0.15
Cysteine	1,269	2,231	409	0.11	<0.01	0.08
Cysteine sulfinic acid	900 ^b	1,490 ^a	166	0.01	<0.01	<0.01
γ-Glutamylcysteine	541	590	98	0.71	<0.01	0.65
Glutathione	10,139	14,178	2,770	0.31	0.01	0.49
Hypotaurine	1,831 ^b	2,527 ^a	174	<0.01	<0.01	0.03
Serine	2,695 ^b	5,517 ^a	246	<0.01	<0.01	0.05
Taurine	41,050 ^b	76,747 ^a	4,674	<0.01	<0.01	<0.01
Tricarboxylic acid cycle						
α-Ketoglutarate	406	461	46	0.41	<0.01	0.25
Fumarate	1,407 ^b	2,761 ^a	130	<0.01	0.09	0.42
Glutamate	32,040 ^b	53,512 ^a	3,524	<0.01	<0.01	<0.01
NAD	13,543 ^b	26,470 ^a	3,366	<0.01	<0.01	<0.01
NADH	5,153 ^b	8,520 ^a	932	<0.01	<0.01	0.01
NAD/NADH	2.77 ^b	3.32 ^a	0.14	0.27	0.91	0.28
Pyruvate	1,116	1,039	90	0.54	<0.01	0.60
Other						
Glutamine	1,551	2,091	394	0.20	0.03	0.56
Bile acids						
Glycocholic acid	6,432 ^b	10,793 ^a	776	<0.01	<0.01	0.64
Glychenodeoxycholic acid	2,356 ^b	4,795 ^a	368.2	<0.01	<0.01	<0.01
Lithocholic acid	10,051 ^b	15,373 ^a	1,539	0.01	0.17	0.03
Taurocholic acid	1,879 ^b	2,707 ^a	235	0.01	<0.01	0.06
Taurochenodeoxycholic acid	988 ^b	1,468 ^a	169	0.03	<0.01	0.42

^{a,b}Means in the same row with different superscripts differ ($P \leq 0.05$).

¹Values are means and pooled SEM; n = 14/treatment.

²The following compounds were undetectable: betaine aldehyde, folic acid, *N*-methylglycine, S-5'-adenosylmethionine, and tetrahydrofolic acid in 1-carbon metabolism; homocysteine, vitamin B₆, and vitamin B₁₂ in transsulfuration pathway; acetyl-CoA, AMP, ATP, citrate/isocitrate, malate, guanosine triphosphate, NADP, NADPH, succinyl-CoA, and uridine diphosphate in the tricarboxylic acid cycle; and trimethyllysine, nicotinic acid, and uridine diphosphate.

³T = maternal treatment; D = day; T × D = maternal treatment × day interaction.

28 d of age in CON calves (Supplemental Figure S2; <https://doi.org/10.3168/jds.2019-16562>).

DNA Methylation. Temporal profiles for all genes are reported in Supplemental Figure S3 (<https://doi.org/10.3168/jds.2019-16562>). Greater overall expression of DNA methyltransferase 3α (*DNMT3A*; $P < 0.01$) was detected due to higher expression at 4 and 14 d of age in calves born to dams supplemented with MET compared with CON (Figure 3).

CDP-Choline Pathway. Temporal profiles for all genes are reported in Supplemental Figure S4 (<https://doi.org/10.3168/jds.2019-16562>). Maternal supplementation with MET led to greater expression ($P = 0.05$) of choline kinase α (*CHKA*) and β (*CHKB*) in MET calves than in CON calves (Table 3). Maternal supplementation with MET led to a T × D interaction ($P = 0.04$) in the abundance of phosphate cytidylyltransferase 1A (*PCYT1A*) and choline/ethanolamine phosphotransfer-

ase 1 (*CEPT1*; $P < 0.01$) due to a gradual increase after 4 d of age in CON calves (Figure 3).

DISCUSSION

1-Carbon and Met Metabolism Pathway

Methionine metabolism via the Met cycle is essential for synthesis of homocysteine, which feeds the transsulfuration pathway and generates GSH and taurine as cellular antioxidants (Stipanuk, 2004). Methionine is also a well-known methyl donor through synthesis of SAM. Thus, supply of Met can play a role in regulating epigenetic mechanisms through DNA methylation (Waterland, 2006; Sinclair et al., 2007). The fact that neither milk replacer intake nor starter intake differed between groups suggests that differences at the mRNA (albeit few) and enzyme activity level of components

of 1-carbon and Met metabolism pathways were partly caused in utero. In support of this idea, we previously reported that enhanced Met supply during late pregnancy increased Met concentration in plasma and placenta mRNA abundance of AA transporters, some of which are transcriptionally regulated (*SLC38A1*, *SLC38A2*, *SLC38A10*, *SLC3A2*, and *SLC7A5*). Thus, supply of Met to the fetus during the latter stages of development in utero can be increased through nutrition of the cow (Batistel et al., 2017a).

The conversion of Met to SAM is accompanied by ATP consumption in a reaction controlled by Met adenosyltransferase (Mato et al., 2008). After demethylation, SAM is converted to SAH, which is subsequently hydrolyzed to homocysteine with the production of adenosine (Lu, 2000). Homocysteine can then be remethylated to Met via MTR or BHMT or used for cystathionine synthesis via CBS (Mato et al., 2008; Williams and Schalinske, 2010). In addition, serine supports the Met cycle through de novo ATP synthesis

Figure 1. Liver metabolite concentrations in calves ($n = 14/\text{group}$) born to dams fed a control diet (CON, ●) or CON supplemented with ethyl-cellulose rumen-protected Met (MET, ○; 0.09% daily diet DMI) during the last 28 d of pregnancy. Data are presented as means $\pm$ SEM. Asterisk indicates that means differ (treatment $\times$ day, $P \leq 0.05$; post hoc means separation using Tukey) between groups (CON and MET).

Figure 2. Hepatic activity of (A) betaine homocysteine methyltransferase (BHMT), (B) cystathionine β -synthase (CBS), and (C) 5-methyltetrahydrofolate homocysteine methyltransferase (MTR) response in calves ($n = 14/\text{group}$) born to dams fed a control diet (CON, $\bullet$) or CON supplemented with ethyl-cellulose rumen-protected Met (MET, $\circ$; 0.09% daily DMI) during the last 28 d of pregnancy. Data are presented as means $\pm$ SEM. Asterisk indicates that means differ (treatment $\times$ day, $P \leq 0.05$; post hoc means separation using Tukey) between groups (CON and MET).

(Maddocks et al., 2016). Choline also supports the Met cycle through its metabolite betaine, which is used to produce Met via BHMT (Li and Vance, 2008). This reaction produces *N,N*-dimethylglycine, which is metabolized to sarcosine and then glycine (Wang et al., 2013). Thus, increased concentrations of adenosine, betaine, choline, glycine, serine, and *N,N*-dimethylglycine in MET calves suggest that maternal supplementation with Met during late gestation (rather than differences in feed intake) primed or programmed the Met cycle in calf liver. Exact mechanisms could not be discerned from the available data.

The similar pattern of activity of MTR and BHMT in CON and MET groups from d 4 to 50 is noteworthy. In lambs, Xue and Snoswell (1986) observed that hepatic activity of BHMT increased with age until a ruminant state was reached, whereas activity of MTR had a pattern that was opposite that of BHMT. A similar pattern in the activity of BHMT was also detected in dairy calves and sheep (Xue and Snoswell, 1986; Jacometo et al., 2017). The patterns of change in BHMT and MTR typically seen as young ruminants age are likely associated with the transition from preruminant to ruminant; establishment of a microbiome in the rumen leading to more vitamin B₁₂ biosynthesis (Kon and Porter, 1954), a cofactor for MTR, might contribute to the increased MTR. Concurrently with rumen development, choline intestinal bioavailability would gradually become limited due to degradation in the rumen (Snoswell and Xue, 1987), which may contribute to the decrease in BHMT activity.

The fact that activity of BHMT and MTR decreased from d 4 to 14 in CON calves and increased from d 4 to 14 in MET calves indicated that other mechanisms besides the transition from preruminant to ruminant regulate 1-carbon metabolism enzyme activity. For instance, the overall upregulation of *MTR* abundance in the MET calves along with the lower postnatal activity of MTR on d 4 and 50 suggest a posttranslational mechanism. An example of such effect could be the intrahepatic concentrations of metabolites; for example, the greater BHMT activity of MET calves at d 14 was associated with greater betaine and choline concentrations. In non-ruminants, SAM is known to regulate CBS activity (Pey et al., 2013). In the absence of differences in feed intake, we speculate that these are examples of maternal effects induced by the greater postruminal supply of Met. The cows giving birth to MET calves used in the present study had increased circulating Met prepartum and greater mRNA abundance of nutrient transporters in the placenta (Batistel et al., 2017a). Although not all nutrient transporters are regulated at the transcriptional level, those data suggest that avail-

Figure 3. Hepatic mRNA abundance (log₂ scale) of betaine aldehyde dehydrogenase (*BADH*), DNA methyltransferase 3α (*DNMT3A*), phosphate cytidyltransferase 1, choline, α (*PCYT1A*), and choline/ethanolamine phosphotransferase 1 (*CEPT1*) in calves (n = 14/group) born to dams fed a control diet (CON, ●) or CON supplemented with ethyl-cellulose rumen-protected Met (MET, ○; 0.09% daily diet DMI) during the last 28 d of pregnancy. Data are presented as means ± SEM. Asterisk indicates that means differ (P ≤ 0.05) between groups (CON and MET).

ability of AA and other nutrients for the developing calf might have been increased. Thus, the lower activity of MTR in MET compared with CON calves at d 4 might have been linked to increased availability of Met in utero, which would limit the need to synthesize Met.

Choline is an indirect methyl donor in the 1-carbon metabolism pathway. The oxidation of choline to betaine is divided into 2 steps that are regulated by choline dehydrogenase and *BADH*, respectively (Slow and Garrow, 2006). In primary bovine liver cells, increased

choline supply resulted in greater mRNA abundance of *BADH* (Zhou et al., 2018b). In the present study, the greater mRNA abundance of *BADH* in MET calves at d 4 may partly be due to greater choline concentration in liver of those calves. However, the greater concentrations of both betaine and choline at d 14 and 28 in MET calves are not explained by differences in *BADH*. Whether protein abundance of *BADH* at those days played a role is unclear. Taken together, these changes provide additional support for our hypothesis

that MET calves might have been better equipped to metabolize choline.

Deoxyribonucleic acid methyltransferases utilize SAM to induce epigenetic changes via DNA methylation (Robertson, 2005). The methyltransferases DNMT3A and DNMT3B establish DNA methylation patterns during embryonic development, whereas DNMT1 maintains methylation of DNA at 5'-C-phosphate-G-3' islands (Li and Zhang, 2014). In the present study, the greater mRNA abundance of *DNMT3A* in the liver of MET calves on d 4 and 14 differs from previous

work with calves born from dams supplemented with RPM, in which abundance of that gene in the liver was lower overall during the first 50 d of age (Jacometo et al., 2017). However, this response agrees with the greater abundance of the gene in the placenta along with greater concentrations of 1-carbon metabolism intermediates in cows fed MET compared with cows fed CON (Batistel et al., 2019). We speculate that greater concentrations of 1-carbon metabolism intermediates in the liver of calves fed MET might have stimulated DNA methylation via *DNMT3A*.

Table 3. Abundance of genes related to methionine metabolism, DNA methylation, glutathione metabolism, and cytidine 5'-diphosphocholine (CDP)-choline pathway in neonatal Holstein calves born to dams fed a control diet (CON) or CON supplemented with ethyl-cellulose rumen-protected methionine (MET; 0.09% daily DMI) during the last 28 d of pregnancy¹

Gene ² (log ₂ scale)	Treatment group			P-value ³		
	CON	MET	SEM	T	D	T × D
Methionine cycle						
<i>MAT1A</i>	0.19	0.30	0.06	0.14	<0.01	0.27
<i>BHMT</i>	0.94	0.88	0.03	0.13	<0.01	0.06
<i>BHMT2</i>	0.21	0.19	0.08	0.84	<0.01	0.67
<i>MTR</i>	0.81	0.98	0.07	0.06	<0.01	0.42
<i>SAHH</i>	0.27	0.30	0.03	0.28	<0.01	0.68
<i>PEMT</i>	0.35	0.44	0.04	0.07	<0.01	0.26
<i>BADH</i>	1.27	1.32	0.05	0.47	<0.01	0.05
<i>CHDH</i>	2.03	1.74	0.14	0.10	<0.01	0.73
<i>SARDH</i>	0.96	1.00	0.03	0.30	<0.01	0.19
DNA methylation						
<i>DNMT1</i>	1.03	1.00	0.02	0.30	<0.01	0.17
<i>DNMT3A</i>	0.92 ^b	1.04 ^a	0.03	<0.01	<0.01	0.04
Transsulfuration						
<i>CBS</i>	0.93	0.86	0.06	0.43	<0.01	0.98
<i>CTH</i>	1.05	1.04	0.10	0.92	0.05	0.77
<i>CSAD</i>	0.62	0.56	0.03	0.16	<0.01	0.23
<i>CDO1</i>	0.83	0.78	0.06	0.63	<0.01	0.93
Glutathione pathway						
<i>GCLC</i>	0.83	0.84	0.05	0.82	<0.01	0.49
<i>GSS</i>	0.86	0.88	0.02	0.51	<0.01	0.12
<i>GSR</i>	0.93	0.88	0.07	0.66	<0.01	0.56
<i>GPX1</i>	0.94	0.87	0.06	0.34	<0.01	0.91
CDP-choline pathway						
<i>CHKA</i>	0.81 ^b	0.94 ^a	0.05	0.05	<0.01	0.61
<i>CHKB</i>	0.87 ^b	0.95 ^a	0.03	0.05	<0.01	0.23
<i>PCYT1A</i>	0.93	0.87	0.08	0.55	<0.01	0.04
<i>PCYT1B</i>	0.61	0.64	0.05	0.65	<0.01	0.96
<i>CEPT1</i>	0.91	0.99	0.09	0.54	<0.01	<0.01

^{a,b}Means in the same row with different superscripts differ ($P \leq 0.05$).

¹Values are means and pooled SEM; n = 14/treatment group.

²*BADH* = betaine aldehyde dehydrogenase; *BHMT* = betaine homocysteine methyltransferase; *CBS* = cystathionine β-synthase; *CDO1* = cysteine dioxygenase 1; *CEPT1* = choline/ethanolamine phosphotransferase 1; *CHDH* = choline dehydrogenase; *CHKA* = choline kinase α; *CHKB* = choline kinase β; *CSAD* = cysteine sulfonic acid decarboxylase; *CTH* = cystathionine gamma ligase; *DNMT1* = DNA methyltransferase 1; *DNMT3A* = DNA methyltransferase 3A; *GCLC* = glutamate-cysteine ligase, catalytic subunit; *GPX1* = glutathione peroxidase 1; *GSR* = glutathione reductase; *GSS* = glutathione synthase; *MAT1A* = methionine adenosyltransferase 1A; *MTR* = 5-methyltetrahydrofolate-homocysteine methyltransferase; *PCYT1A* = phosphate cytidylyltransferase 1, choline, a; *PCYT1B* = phosphate cytidylyltransferase 1, choline, b; *PEMT* = phosphatidylethanolamine methyltransferase; *SAHH* = S-adenosylhomocysteine hydrolase; *SARDH* = sarcosine dehydrogenase.

³T = maternal treatment; D = day; T × D = maternal treatment × day interaction.

Phosphatidylcholine Synthesis

Phosphatidylcholine (**PC**) can be synthesized by the CDP-choline pathway, where choline kinases are key enzymes (Fagone and Jackowski, 2013). In vivo synthesis of PC also occurs via SAM donating 3 methyl groups to phosphatidylethanolamine, a reaction catalyzed by the enzyme PEMT (Zhou et al., 2017). Thus, greater mRNA abundance of *CHKA*, *CHKB*, and *PEMT* in MET calves suggested that enhanced maternal Met supply during late gestation contributed to greater PC synthesis in the liver. Although we did not study aspects of lipoprotein metabolism in the liver or measured triacylglycerol concentration, it is known that PC participates in very-low-density lipoprotein synthesis, which helps prevent fatty liver (Lor et al., 2013; Jia et al., 2019; Zhu et al., 2019). It remains to be determined what, if any, effects a change in hepatic PC concentration can induce in terms of lipoprotein metabolism in preweaned calves. Because of the high level of lipid that calves consume via milk replacer, it seems logical to speculate that PC plays a key role. There is evidence that inadequate supply of Met and choline can reduce PC concentrations in the liver and results in fatty liver (Hopper and Johnson, 1956).

Transsulfuration Pathway

Homocysteine can be converted to cystathionine via CBS, with cystathionine subsequently being utilized to produce cysteine (Stipanuk and Ueki, 2011), which can be utilized to produce the antioxidants GSH and taurine (Stipanuk and Ueki, 2011). It is estimated that almost half of the cellular antioxidant GSH is produced from homocysteine by conversion through the transsulfuration pathway (Mosharov et al., 2000). Greater activity of CBS and concentrations of cystathionine, cysteine sulfinic acid, hypotaurine, and taurine in MET calves suggests that they had greater metabolic rate of transsulfuration, mainly from 4 to 28 d of age. This principal catabolic pathway also requires glycine and serine from the oxidation of Met (Rees et al., 2006). Thus, the increased concentrations of glycine and serine produced from the greater cycling of the Met cycle could also be utilized to support the increased flux of the transsulfuration pathway in MET calves.

Taurine can be synthesized from the oxidation of cysteine via cysteine dioxygenase (**CDO**), which generates cysteine sulfinate that is decarboxylated by cysteine sulfinic acid decarboxylase (**CSAD**; Ueki and Stipanuk, 2009). At least in non-ruminants, both in vivo and in vitro studies demonstrated that increased protein or

sulfur AA supplementation resulted in greater mRNA abundance, protein, and activity of CDO (Kwon and Stipanuk, 2001; Stipanuk et al., 2002; Dominy et al., 2006). In addition, Met supply increased the mRNA abundance of *CSAD* in isolated primary hepatocytes from dairy cows (Zhou et al., 2018a). Because the mRNA abundance of *CDO* and *CSAD* did not differ but taurine concentration was greater overall in MET calves, it is likely that the greater activity of CBS was the major factor controlling taurine. Greater abundance of CDO and CSAD protein also might have played a role.

The peak of taurine at d 14 followed by the gradual decrease with age suggested that it might play an important role during the early neonatal period. This may be related to the critical function that antioxidant defense systems play in several physiological processes during the neonatal stage (Mutinati et al., 2014). Comparing d 6 of age with the first 28 d of life, data indicate that calves have greater oxidative stress indices in serum at 28 d of age (Abuelo et al., 2014). In addition, milk replacers are a poor source of antioxidants (Soberon et al., 2012), as is typical of commercial milk replacers. Thus, increased taurine production in MET calves during this period may help with antioxidant responses. This idea is further supported by data from an in vitro study in which taurine supplementation mitigated the inflammatory activation of blood polymorphonuclear leukocytes from neonatal Holstein calves (Abdelmegeid et al., 2017). The antioxidant status in calves cannot be underestimated because subtle increases in oxidative stress and inflammation could affect important physiologic functions in key organs such as the liver (Li et al., 2015; Du et al., 2018).

In non-ruminants, higher dietary Met supply typically leads to greater CBS activity in the liver (Finkelstein and Mudd, 1967). Despite the greater CBS activity at d 4 in CON calves, the lower mRNA abundance of AA transporters in the placenta from CON cows along with the similar milk replacer and starter intake of the calves makes it unlikely that they had greater Met at d 4. Instead, we speculate that the greater activity of CBS in CON calves might have been related to oxidative status because there is evidence in non-ruminants that as oxidative stress increases, the activity of CBS also increases to promote GSH or taurine synthesis (Mosharov et al., 2000; Zou and Banerjee, 2003). It is also important to consider that in the fetal-to-neonatal transition, important circulatory and respiratory changes in the newborn result in oxidative stress (Sastre et al., 1994; Abuelo et al., 2019). Thus, the lower concentration of taurine coupled with higher enzyme activity of CBS in CON calves at d 4 suggested that those calves

might have spared more Met for antioxidant synthesis to improve redox status in liver.

Taken together, the changes in hepatic metabolites of the transsulfuration pathway suggest that calves were producing more taurine rather than GSH. Although it is not clear why calves were synthesizing taurine rather than GSH, it may be partly linked to concentrations of cysteine. The enzyme CDO is known to be regulated by cysteine supply such that elevated concentrations of cysteine increase CDO activity (Yin et al., 2016). The overall increase in CBS activity in MET calves was associated with numerically greater concentration of cysteine in the liver. Thus, this response might have increased CDO activity, leading to greater taurine production rather than GSH.

Bile Acids

The primary bile acids formed in the liver are cholic and chenodeoxycholic acid, which are subsequently conjugated with glycine or taurine for secretion into bile (Chiang, 2013). Ruminants secrete more taurine-conjugated than glycine-conjugated bile acids (Moore and Christie, 1984). Because feed intake remained the same, the greater concentrations of glycochenodeoxycholic, glycocholic, taurocholic, and taurochenodeoxycholic acids in MET calves suggest enhanced conjugating capacity in those animals. This idea is supported in part by the greater concentrations of glycine and taurine in MET calves. The fact that glycine is also utilized in the synthesis of GSH underscores the potential for competition between pathways; that is, greater utilization of glycine for bile acid conjugation rather than GSH synthesis could explain the greater concentration of taurine in MET calves.

TCA Cycle and Animal Performance

The supply of RUP in beef cows to achieve 128 and 151% of estimated protein requirements during the last third of pregnancy not only led to greater BW of the cows during late pregnancy but also resulted in greater calf birth BW (Sletmoen-Olson et al., 2000). It is also noteworthy that calves from cows fed greater levels of RUP during pregnancy had 11% greater BW at 12 wk of age. The tendency for greater birth BW and greater neonatal BW in MET calves is similar to that in beef calves but also that reported for lambs born to sheep supplemented with RPM during the last month of gestation (Liu et al., 2016). Thus, we speculate that increased circulating Met in dams as well as maternal DMI (Batistel et al., 2017a) would give the fetus access to greater amounts of nutrients.

Because the TCA cycle is a central metabolic pathway connecting carbohydrate, lipid, and protein metabolism, the greater concentrations of its metabolites in MET calves at 4 d of age likely were a reflection of a maternal response. The fact that differences in concentrations of TCA cycle intermediates between groups were evident through at least 28 d of age despite similar rates of feed intake further suggests a maternal priming effect that persisted in early life. Thus, because the neonatal ruminant animal must meet its glucose needs via lactose intake and gluconeogenesis, flux through the TCA cycle is essential for supplying intermediates for gluconeogenesis. We speculate that alterations in concentrations of metabolites in the TCA cycle would reflect the greater activity needed to achieve greater BW by MET calves.

CONCLUSIONS

Overall, enhanced postruminal maternal supply of Met contributes to maintaining Met homeostasis, phosphatidylcholine synthesis, and increased taurine concentrations in the neonatal calf liver via changes in activity of key 1-carbon metabolism enzymes. Greater flux through the TCA cycle along with greater taurine synthesis appear to be important adaptations caused by maternal Met supply that allow calves to carry over a greater birth body mass through the immediate postweaning period. Additional work is needed to fully understand the mechanisms regulating 1-carbon and nutrient metabolism in the developing bovine fetus when the postruminal maternal supply of methyl donors is enhanced.

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