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Maternal rumen-protected methionine supplementation and its effect on blood and liver biomarkers of energy metabolism, inflammation, and oxidative stress in neonatal Holstein calves

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ABSTRACT

In nonruminants, nutrition during pregnancy can program offspring development, metabolism, and health in later life. Rumen-protected Met (RPM) supplementation during the prepartum period improves liver function and immune response in dairy cows. Our aim was to investigate the effects of RPM during late pregnancy on blood biomarkers (23 targets) and the liver transcriptome (24 genes) in neonatal calves from cows fed RPM at 0.08% of diet dry matter/d (MET) for the last 21 d before calving or controls (CON). Blood (n = 12 calves per diet) was collected at birth before receiving colostrum (baseline), 24 h after receiving colostrum, 14, 28, and 50 d (post-weaning) of age. Liver was sampled (n = 8 calves per diet) via biopsy on d 4, 14, 28, and 50 of age. Growth and health were not affected by maternal diet. The MET calves had greater overall plasma insulin concentration and lower glucose and ratios of glucose-to-insulin and fatty acids-to-insulin, indicating greater systemic insulin sensitivity. Lower concentration of reactive oxygen metabolites at 14 d of age along with a tendency for lower overall concentration of ceruloplasmin in MET calves indicated a lesser degree of stress. Greater expression on d 4 of fructose-bisphosphatase 1 (*FBP1*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), and the facilitated bidirectional glucose transporter *SLC2A2* in MET calves indicated alterations in gluconeogenesis and glucose uptake and release. The data agree with the greater expression of the glucocorticoid receptor (*GR*). Greater expression on d 4 of the insulin receptor (*INSR*) and insulin-responsive serine/threonine-protein kinase (*AKT2*) in MET calves indicated alterations in insulin signaling. In that context, the similar expression of sterol regulatory element-binding transcription factor 1 (*SREBF1*) in CON and MET during the preweaning period followed by the marked upregulation regardless of diet after weaning (d 50) support the idea of changes in hepatic insulin sensitivity during early postnatal life. Expression of carnitine palmitoyltransferase 1A (*CPT1A*) was overall greater and acyl-CoA oxidase 1 (*ACOX1*) was lower in MET calves, indicating alterations in fatty acid oxidation. Except forkhead box O1 (*FOXO1*), all genes changed in expression over time. Transcriptome results indicated that calves from MET-supplemented cows underwent a faster maturation of gluconeogenesis and fatty acid oxidation in the liver, which would be advantageous for adapting to the metabolic demands of extrauterine life.

Key words: nutritional programming, nutrition, methyl donors, transcriptomics

INTRODUCTION

During pregnancy, the maternal diet is one important factor that can elicit epigenetic effects in the offspring with long-term metabolic and physiologic consequences (Wu et al., 2004; Barua and Junaid, 2015). Epigenetic alterations can be induced through methylation of DNA and histones, acetylation of histones, or changes in microRNA expression, all of which alter transcription of the target genes (Jaenisch and Bird, 2003). In one of the first studies with ruminants, a low-protein diet fed to pregnant sheep resulted in lower methylation level of CpG islands in the fetal *IGFR2* (insulin-like growth factor 2 receptor) and *H19* genes in longissimus muscle, both of which have key roles in regulating growth and body composition (Lan et al., 2013). Limiting protein intake during pregnancy in rats also reduced

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histone acetylation of the liver-X-receptor α (*NR1H3*) promoter region in the offspring, silencing its expression and increasing the expression of its target glucose 6-phosphatase (*G6PC*), a key player in gluconeogenesis (Vo et al., 2013). Clearly, these data underscored the role of maternal protein nutrition on the programming of metabolic functions in the offspring.

More recent work in nonruminant species has revealed that maternal dietary methyl donors (e.g., Met, folic acid, betaine) play a role in nutritional programming of the offspring; that is, the concept that differences in nutritional experience at critical periods in early life, both pre- and postnatally, can program an individual's development, metabolism, and health for the future (Ji et al., 2016). In the context of gene transcription regulation, methyl donors serve as precursors of S-adenosylmethionine that could be used via methyltransferases to methylate DNA, RNA, and histones (Hollenbeck, 2012; Lin et al., 2014). In newborn piglets, it was demonstrated that maternal folic acid supplementation altered the expression of genes associated with immunity, oxidative stress response, and hepatic energy metabolism (Liu et al., 2013). In addition, supplementing betaine to sows during pregnancy resulted in alterations in the expression of gluconeogenic genes in the liver of newborn piglets partly through changes in DNA methylation (Cai et al., 2014). Whether similar responses occur in dairy calves is unknown, but recent work from our laboratory revealed that supplementation of pregnant cows with organic trace minerals from −30 d to calving (40 mg/kg of diet DM of Zn, 20 mg/kg of Mn, 5 mg/kg of Cu, and 1 mg/kg of Co) was associated with changes in expression of micro RNA in blood neutrophils and also systemic biomarkers of inflammation, oxidative stress, and liver function during the first 21-d of life (Jacometo et al., 2015).

Our general hypothesis was that rumen-protected Met supplementation during late-pregnancy, besides benefitting cows (Osorio et al., 2013, 2014a,b), would also be associated with changes in neonatal calf liver gene expression. Thus, the specific objectives of the present study were to determine the expression of genes related to energy metabolism, insulin signaling pathway, growth hormone-IGF-1 axis, glucocorticoid and adrenergic receptors, and also concentrations of immunometabolic biomarkers of metabolism, liver function, inflammation, and oxidative stress.

MATERIALS AND METHODS

All the 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 #13023).

Maternal Treatments

Calves in the present study were from cows randomly assigned to receive rumen-protected Met (**MET**, n = 21; Smartamine M, Adisseo NA, Alpharetta, GA) at 0.08% of diet DM/d (~2.8:1 Lys:Met) or no supplemental Met (**CON**; n = 20, ~3.6:1 Lys:Met) from −21 ± 2 before expected calving date until 30 DIM. The MET supplement was top-dressed once daily at the morning feeding using approximately 50 g of ground corn as carrier for all treatments. The TMR DM for the close-up and lactation diets was measured weekly for estimation of daily TMR DM offered. Supplementation of Met (0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Met, physically protected by a pH-sensitive coating, which is considered to have a Met bioavailability of 80% (Graulet et al., 2005); therefore, per 10 g of Smartamine M, the cows received 6 g of metabolizable Met. The Met supplement was top-dressed on the TMR. Ingredient and chemical composition of the diets is in the Supplemental Table S1 (http://dx.doi.org/10.3168/jds.2016-11018). Cow BW (773 ± 11 kg) and BCS (3.51 ± 0.05) did not differ. After birth, calves were fed the same milk replacer and starter, and managed similarly.

Animal Management and Calf Enrolment Criteria

During the dry period, cows were housed in a ventilated, sand-bedded freestall barn, with a photoperiod of 8 h of light and 16 h of dark. Diets were fed for ad libitum intake as a TMR once daily using an individual gate feeding system (American Calan, Northwood, NH) and DMI was recorded daily. As cows began demonstrating signs of impending parturition, they were moved to an individual maternity pen bedded with straw. On average, cows remained in the maternity pen for 3.69 ± 3.61 d. After parturition, cows were milked at the end of the farm's next milking period (0400, 1200, or 2000 h). Colostrum volume was recorded and IgG content was estimated based on specific gravity with a bovine colostrometer (Nasco, Fort Atkinson, WI; Cat. no. C10978N).

Calves were kept in the experiment if they fulfilled all the following criteria: (1) single calf; (2) calving difficulty score <3; (3) dam's colostrum quality assessed by a bovine colostrometer of >50 mg/L of IgG; (4) dam produced at least 3.8 L of a good-quality first

colostrum; and (5) calf birth weight >36 kg (Johnson et al., 2007). After birth, calves were weighed, had the navel disinfected with a 7% tincture of iodine solution (First Priority Inc., Elgin, IL), were vaccinated with TSV II (Pfizer Inc., New York, NY) via nostril application, and received 3.8 L of first-milking colostrum from the respective dam within 8 h after birth. If voluntary colostrum intake had not reached the 3.8 L required, calves were force-fed via esophageal tube. Calves were housed in individual outdoor hutches bedded with straw, fed twice daily with a milk replacer (Advance Excelerate, Milk Specialties, Carpentersville, IL; 28.5% CP, 15% fat) at rates of 520 g/d from 1 to 10 d of age, 680 g/d from 11 to 20 d of age, 840 g/d from 21 to 35 d of age, and 420 g/d from 36 to 42 d of age in a single feeding; cows had ad libitum access to a starter grain mix (19.9% CP, 13.5% NDF). Health checks, including fecal score (scale 1–4) and respiratory score (scale 1–4), were recorded daily until weaning (Osorio et al., 2012), whereas rectal temperature was recorded daily until 21 d of age. Growth performance including BW and withers height were recorded weekly. Calves were weaned at 42 d of age. All calves remained clinically healthy during the study.

Sample Collection

Blood samples were collected from the jugular vein using 20-gauge BD Vacutainer needles (Becton Dickinson, Franklin Lakes, NJ) before receiving colostrum (baseline), 24 h after receiving colostrum, and at 14, 28, and 50 d (n = 12/group). At each time point, a total of 40 mL of blood were collected in vacutainer tubes (10 mL, BD Vacutainer, Becton Dickinson) containing serum clot activator or lithium heparin. After blood collection, tubes with lithium heparin were placed on ice whereas tubes with clot activator were kept at room temperature until centrifugation (~30 min). Serum and plasma were obtained by centrifugation of clot activator and lithium heparin tubes, respectively, at 1,900 × *g* for 15 min at 4°C. Serum and plasma were aliquoted and stored at −80°C until further analysis.

Liver was sampled via puncture biopsy (Swanson et al., 2000) from calves under local anesthesia before the afternoon feeding, at approximately 1500 h on d 4, 14, 28, and 50 of age (n = 8/group). Tissue specimens were stored in liquid N_2 until further analysis.

Apparent Efficiency Absorption of Immunoglobulin

Apparent efficiency of absorption was estimated by measuring the IgG content in both first-milking colostrum and calf serum at 24 h after colostrum ingestion. Apparent efficiency of absorption was calculated as plasma IgG (g/L) × plasma volume (L)/IgG intake (g) (Quigley et al., 2002).

Blood Metabolites, Acute-Phase Proteins, and Oxidative Stress Biomarkers

Blood samples were analyzed for the target biomarkers using the same kits and procedures described in (Jacometo et al., 2015). Biomarkers of interest were albumin, cholesterol, bilirubin, creatinine, urea, aspartate aminotransferase (**AST**), gamma-glutamyl transferase, glucose, fatty acids, BHB, haptoglobin, ceruloplasmin, paraoxonase, myeloperoxidase, antioxidant potential [ferric-reducing antioxidant power (**FRAP**)], reactive oxygen metabolites (**ROMt**), retinol, tocopherol, IL-6, and IL-1β. Insulin was analyzed using a bovine ELISA kit (Cat No. 10–1201–01, Mercodia AB, Uppsala, Sweden). Immunoglobulin G concentration (colostrum and serum) was measured using a quantitative bovine IgG ELISA kit (ZeptoMetrix Corporation, Buffalo, NY).

RNA Isolation

For liver total RNA extraction, the miRNeasy kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocols. Samples were treated on-column with DNaseI (Qiagen), concentration was measured using the NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE), and RNA quality was measured using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Samples used in the analysis had a mean RNA integrity number of 7.2 ± 0.2.

cDNA Synthesis and Quantitative Real-Time PCR

The protocols used for cDNA synthesis and quantitative real-time PCR were exactly the same as those reported by Jacometo et al. (2015). Primer design and testing also was performed exactly as described by Jacometo et al. (2015). For this study, *GAPDH*, ubiquitously expressed prefoldin-like chaperone (*UXT*), and ribosomal protein S9 (*RPS9*) were used as internal control genes, and their geometric mean was used to normalize the expression data. All evaluated genes and primer information are reported in the supplemental material (Supplemental Tables S2–S6; http://dx.doi.org/10.3168/jds.2016-11018).

Statistical Analysis

Data were analyzed with the Proc MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Fixed ef-

fects in the model were treatment (T), day (D) or week (wk), and their interaction (T × D, T × wk). Random effect was calf within diet. The exponential correlation covariance structure SP for repeated measures was used for analysis of blood metabolites and gene expression. Blood metabolites and gene expression results were $\log_2$-scale transformed if needed to comply with normal distribution of residuals, and subsequently back-transformed. Least squares means separation between time points was performed using the PDIFF statement. Statistical significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Apparent Efficiency of IgG Absorption, Growth Performance, and Health

Colostrum IgG concentration (n = 12 per diet) was not affected by maternal diet and averaged 75.6 ± 6.6 mg/dL for CON and 81.8 ± 6.7 mg/dL for MET cows. Similarly, calf apparent efficiency of IgG absorption (n = 12 per diet) was not affected, averaging 26.1 ± 3.2% for CON and 30.3 ± 3.2% for MET calves. Calf birth BW (n = 12 per diet) was similar between groups (CON, 42.9 ± 1.5 kg; MET, 44.2 ± 1.6 kg). Weekly BW, weekly gain, and withers height until 7 wk of life also did not differ between groups (Supplemental Table S6; http://dx.doi.org/10.3168/jds.2016-11018); however, a linear increase ($P < 0.001$) was observed for all parameters in both groups (Supplemental Table S6). Regardless of maternal diet, starter intake increased ($P < 0.001$) over time (Supplemental Table S7: http://dx.doi.org/10.3168/jds.2016-11018). In addition, rectal temperature, respiratory score, and fecal score were not affected by maternal treatment. However, both groups had a higher fecal score at wk 2 and then decreased until weaning time ($P < 0.001$; Supplemental Table S7).

Blood Immunometabolic Biomarkers

Metabolism and Liver Function. A treatment by day interaction ($P = 0.04$) was detected for glucose concentration, where MET calves had lower ($P = 0.007$) glucose at birth (Table 1); however, no overall maternal diet effect was noted. Both groups had a marked increase in glucose ($P < 0.001$) concentration from birth to 24 h after colostrum intake, and then decreased ($P < 0.001$) at 14 d and remained constant until 50 d. Similarly, insulin concentration had a peak 24 h after colostrum intake ($P < 0.001$), and was higher overall in MET calves ($P = 0.011$) (Table 1). Concentrations of fatty acids were not affected by maternal diet, but it decreased ($P < 0.001$) over time, with a marked reduction from birth to 24 h after colostrum intake (Table 1). The ratios of fatty acids to insulin and glucose to insulin were overall lower ($P < 0.05$) in MET calves.

The hepatic enzymes AST and gamma-glutamyl transferase had a time effect ($P < 0.001$), mainly due to the increased concentration at 24 h after colostrum intake (Table 1). Concentration of AST also had a treatment by day interaction ($P = 0.05$). At birth, MET calves had lower ($P = 0.009$) AST concentration. Calves in MET also had greater ($P = 0.01$) creatinine concentration at birth compared with CON, but no overall maternal diet effect was detected (Table 1). Creatinine concentration had a marked decrease ($P < 0.001$) after colostrum intake. Neither cholesterol nor BHB concentrations were affected by maternal diet, but both increased over time ($P < 0.001$) with peak concentrations at 50 d of age (Table 1).

Bilirubin concentration peaked at 24 h after colostrum intake ($P < 0.001$) and then decreased markedly through 50 d of age, with no maternal diet effect (Table 1). A treatment by day interaction ($P = 0.002$) was detected for urea concentration because MET calves compared with CON calves had higher ($P = 0.02$) concentration at 24 h and then lower concentration at 14 d of age. A day effect ($P < 0.001$) was observed, mainly due to a marked increase in concentration from birth to 50 d of age (Table 1).

Inflammation. No overall maternal treatment effect was detected for any of these biomarkers (Table 1). Haptoglobin had a day effect ($P < 0.001$) mainly due to a peak in concentration at 28 d of age. Paraoxonase, albumin, and ceruloplasmin concentrations increased ($P < 0.001$) over time, with a tendency ($P = 0.09$ and 0.11) for albumin and ceruloplasmin to be overall lower in MET calves. Ceruloplasmin also had a treatment by day interaction ($P = 0.003$), with a lower ($P = 0.004$) concentration in MET calves at 14 d of age. Concentrations of IL-1β and IL-6 decreased markedly ($P < 0.001$) from birth to 24 h after colostrum intake. The concentration of IL-1β was highest at birth, whereas IL-6 was highest at 14 d of age.

Oxidative Stress. No overall maternal treatment effect was observed for these biomarkers (Table 1). A treatment by day intercation was detected for tocopherol ($P = 0.05$) and ROMt ($P = 0.06$), with lower concentrations ($P = 0.02$ and 0.001, respectively) in MET than CON calves at 14 d of age. Regardless of maternal treatment, ROMt and myeloperoxidase had peak concentrations at 14 d of age, whereas FRAP had a nadir at the same time ($P < 0.001$). Tocopherol had a peak in concentration at 28 d ($P < 0.001$), and retinol had a consistent increase over time ($P < 0.001$).

Table 1. Blood immunometabolic biomarkers related to energy metabolism, liver function, inflammation, and oxidative stress in calves born to dams fed a conventional diet (CON) or CON supplemented with rumen-protected Met (MET) during the last 21 d prepartum

		Day of age						*P*-value[2]		
Item	Treatment	0	1	14	28	50	SEM[1]	Trt	Day	T × D
Energy metabolism and liver function										
Glucose, mmol/L	MET	4.05^{B,d}	8.91^{a}	5.70^{c}	6.80^{b}	6.07^{bc}	0.32	0.182	<0.001	0.045
	CON	4.74^{A,c}	9.73^{a}	6.18^{b}	6.35^{b}	5.96^{b}	0.32			
Fatty acids, mmol/L	MET	1.11	0.24	0.26	0.21	0.13	0.06	0.910	<0.001	0.712
	CON	1.08	0.29	0.21	0.19	0.14	0.06			
Insulin, μg/L	MET	0.26	1.46	0.19	0.93	0.32	0.24	0.011	<0.001	0.161
	CON	0.09	1.43	0.17	0.13	0.12	0.23			
Glucose-to-insulin ratio	MET	159.7	10.6	84.2	29.3	31.5	39.8	0.007	<0.001	0.152
	CON	113.4	23.6	186.9	180.6	94.3	39.8			
Fatty acids-to-insulin ratio	MET	32.8	0.33	3.76	1.58	0.69	5.01	0.022	<0.001	0.184
	CON	31.6	0.71	6.11	6.94	2.94	5.26			
BHB, mmol/L	MET	0.10	0.16	0.13	0.16	0.33	0.04	0.161	<0.001	0.465
	CON	0.05	0.05	0.07	0.11	0.30	0.04			
Creatinine, μmol/L	MET	208.9^{A,a}	112.7^{b}	89.3^{c}	83.7^{c}	118.7^{b}	4.35	0.348	<0.001	0.015
	CON	180.9^{B,a}	113.2^{b}	86.1^{c}	81.2^{c}	119.8^{b}	4.19			
Urea, mmol/L	MET	4.62^{b}	4.16^{A,bc}	2.74^{B,d}	3.63^{c}	7.05^{a}	0.28	0.818	<0.001	0.002
	CON	4.45^{b}	3.39^{B,c}	3.57^{A,c}	3.56^{c}	6.46^{a}	0.28			
Bilirubin, μmol/L	MET	12.6	16.6	4.51	3.48	1.15	1.02	0.703	<0.001	0.981
	CON	12.9	15.0	3.95	3.02	1.04	1.06			
AST,[3] U/L	MET	36.4^{B,d}	99.4^{a}	49.4^{c}	47.4^{c}	70.7^{b}	3.93	0.130	<0.001	0.045
	CON	45.0^{A,d}	110.7^{a}	50.5^{cd}	54.1^{c}	62.7^{b}	4.08			
GGT,[4] U/L	MET	12.5	1,884.3	139.9	55.0	22.3	140.7	0.169	<0.001	0.152
	CON	9.95	3,026.2	186.8	67.2	24.1	140.7			
Cholesterol, mmol/L	MET	0.59	0.85	1.51	2.49	2.49	0.16	0.940	<0.001	0.408
	CON	0.60	0.88	1.74	2.28	2.48	0.16			
Albumin, g/L	MET	29.0	26.2	29.4	32.0	34.2	0.42	0.098	<0.001	0.353
	CON	28.9	26.8	30.5	33.1	34.9	0.41			
Inflammation										
Ceruloplasmin, μmol/L	MET	0.26^{c}	0.87^{b}	2.53^{a}	2.66^{a}	3.57^{a}	0.17	0.110	<0.001	0.003
	CON	0.17^{c}	1.01^{b}	3.24^{a}	2.98^{a}	3.89^{a}	0.17			
Haptoglobin, g/L	MET	0.24	0.23	0.24	0.56	0.22	0.06	0.121	<0.001	0.566
	CON	0.17	0.16	0.30	0.47	0.18	0.06			
Paraoxonase, U/mL	MET	12.5	10.0	26.6	48.4	68.2	4.88	0.287	<0.001	0.868
	CON	14.8	10.1	29.4	42.9	67.2	4.30			
IL-1β, pg/mL	MET	69.8	31.5	35.7	24.5	20.3	6.81	0.962	<0.001	0.172
	CON	71.4	28.4	31.4	13.6	39.6	6.47			
IL-6, pg/mL	MET	1,946.4	1,052.1	2,645.5	1,812.7	1,963.8	338.5	0.373	<0.001	0.977
	CON	1,710.8	1,104.1	2,287.7	1,157.8	2,062.5	350.8			
Oxidative stress and antioxidants										
Myeloperoxidase, U/L	MET	274.4	431.1	466.1	457.8	381.9	28.6	0.823	<0.001	0.113
	CON	228.2	442.1	544.0	431.4	392.3	27.4			
ROMt,[5] mg of H_2O_2/100 mL	MET	7.40^{c}	11.7^{ab}	12.4^{B,a}	12.7^{a}	10.8^{b}	0.67	0.140	<0.001	0.061
	CON	7.31^{d}	11.7^{c}	15.5^{A,a}	13.6^{b}	11.0^{c}	0.65			
FRAP,[6] μmol/L	MET	201.5	165.1	114.8	134.1	127.8	6.19	0.383	<0.001	0.847
	CON	194.9	158.9	117.0	128.1	127.0	5.90			
Retinol, μg/100 mL	MET	11.9	13.8	19.1	22.5	46.7	2.26	0.371	<0.001	0.113
	CON	10.4	15.8	24.0	28.1	46.9	2.20			
Tocopherol, μg/mL	MET	0.39^{c}	1.32^{b}	1.31^{B,b}	4.37^{a}	1.88^{b}	0.26	0.401	<0.001	0.048
	CON	0.46^{d}	1.61^{c}	2.19^{A,b}	3.91^{a}	2.13^{b}	0.26			

^{A,B}Differences ($P < 0.05$) between treatments within time point.

$^{a–d}$Differences ($P < 0.05$) between time points within treatment.

[1]Greatest SEM is shown.

[2]*P*-value for treatment (Trt), day, and their interaction (T × D).

[3]Aspartate aminotransferase.

[4]Gamma-glutamyl transferase.

[5]Reactive oxygen metabolites.

[6]Ferric reducing antioxidant power.

Table 2. Expression of genes related to gluconeogenesis, fatty acid oxidation, lipoprotein metabolism, growth hormone (GH)-IGF-1 axis, insulin signaling pathway, inflammatory response, and glucocorticoid and adrenergic receptors in calves born to dams fed a conventional diet (CON) or CON supplemented with rumen-protected Met (MET) during the last 21 d prepartum

		Day of age					*P*-value[2]		
Item[3]	Treatment	4	14	28	50	SEM[1]	Trt	Day	T × D
Gluconeogenesis									
PCK1	MET	1.20^{A,c}	0.89^{c}	1.18^{A,b}	1.61^{B,a}	0.11	0.050	<0.001	<0.001
	CON	0.65^{B,c}	0.99^{b}	0.85^{B,b}	1.73^{A,a}	0.10			
PC	MET	1.12	0.94	0.80	0.77	0.07	0.007	<0.001	0.367
	CON	1.15	0.98	0.93	0.96	0.07			
G6PC	MET	1.34	1.09	1.30	1.67	0.11	0.258	<0.001	0.415
	CON	1.29	1.07	1.03	1.73	0.10			
FBP1	MET	1.25^{A,a}	1.06^{b}	1.03^{b}	1.26^{a}	0.07	0.020	<0.001	0.034
	CON	0.90^{B,b}	1.00^{b}	0.90^{b}	1.30^{a}	0.06			
Fatty acid oxidation									
PPARA	MET	1.24	1.13	1.16	1.34	0.07	0.478	<0.001	0.685
	CON	1.21	1.15	1.07	1.30	0.07			
ACOX1	MET	0.96^{b}	0.94^{b}	0.84^{B,c}	1.14^{a}	0.04	0.010	<0.001	0.004
	CON	0.91^{c}	1.03^{b}	1.09^{A,ab}	1.19^{a}	0.04			
CPT1A	MET	1.28	1.21	1.10	1.29	0.04	<0.001	<0.001	0.107
	CON	1.09	1.11	0.83	1.07	0.04			
HMGCS2	MET	1.54	1.04	1.13	0.91	0.10	0.885	<0.001	0.093
	CON	1.29	0.96	1.08	1.09	0.10			
Lipoprotein metabolism									
APOB	MET	1.03	1.18	1.53	1.41	0.11	0.578	<0.001	0.198
	CON	1.07	1.29	1.27	1.62	0.11			
MTTP	MET	1.04	1.10	1.08	1.19	0.05	0.188	<0.001	0.383
	CON	0.94	1.04	1.09	1.13	0.05			
GH-IGF1 axis									
GHR1A	MET	0.76	0.99	1.52	1.07	0.12	0.270	0.006	0.776
	CON	0.69	0.86	1.37	0.95	0.11			
IGF1	MET	0.87	0.71	1.27	1.17	0.08	0.660	<0.001	0.259
	CON	0.73	0.82	1.32	1.21	0.08			
Insulin signaling pathway									
INSR	MET	1.01^{A,b}	0.97^{bc}	0.86^{c}	1.31^{a}	0.05	0.398	<0.001	0.004
	CON	0.83^{B,c}	1.08^{b}	0.87^{c}	1.31^{a}	0.04			
IRS1	MET	1.13	1.20	1.04	1.35	0.06	0.330	<0.001	0.398
	CON	1.12	2.10	0.91	1.31	0.05			
AKT2	MET	1.18^{A,b}	1.10^{c}	1.05^{c}	1.31^{A,a}	0.05	0.002	<0.001	0.004
	CON	0.87^{B,b}	1.04^{a}	0.94^{b}	1.05^{B,a}	0.05			
SREBF1	MET	0.98	0.81	0.73	2.36	0.12	0.385	<0.001	0.554
	CON	0.96	0.74	0.72	1.97	0.11			
SLC2A2	MET	2.33^{A,a}	1.79^{b}	1.62^{A,b}	1.58^{b}	0.12	<0.001	<0.001	<0.001
	CON	1.44^{B,a}	1.67^{a}	0.93^{B,b}	1.36^{a}	0.10			
FOXO1	MET	1.14	1.09	1.12	1.20	0.03	0.364	0.147	0.465
	CON	1.16	1.14	1.15	1.17	0.03			
Inflammatory response									
NFKB1	MET	1.10	1.03	1.16	1.16	0.03	0.009	0.051	0.079
	CON	1.04	1.03	1.00	1.05	0.03			
SOD1	MET	0.80	1.07	1.05	1.49	0.04	0.086	<0.001	0.155
	CON	0.70	1.04	1.05	1.43	0.04			
SOD2	MET	1.06^{A,a}	0.95^{A,b}	0.89^{b}	1.14^{A,a}	0.04	<0.001	<0.001	<0.001
	CON	0.84^{B,b}	0.73^{B,c}	0.97^{a}	0.89^{B,ab}	0.04			
Glucocorticoid and adrenergic receptors									
GR	MET	1.00	1.10	1.19	1.29	0.04	0.009	<0.001	0.464
	CON	0.87	1.01	1.09	1.21	0.04			
ADRA1	MET	1.12	1.15	0.97	1.17	0.06	0.173	<0.001	0.221
	CON	0.95	1.16	0.86	1.11	0.06			
ADRB2	MET	0.68^{A,c}	0.80^{c}	1.13^{b}	2.11^{a}	0.13	0.188	<0.001	<0.001
	CON	0.43^{B,c}	0.88^{b}	0.97^{b}	1.68^{a}	0.11			

^{A,B}Differences ($P < 0.05$) between treatments within time point.
$^{a–c}$Differences ($P < 0.05$) between time points within treatment.
[1]Greatest SEM is shown.
[2]*P*-value for treatment (Trt), day, and their interaction (T × D).
[3]Gene symbols: *PCK1* = phosphoenolpyruvate carboxykinase 1; *PC* = pyruvate carboxylase; *G6PC* = glucose-6-phosphatase catalytic subunit; *FBP1* = fructose-bisphosphatase 1; *PPARA* = peroxisome proliferator activated receptor α; *ACOX1* = acyl-CoA oxidase, palmitoyl; *CPT1A* = carnitine palmitoyl-transferase 1A; *HMGCS2* = 3-hydroxy-3-methylglutaryl-CoA synthase 2; *APOB* = apolipoprotein B; *MTTP* = microsomal triglyceride transfer protein; *GHR1A* = growth hormone receptor 1A; *IGF1* = insulin-like growth factor 1; *INSR* = insulin receptor; *IRS1* = insulin receptor substrate 1; *AKT2* = v-akt murine thymoma viral oncogene homolog 2; *SREBF1* = sterol regulatory element binding transcription factor 1; *SLC2A2* = solute carrier family 2 member 2; *FOXO1* = forkhead box O1; *NFKB1* = nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; *SOD1* = superoxide dismutase 1, soluble; *SOD2* = superoxide dismutase 2, mitochondrial; *GR* = glucocorticoid receptor; *ADRA1* = adrenoceptor α 1A; *ADRB2* = adrenoceptor β 2.

Hepatic Gene Expression

Gluconeogenesis. Calves in MET had overall higher *FBP1* ($P = 0.02$) and *PCK1* ($P = 0.05$) and lower ($P = 0.007$) *PC* expression (Table 2). Regardless of maternal diet, *PCK1*, *G6PC*, and *FBP1* expression increased over time ($P < 0.001$), whereas *PC* expression decreased ($P < 0.001$) over time.

Fatty Acid Oxidation. Maternal supplementation with Met led to lower ($P = 0.001$) overall *ACOX1* and greater ($P < 0.001$) *CPT1A* expression (Table 2). Furthermore, expression of *CPT1A* and *PPARA* decreased ($P < 0.001$) from 4 until 28 d and then increased ($P < 0.001$) at 50 d of age. The expression of *ACOX1* increased consistently over time ($P = 0.02$), whereas expression of *HMGCS2* decreased ($P < 0.001$) from 4 to 14 d of age.

Lipoprotein Metabolism. No maternal diet effect was detected for these genes, and only an overall increase in expression of *APOB* ($P < 0.001$) and *MTTP* ($P = 0.001$) over time was detected regardless of maternal diet (Table 2).

Growth Hormone-IGF1 Axis. No maternal diet effect was observed for these genes, but we observed an increase in expression from 4 to 28 d of age regardless of maternal diet for *IGF1* ($P < 0.001$) and *GHR1A* ($P = 0.006$). The expression of *GHR1A* then decreased ($P < 0.01$) from 28 to 50 d of age (Table 2).

Insulin Signaling Pathway. Maternal supplementation with Met resulted in greater *AKT2* ($P = 0.002$) and *SLC2A2* ($P < 0.001$) expression (Table 2). However, regardless of maternal diet, *INSR*, *IRS1*, *AKT2* and *SREBF1* expression increased ($P < 0.05$) over time, whereas *SLC2A2* decreased ($P < 0.001$). The expression of *FOXO1* was not affected (Table 2).

Inflammatory Response. Maternal supplementation with Met led to greater *NFKB1* ($P = 0.009$) and *SOD2* ($P < 0.001$) expression, and tended ($P = 0.08$) to increase the overall expression of *SOD1* (Table 2). The expression of *SOD1* and *NFKB1* increased over time ($P < 0.001$ and $P = 0.05$) whereas *SOD2* had a treatment by day interaction ($P = 0.03$) because of a decrease ($P < 0.001$) from 4 to 14 d of age followed by an increase ($P < 0.001$) from 28 to 50 d of age (Table 2).

Glucocorticoid and Adrenergic Receptors. Maternal supplementation with Met led to greater overall ($P = 0.009$) *GR* expression (Table 2). A treatment by day interaction ($P < 0.001$) for *ADRB2* was detected, with higher expression for MET than CON calves at 4 and 50 d of age ($P < 0.001$). Regardless of maternal treatment, *GR* and *ADRB2* expression increased over time ($P < 0.001$). Expression of *ADRA1* had a time effect ($P < 0.001$) mainly due to a marked reduction from birth to 28 d of age (Table 2).

DISCUSSION

Fetal growth is greatly increased during the final third trimester of gestation, and proper placental transfer of nutrients is required to ensure adequate development (Borowicz et al., 2007). Besides blood flow, the expression and activity of specific transporters in the placenta (e.g., glucose and AA) can limit nutrient delivery to the fetus (Regnault et al., 2005; Jones et al., 2007). Placental AA transport is dependent on maternal circulating AA profiles and transport capacity, both of which are affected by the composition and amount of AA in the diet (Brown et al., 2011). A recent study from our laboratories (Zhou et al., 2016) revealed that Met concentration in cow plasma decreased markedly between −21 and 10 d relative to parturition, thus indicating that Met could be a limiting AA for cow and calf during the last 3 wk prepartum. Although in the present study maternal supplementation with rumen-protected Met during the last 3 wk of pregnancy did not greatly alter calf growth during the first 7 wk of life, some of the blood biomarker and hepatic gene expression data seem to indicate a residual effect on the neonatal calf.

The changes in biomarker profiles in MET calves starting at 24 h after colostrum feeding were indicative of effects of maternal Met supplementation through components (e.g., EAA, insulin, cortisol, prolactin, IGF-1, and progesterone) in the colostrum and the calf response to those components (Blum, 2006). Compared with colostrum, the absence of bioactive compounds such as growth factors (e.g., IGF-1, IGF-2, and transforming growth factor-β1 and -β2) and hormones (e.g., insulin, prolactin, leptin, and progesterone) in a milk-based formula fed to neonatal calves was associated with lower plasma concentrations of IGF-1, leptin, 3,5,3-triiodothyronine (T3), thyroxine (T4), and insulin, as well as greater cortisol (Schäff et al., 2014). The same study indicated greater activity of glucocorticoid and adrenergic receptors (assessed through receptor saturation binding assays) in colostrum-fed calves, confirming the involvement of both pathways in the maturation of postnatal energy metabolism (Schäff et al., 2014).

Blood glucose concentration at calving could potentially be affected by factors such as time during the birth process, handing, and lag between birth and sampling. However, the fact that cortisol is positively and directly correlated with glucose (Vannucchi et al., 2015) offers support to the idea that the lower concentration of glucose at birth in MET calves, which was within the normal physiological range (Bertoni et al., 2009), was associated with a reduction in stress around calving. The lower concentration of AST in MET on d

4 also supports the idea of MET calves being under a reduced state of stress (Sánchez et al., 2002).

The higher overall insulin in MET calves along with the lower glucose and glucose-to-insulin and fatty acids-to-insulin ratios was indicative of higher insulin sensitivity. Furthermore, the greater overall concentration of creatinine in MET calves indicated a lower degree of muscle mass catabolism (Kokkonen et al., 2005; Pires et al., 2013) and supports the idea of greater insulin sensitivity. Dietary AA are potent stimulators of insulin secretion (Milner et al., 1972; Patti, 1999). If, indeed, the MET calves had higher postnatal insulin sensitivity in tissues such as skeletal muscle, supplementation with EAA might have allowed them to achieve greater rates of growth.

The combination of lower ceruloplasmin (a positive acute-phase protein), lower ROMt (metabolites that are produced during oxidative stress), lower myeloperoxidase (enzyme produced in response to ROMt), and lower tocopherol (an antioxidant) in MET calves particularly at 14 d of age was physiologically relevant because all calves experienced an increase in fecal score at that stage of life, partly because of the increase in milk replacer intake. Therefore, the changes in blood biomarkers in MET calves at a time when they were consuming greater amounts of milk replacer were indicative of a more controlled physiological response during a period of stress at birth followed by the adaptation to extrauterine life and the gradual microbial colonization of the gut (Rey et al., 2014). Overall, these changes observed reflect a better health status in MET calves (Bertoni et al., 2009; Ranade et al., 2014).

The upregulation on d 4 of *PCK1* and *FBP1*, *CPT1A*, *SLC2A2*, *AKT2*, and *GR* in MET calves underscored a faster maturation of gluconeogenesis and fatty acid oxidation along with insulin sensitivity. Such a response would be advantageous for postnatal calf development (Hammon et al., 2012). Immediately after birth, the neonate must activate glycogenolysis and especially gluconeogenesis to maintain normal glycemia to fully meet its requirements for glucose (Girard, 1990). During the first 3 d of life gluconeogenesis from lactate and AA (especially Ala) accounts for 60% of the total endogenous glucose production in calves, the remainder being from glycogenolysis (Hammon et al., 2012). At birth, these pathways are immature, and the maturation process is necessary to achieve normal metabolism; hence, the faster the calf acquires the ability to generate energy endogenously the better they can deal with the extrauterine demands of the body (Steinhoff-Wagner et al., 2011a). As calves grow and receive increasing amounts of solid feed, the development of a rumen microbial population ensures that propionate becomes the predominant gluconeogenic substrate (Donkin and Hammon, 2005).

Glucocorticoids promote gluconeogenesis (Hanson and Reshef, 1997) and impair the metabolic actions of insulin (Dimitriadis et al., 1997). Adrenergic signaling via glucocorticoid receptor also stimulates endogenous glucose production (Coker et al., 1997), a key process in neonatal calf liver (Schäff et al., 2014). Therefore, the greater overall *GR* expression in MET calves could have been a factor contributing to the faster maturation of the metabolic pathways (Schäff et al., 2015). This idea is supported by the fact that preterm calves had lower rates of gluconeogenesis at least in part due to lower plasma cortisol and hepatic PCK1 mRNA and enzyme activity (Steinhoff-Wagner et al., 2011a). Our data provide evidence that maternal diet could enhance the maturation of pathways associated with energy metabolism and insulin signaling and, hence, contribute to optimal calf adaptations to extrauterine life.

Genes encoding enzymes in the hepatic one-carbon metabolism and gluconeogenesis pathways can be programmed through DNA and histone methylation and micro RNA (Jia et al., 2012). For instance, maternal dietary protein restriction induced hypomethylation of the *G6PC* gene promoter in newborn piglets (Jia et al., 2012). Certain regions of the promoters of *PCK2* and *FBP1* were hypomethylated in the liver of newborn piglets from sows fed a diet supplemented with the methyl donor betaine (3 g/d during gestation; Cai et al., 2014). Although we are unaware of such data in calves, rumen-protected Met supplementation from −21 to 30 d around parturition in dairy cows was associated with reduced global hepatic DNA methylation and increased methylation in a *PPARA* promoter region (Osorio et al., 2016). These data offer support to the idea that the upregulation of *FBP1* expression on d 4 in MET calves could have encompassed changes in methylation status in the promoter region.

It is noteworthy that, among the genes measured, those related to lipid metabolism accounted for more than 40% of the total mRNA expression. Their greater overall abundance could have been triggered by the high plasma fatty acid concentration at birth, likely from maternal transfer, which underscored that the neonatal liver is metabolically challenged before birth and colostrum intake. Postnatally, the high content of fat in the colostrum and milk replacer fed likely contributed to the high-abundance of these genes but does not explain the overall temporal decrease (albeit small) in *PPARA*, *CPT1A*, and *HMGCS2*. The fact that plasma fatty acids decreased and BHB increased was lower in MET and CON through at least d 28 indicates a sustained ketogenic response, which is known to occur

in the neonate until after weaning (Girard 1990). The temporal decrease in *ACOX1* expression at the same time that *CPT1A* expression increased in MET calves could have been a cellular mechanism to regulate fatty acid oxidation when an overload of fatty acid uptake from milk replacer occurred that could overwhelm oxidation capacity (Jacometo et al., 2014). Some evidence points to epigenetic regulation of lipid metabolism-related gene transcription as a function of dietary methyl donor intake. For instance, betaine supplementation in apolipoprotein E-deficient mice decreased *PPARA* promoter methylation and upregulated *PPARA* expression leading to decreased liver triacylglycerol accumulation, a response those authors speculated was due partly to activation of fatty acid oxidation, which is known to be controlled by peroxisome proliferator-activated receptor-α signaling (Wang et al., 2013).

The transcription of lipogenic enzymes in liver of species such as rodents where the liver is the main lipogenic organ is closely regulated by insulin and glucose in large part through the transcription regulator SREBF1 (Wong and Sul, 2010). The postreceptor insulin signaling cascade encompasses several molecular mechanisms (Kahn and Folli, 1993), some of which remain to be elucidated from a nutritional standpoint. In the present study, it was noteworthy that expression of sterol regulatory element-binding transcription factor 1 remained largely unchanged in CON and MET until after weaning. Such a response could partly be attributed to a combination of greater insulin responsiveness after weaning along with the shift from milk replacer (higher fat content) to solid feed (lower fat content). In that context, the markedly low mRNA expression of *FOXO1* relative to other genes measured and the absence of treatment and time effects seemed to indicate an inherently low capacity of this transcription regulator to inhibit insulin signaling in calf liver as it occurs in monogastrics (Kwon and Harris, 2004).

In nonruminant hepatocytes, glucose transporter isotype *SLC2A2* (a.k.a. GLUT2) is primarily required for control of glucose uptake and glucose-sensitive gene expression (Thorens, 2015). Therefore, at least in rodents, in the absence of *SLC2A2* hepatic glucose uptake is markedly reduced but output remains unchanged (Thorens, 2015). The greater *SLC2A2*, *AKT2*, and *INSR* expression on d 4 in MET calves indicated a better ability to uptake glucose from the circulation during early postnatal life. Although expression decreased from d 4 to 14 of age, the lack of clear change in expression of these genes on d 14 and 28 in MET calves indicates a sustained ability for glucose uptake at least in part driven by greater insulin sensitivity. The gradual decrease in expression of *SLC2A2* over time after 14 d of age in MET calves agrees with gluconeogenesis becoming a more predominant pathway as the calf rumen developed. A greater degree of hepatic glucose uptake soon after birth is not unreasonable because previous work revealed that calves fed colostrum compared with formula had a higher efficiency of intestinal glucose uptake (Steinhoff-Wagner et al., 2011b).

The greater overall expression of *NFKB1* and *SOD2* in MET calves was indicative of an inflammatory state. Although no published data exist for calves, dairy cows fed rumen-protected Met during the transition period also had a greater hepatic expression of *NFKB1* and *SOD1* (Osorio et al., 2014a). It would appear that in cows any effect of Met supplementation on SOD isotypes was partly compartmentalized to the cytosol (*SOD1*) rather than mitochondria (*SOD2*; Osorio et al., 2014a). This underscores the existence of different effects of exogenous Met on inflammation and oxidative stress between dam and offspring. An in vitro study with human adipocytes demonstrated that betaine was effective in reducing the expression of inflammatory adipokines during hypoxia, underscoring the anti-inflammatory potential of certain methyl donors (Olli et al., 2013). The mechanistic reason for the greater expression of these genes in MET calves is not readily apparent. It could be possible that a greater inflammatory status would be beneficial for a timely response to exogenous stressors (e.g., pathogens or microbes colonizing the gut).

It is already well-established in nonruminants that varying the intake of methyl donors during pregnancy can elicit mechanistic changes in the offspring at least in part through the control of gene transcription (Burdge et al., 2012). Great advances have been achieved in human studies in terms of defining better the doses and physiological stages during which supplementation can elicit epigenetic consequences (Shorter et al., 2015). However, the effect of methyl donor supplementation in farm animals is still not well understood. Data on rumen-protected Met supplementation during the preimplantation period (1 through ~70 d postpartum) in dairy cows (Peñagaricano et al., 2013) clearly indicated an effect on gene transcription in the developing embryo. Although it is likely that most of the profound epigenetic changes would be induced during early embryo development, the data from the present study provides some evidence that maternal Met supplementation during late pregnancy can elicit changes related to maturation of the metabolic pathways in the liver. Based on the pattern of gene transcription, such changes could benefit calf metabolism from birth through weaning. Clearly, additional research in this area to clarify the underlying mechanisms is warranted. Such research could encompass omics technology, including the transcriptome and methylome among others.

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