Jersey calf performance in response to high-protein, high-fat liquid feeds with varied fatty acid profiles: Blood metabolites and liver gene expression

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ABSTRACT

Most available Jersey calf milk replacers (CMR) use edible lard as the primary fat source, which lacks medium-chain fatty acids (MCFA). However, Jersey cow milk consists of over 10% MCFA. The objective of this trial was to determine whether altering the fatty acid profile of CMR by increasing the amount of MCFA would alter liver lipid infiltration, liver gene expression, and blood metabolites when fed to Jersey calves. Fifty Jersey calves were fed 1 of 4 diets: pasteurized saleable whole milk (pSWM) from Jersey cows [27.9% crude protein (CP), 33.5% fat, dry matter (DM) basis]; CMR containing 100% of fat as edible lard (100:00; 29.3% CP, 29.1% fat, DM basis); CMR containing 20% of fat as coconut oil (CO; 80:20; 28.2% CP, 28.0% fat); or CMR containing 40% of fat as CO (60:40; 28.2% CP, 28.3% fat). Liquid diet DM intake averaged 0.523, 0.500, 0.498, and 0.512 kg/d for pSWM, 100:00, 80:20, and 60:40, respectively. Calves were fed their assigned liquid diet daily at 0600 and 1800 h from 2 d of age until 7 wk of age, and once daily until 8 wk of age. Calves were taken off trial at 9 wk of age. Calves had access to water and grain (23.8% CP, 2.71% fat, DM basis). Grain DM intake averaged 0.386, 0.439, 0.472, and 0.454 kg/d for pSWM, 100:00, 80:20, and 60:40, respectively. Liver biopsy cores were obtained from 15 calves at 42 d of age (pSWM, $n = 4$; 100:00, $n = 4$; 80:20, $n = 3$; 60:40, $n = 4$) and from 4 baseline calves <2 d of age. Liver biopsy cores were used for histological appraisal of lipid infiltration and gene expression analyses of short-, medium-, and long-chain acyl-coenzyme A dehydrogenases, sterol regulatory element binding transcription factor 1, acetyl coenzyme A carboxylase, and fatty acid synthase. Lipid infiltration and expression of selected genes were not different among diets. After an overnight fast, weekly blood samples were taken immediately before feeding at 0600 h via jugular

venipuncture in all calves. Serum and plasma obtained from blood samples were used in the analyses of total protein, glucose, triglycerides, nonesterified fatty acids, and plasma urea nitrogen (PUN). Nonesterified fatty acids and PUN were the only blood metabolites affected solely by diet. Nonesterified fatty acids decreased in a linear manner with increased dietary CO inclusion. Calves fed pSWM had higher PUN than calves fed 80:20. In this trial, altering the fatty acid profile of CMR with the addition of medium-chain fatty acids from CO had minimal effects on liver lipid infiltration, liver gene expression, and blood metabolites when fed to Jersey calves.

Key words: fatty acid, Jersey calf, coconut oil, milk replacer

INTRODUCTION

Many Jersey calves are currently fed calf milk replacer (CMR) that is formulated to match the needs of Holstein calves. These products typically contain 20 to 28% CP and 20% fat (DM basis). Inferior diets can negatively affect growth; for example, Bascom et al. (2007) observed that Jersey calves fed a 20% CP, 20% fat (DM basis) CMR at 15% of BW (adjusted weekly) had only minimal growth and the feeding method was not recommended. They also determined that the fat percentage of CMR intended for Jersey calves should be between 16 and 33% (DM basis; Bascom et al., 2007).

In the United States, Jersey cow milk averages 4.69% fat (fluid basis), whereas Holstein cow milk averages only 3.65% fat (fluid basis; USDA-AIPL, 2011). In addition to the difference in milk fat percentage between Jersey and Holstein cows, milk from Jersey cows contains more medium-chain FA (MCFA) than does Holstein cow's milk. On an equal volume basis, milk fat from Jersey cows contains more C10:0 and C12:0 (both MCFA) when compared with milk fat from Holstein cows (Morales et al., 2000).

Currently, the most popular fat source used in CMR for both Jersey and Holstein calves is edible lard, which has a different FA profile than found in either Jersey

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or Holstein cow milk. Edible lard consists of long-chain FA (**LCFA**), and lacks both short-chain FA (**SCFA**) and MCFA. Coconut oil (**CO**), which consists of approximately 47% MCFA (Palmquist, 1988), may be an additional fat source in CMR formulations intended for Jersey calves.

Coconut oil has been formulated into CMR and used in prior experiments (Jenkins et al., 1985; Piot et al., 1999; Graulet et al., 2000; Mills et al., 2010), but not in CMR for Jersey calves. Mills et al. (2010) found evidence of fatty liver development when preweaned Holstein calves were fed a CMR (28% CP, 23% fat; DM basis) containing CO with a total FA profile of 32% medium-chain triglycerides (**TG**). Mills et al. (2010) suspected that this type of fatty liver most likely represents the calf liver's inability to metabolize dietary fat in a timely manner.

Carbon chain length of dietary FA is important because it determines when and via which route the FA reaches the liver (Hocquette and Bauchart, 1999). The SCFA and MCFA up to 8 carbons in length can be absorbed in the abomasum, and MCFA up to 12 carbons in length can be absorbed in the small intestine. In these scenarios, NEFA first enter the portal vein, and then travel directly to the liver. In contrast, LCFA are absorbed into enterocytes, re-esterified into TG, and then incorporated into a chylomicron before release into the lymphatic system. Chylomicrons absorbed from the diet arrive at the liver after travel through the lymphatic system, arterial system, capillary beds, and venous system. Given the different routes, dietary SCFA and MCFA reach the liver faster in comparison to LCFA (Singh, 1997).

Because of the association between MCFA intake and liver metabolism, we studied Jersey calves fed liquid diets with varying amounts of MCFA from CO and assessed liver histology to evaluate degree of fat infiltration and liver gene activity. Genes of interest were short-chain acyl-CoA dehydrogenase (**SCAD**), medium-chain acyl-CoA dehydrogenase (**MCAD**), long-chain acyl-CoA dehydrogenase (**LCAD**), sterol regulatory element-binding transcription factor 1 (**SREBF-1**), fatty acid synthase (**FASN**), and acetyl-CoA carboxylase (**ACACA**). It was also of interest to study select blood metabolites involved with both lipid metabolism (NEFA and TG) and overall metabolism [glucose, plasma urea nitrogen (**PUN**), and total protein (**TP**)] to track pattern changes due to diet, time, or both.

The first hypothesis of this study was that feeding CMR diets containing 0, 20, or 40% of total fat as CO to Jersey calves would result in either linear or quadratic effects on lipid deposition in the liver, gene expression in the liver, and blood metabolites. The sec-

ond hypothesis was that feeding a CMR diet containing 20% of total fat as CO to Jersey calves would result in similar lipid deposition in the liver, gene expression in the liver, and blood metabolites when compared with Jersey calves fed pasteurized Jersey saleable whole milk. Results of diet effects on DMI, total fat intake, individual FA intake, body growth, and health measures for these same animals are reported in detail in a companion paper (Bowen Yoho et al., 2013). Tables 1 to 3 summarize those data.

MATERIALS AND METHODS

Animals and Treatments

Animal procedures described herein were approved by The Ohio State University Institutional Animal Care and Use Committee (Protocol #2010A00000186). Calves were enrolled in the study as they were born until a total of 50 calves was reached (male, $n = 18$; female, $n = 32$). Calves were individually housed and fed at the Waterman Dairy Farm at The Ohio State University. The trial was conducted from November 2010 to August 2011. A randomized complete block design with 4 treatments was used. Calves were blocked by sex, parity of dam, and date of birth, and assigned to 1 of 4 liquid feeds. One liquid feed was pasteurized (72°C for 30 s) saleable whole milk (**pSWM**; $n = 12$) from the Jersey herd at Waterman Dairy. The pSWM was 27.9% CP, 33.5% fat (DM basis). The other 3 liquid feeds were CMR varying in FA profile. These 3 CMR were intended to be isonitrogenous and isocaloric, with dried whey, whey protein concentrate, dried whey product, dried skim milk, and dried milk protein included as protein sources. The CMR diets differed only in FA profile, which was a result of the fat sources used. Diet 100:00 ($n = 13$) contained only edible lard as the fat source (Cow's Match-Jersey Blend, 29.3% CP, 29.1% fat (DM basis); Land O'Lakes Animal Milk Products, Shoreview, MN). Diet 80:20 ($n = 13$) contained edible lard (80%) and CO (20%) as fat sources (this was 28.2% CP, 28.0% fat; DM basis). Diet 60:40 ($n = 12$) contained edible lard (60%) and CO (40%) as fat sources (28.2% CP, 28.3% fat; DM basis). All CMR diets were formulated and blended by Land O'Lakes Animal Products Company and were nonmedicated. Composition of diets is summarized in Table 1. The CMR was reconstituted to 15% milk powder (ca. 13.8% solids) and fed at 40.0 to 43.0°C. Calves were fed their respective liquid diet twice daily (0600 and 1800 h) from 2 d of age until 7 wk of age, and once daily until weaning (8 wk of age). During the first week of life, all calves were fed 1.9 L of liquid at each feeding. During wk 2 through 8 of life, calves were fed 2.27 L of liquid

Table 1. Summary of feed composition data¹

Variable	Grain	pSWM ¹	Milk replacer ²		
			100:00	80:20	60:40
DM, %	94.9	14.1	92.4	91.8	92.0
CP, % of DM	23.8	27.9 (3.70)	29.3	28.2	28.2
Fat, % of DM	2.71	33.5 (4.72)	29.1	28.0	28.3
FA, % of fat					
SCFA ³	—	3.66	1.67	1.82	1.75
MCFA ⁴	2.87	12.54	1.26	10.14	18.31
LCFA ⁵	94.44	83.52	94.66	85.55	77.73
Other	3.17	0.34	2.46	2.43	2.16

¹Adapted from Bowen Yoho et al. (2013). Numbers in parentheses are on an as-fed basis and as reported by DHI; true protein values were determined by DHI and adjusted to a CP value assuming 6% nonprotein nitrogen (DePeters and Ferguson, 1992). pSWM = pasteurized saleable whole milk.

²100:00 = 100% edible lard, 0% coconut oil milk replacer (CMR; Cow's Match-Jersey Blend, Land O'Lakes Animal Milk Products, Shoreview, MN); 80:20 = 80% edible lard, 20% coconut oil CMR; and 60:40 = 60% edible lard, 40% coconut oil CMR.

³SCFA = short-chain FA (4 carbon FA).

⁴MCFA = medium-chain FA (6–12 carbon FA).

⁵LCFA = long-chain FA (sum of 14:0, 14:1, 15:0, 16:0, 16:1, 18:0, 18:1 *trans*, 18:1 *cis*, 18:2, 18:3, and 20:1).

at each feeding. Calves had ad libitum access to water at all times. In addition, all calves were given access to a common texturized grain (23.8% CP, DM basis; AM-PLI-Calf Jersey 22; Land O'Lakes Purina Feed LLC, St. Louis, MO). To avoid any confounding effects of grain on blood metabolite concentrations, calves were not offered grain on evenings preceding blood collection days (d 7, 14, 21, 28, 35, 42, 49, 56, and 63). Grain was offered the next morning following a single blood draw, except in the cases of d 7, 21, and 42, when 4 blood samples were taken throughout a 12-h period and grain was withheld for a full 24 h for 34 calves (described later). Otherwise, calves had ad libitum access to grain.

Grain orts were recorded daily at the evening feeding and liquid feed orts (if any) were recorded at both feedings. Full details regarding animals, treatments, growth, and health data are reported in a companion paper (Bowen Yoho et al., 2013).

Liver Biopsies and Analyses

Liver Tissue Collection and Processing. Liver tissue biopsies were obtained from 15 calves (n = 4 each for pSWM, 100:0, and 60:40; n = 3 for 80:20) when they were approximately 42 d of age to assess degree of lipid infiltration and for gene expression stud-

Table 2. Summary of calf performance and feed intake data¹

Variable ²	Diet ³				SEM	Contrast ⁴		
	pSWM (n = 12)	100:00 (n = 13)	80:20 (n = 13)	60:40 (n = 12)		pSWM vs 80:20	Linear	Quadratic
Birth BW, kg	26.8	26.8	26.5	26.4	0.9	0.82	0.74	0.95
Weaning BW, kg	56.6	53.0	54.9	55.8	1.6	0.39	0.16	0.76
Weaning age, d	58.2	57.3	57.0	57.3	0.8	0.33	0.99	0.75
Final BW, kg	62.6	60.0	61.4	60.7	1.8	0.64	0.78	0.62
ADG, kg/d	0.584	0.539	0.560	0.550	0.030	0.54	0.76	0.62
Gain:feed	0.643	0.576	0.582	0.574	0.019	0.02	0.94	0.74
Liquid diet DMI, kg/d	0.523	0.500	0.498	0.512	0.006	0.01	0.20	0.35
Birth to weaning grain DMI, kg/d	1.63	1.78	1.76	1.74	0.12	0.46	0.83	0.98
Weaning to wk 9 grain DMI, kg/d	1.70	1.81	1.78	1.87	0.10	0.59	0.68	0.66
Grain DMI, kg/d	0.386	0.439	0.472	0.454	0.080	0.42	0.89	0.78
Total DMI, kg/d	0.909	0.938	0.970	0.966	0.067	0.49	0.76	0.81

¹Adapted from Bowen Yoho et al. (2013).

²ADG = ADG from birth to d 63 unless noted otherwise; gain:feed = ADG/total DMI.

³pSWM = pasteurized saleable whole milk; 100:00 = 100% edible lard, 0% coconut oil milk replacer (CMR; Cow's Match-Jersey Blend, Land O'Lakes Animal Milk Products, Shoreview, MN); 80:20 = 80% edible lard, 20% coconut oil CMR; and 60:40 = 60% edible lard, 40% coconut oil CMR.

⁴pSWM vs. 80:20 = comparison of means of pSWM and 80:20; and linear and quadratic effects of coconut oil (0, 20%, and 40%).

Table 3. Fat intakes from grain and liquid portion of the diets and total intakes of fat and FA during the entire trial for calves fed the 4 different liquid diets¹

Item	Diet ²				Contrast ³			
	pSWM (n = 12)	100:00 (n = 13)	80:20 (n = 13)	60:40 (n = 12)	SEM	pSWM vs. 80:20	Linear	Quadratic
Days on trial	65.2	64.3	63.9	64.2	0.84	—	—	—
FA intake, kg								
Grain fat intake	0.80	0.80	0.84	0.81	0.07	0.30	0.86	0.76
Liquid diet fat intake	11.8	9.19	8.79	9.12	0.11	<0.01	0.63	0.01
Total fat intake	12.6	9.99	9.63	9.93	0.12	<0.01	0.73	0.02
C4:0	0.40	0.15	0.16	0.16	0.002	<0.01	0.01	0.16
C6:0	0.34	0.03	0.05	0.06	0.002	<0.01	<0.01	0.03
C8:0	0.18	0.02	0.15	0.27	0.002	<0.01	<0.01	0.17
C10:0	0.40	0.03	0.11	0.18	0.002	<0.01	<0.01	0.33
C12:0	0.47	0.04	0.59	1.16	0.01	<0.01	<0.01	0.55
C14:0	1.35	0.21	0.44	0.67	0.008	<0.01	<0.01	0.58
C14:1	0.90	0.00	0.00	0.00	0.04	<0.01	0.98	0.99
C15:0	0.13	0.02	0.02	0.02	0.001	<0.01	0.99	0.99
C16:0	3.96	2.38	2.13	2.00	0.03	<0.01	<0.01	0.05
C16:1	0.17	0.16	0.13	0.12	0.002	<0.01	<0.01	0.01
C18:0	1.29	1.25	1.08	1.01	0.001	<0.01	<0.01	0.01
C18:1 <i>trans</i>	0.27	0.08	0.09	0.10	0.001	<0.01	<0.01	0.33
C18:1 <i>cis</i>	2.11	3.48	2.88	2.58	0.03	<0.01	<0.01	<0.01
C18:2	0.56	1.77	1.48	1.32	0.03	<0.01	<0.01	0.06
C18:3 and 20:1	0.04	0.12	0.08	0.06	0.002	<0.01	<0.01	<0.01
Other	0.06	0.25	0.23	0.22	0.002	<0.01	<0.01	0.68

¹Table 6 in Bowen Yoho et al. (2013).²pSWM = pasteurized saleable whole milk; 100:00 = 100% edible lard, 0% coconut oil milk replacer (CMR; Cow's Match-Jersey Blend, Land O'Lakes Animal Milk Products, Shoreview, MN); 80:20 = 80% edible lard, 20% coconut oil CMR; and 60:40 = 60% edible lard, 40% coconut oil CMR.³pSWM vs. 80:20 = comparison of means of pSWM and 80:20; and linear and quadratic effects of coconut oil (0, 20%, and 40%).

ies. Baseline liver biopsies were also obtained from 4 male calves on d 2 of life and processed identically to those obtained at 42 d of age. However, baseline calves were not enrolled in the study nor were they biopsied at 42 d of age. For biopsies, calves were taken from hutches to a nearby barn (within 25 m of hutch), and placed in a restraint chute. The liver biopsy procedure used was similar to the protocol described by Kahl et al. (1997). Briefly, the intended liver biopsy site was examined by ultrasound (Aloka model SSD-900V; Hitachi Aloka, Wallingford, CT; 3.5-MHz sector probe) to ensure that the biopsy site was centered over liver tissue and to assess the thickness of the lateral body wall and locations of organs. The hair over the desired location on the calf's right side was clipped, and the area was scrubbed. Five milliliters of lidocaine (2%) were infused to desensitize the skin, intercostal tissues, and peritoneum. A 1-cm incision was made through the skin. Transcutaneous biopsy samples were obtained (~100 mg/sample, 5 samples) with an EZ Core Biopsy instrument (Products Group International, Lyons, CO; 16 gauge 1.6 mm × 9 cm trocar or 14 gauge 2.1 mm × 9 cm trocar). After liver biopsy samples were obtained, the puncture site was infused with procaine penicillin (300,000 units/mL) and the skin was sutured using 2–0 polypropylene suture with 1 to 2 simple interrupted

sutures. Sutures were removed 10 d later. For each calf, all but one liver biopsy core samples were snap frozen in liquid nitrogen and stored at –80°C for later analysis. The remaining liver biopsy core was fixed in 10% formalin for 24 h and then placed in 70% ethanol until the time of embedding in paraffin.

Liver Histology. After embedding and cutting, the 5-μm thick liver samples underwent general hematoxylin and eosin-Y staining. Stained slides were viewed on an Olympus IX81 microscope (Olympus Corporation, Shinjuku-ku, Tokyo, Japan) and 3 digital images per animal were captured at 320× magnification with an Olympus DP-72 camera operating in 24-bit RGB mode. Each image was centered on a central vein. Digital images were viewed by a single observer blinded to treatment. The observer was trained to look for signs of fatty liver and was to score each image as either positive or negative for lipid infiltration.

Liver Gene Expression. Snap frozen liver samples from each animal equaling up to 30 mg were lysed using a TissueLyser LT (Qiagen, Valencia, CA). Each sample was placed in a 2-mL microcentrifuge tube containing a 1.7-mm stainless steel bead and 600 μL of buffer RLT (Qiagen) and then lysed for 5 min at 50 Hz. Tissue lysates were then processed using RNeasy mini kits (Qiagen) to extract total RNA from biopsy samples

Table 4. Primer sequences (5' to 3') for quantitative reverse-transcription PCR¹

Accession no.	Gene ²	Sequence (5'–3')	Product size, bp	Reference
BC103365.1	<i>SCAD</i>	Forward: CCTCTACTTAGGGCCCATCC Reverse: ACTCAGGACCCACGAGTCAC	178	
BC102989.1	<i>MCAD</i>	Forward: GTGGAGGTCTTGGACTTGGA Reverse: CCTCAGTCATTCTCCCCAAA	171	
BC123880.1	<i>LCAD</i>	Forward: CACTAATGGGTGGCTGTGTG Reverse: TCTGCAGTATCCTGGGCTTT	168	
NM_001113302.1	<i>SREBF-1</i>	Forward: CCAGCTGACAGCTCCATTGA Reverse: TGC GCGCCACAAGGA	67	Loor et al., 2005
AJ132890.1	<i>ACACA</i>	Forward: CGGCATTACGAAACTACCT Reverse: GGGCAGTGATCTCTTTCTGC	143	
AY343889.1	<i>FASN</i>	Forward: CTGAGTCGGAGAACCTGGAG Reverse: CGAAGAAGGAAGCGTCAAAC	156	
DT860044	<i>RPS9</i>	Forward: CCTCGACCAAGAGCTGAAG Reverse: CTCCAGACCTCACGTTTGTTC	108	Loor et al., 2005

¹Primer sequences not referenced were designed using Primer3 (v. 0.4.0; <http://frodo.wi.mit.edu/>).

²*SCAD* = short-chain acyl-CoA dehydrogenase; *MCAD* = medium-chain acyl-CoA dehydrogenase; *LCAD* = long-chain acyl-CoA dehydrogenase; *SREBF-1* = sterol regulatory element binding transcription factor 1; *ACACA* = acetyl CoA carboxylase; *FASN* = fatty acid synthase; *RPS9* = ribosomal protein s9 (endogenous control).

using the procedures recently described by Rinaldi et al. (2010). The RNA quantity was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE); the 260 to 280 ratio averaged 2.06 for liver RNA.

Single-stranded cDNA was reverse transcribed from total RNA by denaturing 0.5 µg of RNA in DNase/RNase-free water, totaling 11.3 µL at 70°C for 12 min, and then placed on ice for 3 min. Reverse transcription master mix (8.7 µL) contained 4 µL of M-MLV RT 5× buffer (Promega, Madison, WI), 2 µL of 0.1 M dithiothreitol, 1 µL of 10 mM dNTP mix (Promega), 1 µL of oglio(dT)15 primer, 0.5 µL M-MLV reverse transcriptase (Promega), and 0.2 µL of RNase inhibitor (Promega). The entire volume of master mix was added to each well of denatured RNA in a 96 well PCR plate for a final reaction volume of 20 µL (Bio-Rad, Hercules, CA). Reverse transcription was carried out in an iQ5 Multicolor reverse transcription PCR Detection System (Bio-Rad) with cycle conditions at 40°C for 1 h, followed by 95°C for 10 min. Resultant cDNA was then diluted 1:1 with addition of 20 µL of DNase/RNase-free water. The cDNA was stored at –20°C until further use.

Primers were designed with Primer3 (v. 0.4.0; <http://frodo.wi.mit.edu/>) for genes *SCAD*, *MCAD*, *LCAD*, *ACACA*, and *FASN* (Table 4). Primer sequences for *SREBF-1* and the endogenous control gene, ribosomal protein s9, were the same as in Loor et al. (2005; Table 4). Primer efficiencies were determined for each primer pair by the use of serial dilutions of cDNA, using the following equation: percent efficiency = $(10 - 1/\text{slope} - 1) \times 100$. Primer efficiencies averaged 102.1%.

Quantitative reverse transcription PCR (qPCR) reactions were carried out using a master mix consist-

ing of 10.5 µL of RNase/Dnase-free water, 12.5 µL of iQ SYBR Green Supermix (Bio-Rad), and 0.5 µL of each respective forward and reverse primer. Twenty-four microliters of master mix were then added to 2 µL of first-strand cDNA, yielding a 26 µL reaction volume for qPCR. Amplification was carried out in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with conditions set at 1 cycle at 95°C for 5 min, 40 repeating cycles at 95°C for 10 s, and then at 57.4°C for 45 s. Negative assay controls included no template cDNA controls and no reverse transcriptase controls. Immediately after the qPCR experiment, a melt curve analysis was conducted to verify the purity of amplicons and absence of primer dimers. For this, plate temperature was increased from 65 to 95°C in 1°C increments.

After qPCR, representative samples of PCR products for each primer pair were electrophoresed on a 2% agarose gel to confirm purity and size of each amplicon. All PCR products were single bands that corresponded with predicted product size (data not shown).

Relative mRNA abundance of target genes is expressed as the fold differences of target genes relative to the expression of the endogenous reference gene ribosomal protein s9 and baseline animals. Specifically, relative mRNA abundance for each target gene was determined by first subtracting the cycles to threshold (Ct) value of the endogenous reference gene from the Ct value for each target gene, thus yielding ΔCt values. Next, ΔCt values for baseline animals ($n = 3$; all data from one baseline animal were discarded on the basis of nonconformity for all genes) were averaged, resulting in a $\Delta\text{Ct}_{\text{baseline average}}$ for each target gene; $\Delta\text{Ct}_{\text{baseline average}}$ was subtracted from $\Delta\text{Ct}_{\text{target gene}}$ values for all other observations, yielding $\Delta\Delta\text{Ct}$ values. For

ease of interpretation, data are presented as $2^{(-\Delta\Delta Ct)}$ values.

Blood Collection and Analysis

Weekly Blood Collection and Processing. All calves were bled weekly (0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 wk of life) at 0600 h (after an overnight fast) to track changes in blood metabolite concentrations over time, by diet, or both. Three vials of blood (total volume of blood collected ≤ 26 mL) were obtained via jugular venipuncture at each time point into evacuated tubes, 2 tubes with no anticoagulant (serum tubes), and 1 tube with sodium fluoride (plasma tube). All samples were kept on ice after collection and transported to a laboratory within 1 h for further processing. A subset of animals on each treatment (pSWM, $n = 8$; 100:00, $n = 9$; 80:20, $n = 9$; 60:40, $n = 8$) underwent more intensive blood sampling at 1, 3, and 6 wk of life to track pattern changes in metabolite concentrations in relation to feeding and age. In these selected animals, 4 blood collection incidents (totaling no more than 26 mL of blood per incident; same size and type of tubes as used in 0600 h sample collection) took place at 0600 (fasted sample), 0800, 1200, and 1800 h (results not shown).

Blood Metabolite Analyses. Plasma was used for analysis of glucose and PUN; serum was used for analysis of TP, NEFA, and TG. All blood samples were processed and analyzed using methods similar to Daniels et al. (2008) for glucose, PUN, TP, and TG. The HR Series NEFA (2) reagent set (Wako Pure Chemicals Industries Ltd., Richmond, VA) was used to determine NEFA concentrations of serum. An aqueous NEFA standard was used for this assay (1.0 mmol/L; Wako Pure Chemicals Industries Ltd.). Concentrations were determined at 550 nm using an ELX 808 Ultra Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT). Intra- and interassay CV for all metabolite assays averaged 1 and 3%, respectively.

Statistical Analyses

Histology data for liver biopsy samples that underwent hematoxylin and eosin staining were not analyzed statistically, as no lipid infiltration was noted in any samples. All other data were analyzed using SAS (SAS Version 9.1.3, SAS Institute Inc., Cary, NC).

Prior to ANOVA, normality of data distribution for each treatment and gene combination in the qPCR data set were examined using the Shapiro-Wilk test in the univariate procedure of SAS. The null hypothesis that data were from a random sample from a normal distribution was rejected when $P \leq 0.05$; this occurred

in 3 instances. Because of that, a more conservative α was selected for later ANOVA testing of qPCR data; differences were declared significant at $P \leq 0.02$. Additionally, absence of heterogeneity of variance was confirmed with Hartley F-max testing (5 variances, 2 df, 0.05 α). The qPCR data were analyzed using the Mixed Procedure of SAS; data submitted to SAS consisted of $2^{(-\Delta\Delta Ct)}$ values. Treatment was the single fixed effect in the model, and the random effect was calf within treatment.

Weekly blood metabolite data were analyzed using the mixed procedure of SAS with repeated measures. The repeated measure was week and the subject used in tests was block; a compound symmetry covariance structure was specified for all tests. No denominator degrees of freedom approximation was specified. Preplanned, nonorthogonal contrast statements were used to examine diet differences, when present: pSWM versus 80:20; linear effect of CO (0, 20, and 40%); and quadratic effect of CO (0, 20, and 40%). Orthogonal polynomial contrasts were used to test for linear and quadratic responses over time. All data are reported as LSM $\pm$ SE of the mean. Significance was declared at $P \leq 0.05$ except for in qPCR testing, as noted above.

RESULTS AND DISCUSSION

Intake and Performance

Calf intake and performance data are described in detail in a companion paper by Bowen Yoho et al. (2013). Briefly, initial and final BW did not differ by diet, nor did weaning age. Calves fed pSWM compared with 80:20 had a higher liquid diet DMI (0.523 vs. 0.498 kg/d, respectively) and similar grain DMI (0.386 vs. 0.472 kg/d, respectively). Coconut oil inclusion did not affect liquid diet DMI, grain DMI, or total DMI. Fat intake from grain did not differ between diets. Differences in liquid diet fat intake were observed. Calves fed pSWM consumed 11.8 kg of fat from the liquid diet; whereas calves fed 80:20 consumed only 8.79 kg of fat from the liquid diet ($P < 0.01$). A quadratic effect of CO on consumption of fat from the liquid portion of the diet ($P = 0.01$) was also noted; liquid diet fat consumption was 9.19, 8.79, and 9.12 kg for 100:00, 80:20, and 60:40, respectively. Total fat intake was higher in calves fed pSWM than in calves fed 80:20 (12.6 vs. 9.63 kg, respectively). Coconut oil inclusion had a quadratic, but not linear, effect on total fat intake. Total fat intakes were 9.99, 9.63, and 9.93 kg for 100:00, 80:20, and 60:40, respectively.

Individual FA intake differed by diet in several instances. Most relevant to the current study is intake of MCFA. Intake of C6:0, C8:0, and C10:0 were higher in

Figure 1. Representative hematoxylin and eosin stained liver biopsies. Baseline samples obtained at 2 d of age, all other samples obtained at 42 d of age. Diets were pasteurized saleable whole milk (pSWM; 27.9% CP, 33.5% fat, DM basis); calf milk replacer (CMR; Cow's Match-Jersey Blend, Land O'Lakes Animal Milk Products, Shoreview, MN) containing 100% of fat as edible lard (100:00; 29.3% CP, 29.1% fat); CMR containing 20% of fat as coconut oil (CO; 80:20; 28.2% CP, 28.0% fat); and CMR containing 40% of fat as CO (60:40; 28.2% CP, 28.3% fat). No difference in lipid infiltration of the liver was noted by visual appraisal. Scale bar = 20 μ m.

calves fed pSWM compared with those fed 80:20 (0.92 vs. 0.31 kg, respectively); calves fed 80:20, however, had a higher intake of C12:0 compared with calves fed pSWM (0.59 vs. 0.47 kg, respectively). Overall, calves fed pSWM had a higher intake of MCFA than calves fed 80:20. In addition, a linear increase in total MCFA consumption with CO inclusion in CMR was observed (Bowen Yoho et al., 2013).

Liver Histology

One objective of this trial was to examine the effects of CO inclusion in CMR on FA metabolism in Jersey calves. Hematoxylin and eosin stained liver samples showed no noticeable difference in the amount of fat present by diet or when visually compared with baseline animals. Representative samples from each

diet plus the baseline are presented in Figure 1. Given that observation, increasing the amount of CO in the diet did not appear to increase the amount of lipid infiltration in the liver. An increase in the amount of lipid infiltration would have most likely indicated that the calf's hepatocytes had reduced FA oxidation or increased incorporation of FA into TG in excess of what was being exported to peripheral tissues. Coconut oil included in CMR as the sole source of fat has been shown to increase the amount of TG present in the liver of Holstein-Friesian calves when fed for 19 d; TG content of wet liver weight was determined to be 4.1% compared with 0.2% for those not fed CO (Piot et al., 1999). Piot et al. (1999) showed that CO fed at high levels can increase lipid infiltration in the liver. However, even at the highest level of CO inclusion in this trial (40% of total fat), no increase in liver lipid infiltration was seen. Another study revealed that feeding a CMR containing 32% medium-chain TG from CO to Holstein calves also led to increased liver lipid infiltration; TG content was 6.5% of wet liver weight for those fed CO compared with 5.3% for those fed CMR that used animal fat as the sole fat source (Mills et al., 2010). The highest percentage of MCFA intake on this trial was 18.3% for diet 60:40 (Table 3); this percentage is just over half of the amount fed in Mills et al. (2010). It seems that neither CO nor MCFA were included at a high enough level in CMR to elicit an increase in liver lipid infiltration in this trial.

Liver Gene Expression

A second objective was to test whether CO inclusion alters a calf's metabolism is through examination of gene expression associated with FA metabolism. Fatty acid metabolism in the liver consists of 3 main processes: FA oxidation, FA synthesis and TG assembly.

Fatty acid oxidation in the liver occurs mainly through β -oxidation, where FA in mitochondria are either completely oxidized to acetyl-CoA or partially oxidized to ketones. Once inside the mitochondrion, carbon chain-length specific enzymes, known as acyl-CoA dehydrogenases, catalyze the next step of β -oxidation. Specifically, 3 members of this family are involved in β -oxidation of FA: SCAD acts on acyl-CoA with carbon chain lengths of 4 to 6 (Ikeda et al., 1985); MCAD will act on acyl-CoA with carbon chain lengths of 4 to 16, with preferred substrates that have carbon chain lengths of 6 to 12 (Zeng and Li, 2005); and LCAD acts on acyl-CoA with carbon chain lengths of 6 to 16, with preferred substrates that have carbon chain lengths of 8 to 14 (Ikeda et al., 1985). In this study, CMR with 20 and 40% CO inclusion had FA profiles that contained larger amounts of MCFA than did the CMR containing

only edible lard. For this reason, it can be assumed that calves fed diets with greater amounts of MCFA absorbed more intact MCFA. Increased β -oxidation of MCFA would have been a potential adaptive change in lipid metabolism in the liver to deal with increased intake of MCFA. However, no differences in gene expression by diet for SCAD, MCAD, or LCAD were observed in this trial (Table 5).

Fatty acid synthesis and TG assembly both occur in the liver. Fatty acid synthase is involved with de novo FA synthesis (Strable and Ntambi, 2010). If FA are not oxidized in liver, they can undergo TG assembly. In this case, FA are esterified onto glycerol to form TG and are normally transported out of the liver for storage or use in peripheral tissues. Sterol regulatory element-binding transcription factor 1 is at least partially responsible for regulating FA synthesis prior to FA esterification onto glycerol (McFadden and Corl, 2009). The lipid may pool in hepatocytes unless FA are oxidized or TG are exported as very low density lipoproteins (Nguyen et al., 2008). Graulet et al. (2000) noted that 15-d-old Holstein $\times$ Friesian calves fed CMR (22.4% fat) that contained 47% MCFA as total fat for 19 d had increased hepatic TG formation, but noted no increase in very low density lipoprotein production. As a result, these calves developed fatty liver (Graulet et al., 2000). Acetyl-CoA carboxylase catalyzes the synthesis of malonyl-CoA, which donates 2 carbons per cycle during de novo synthesis of FA; malonyl-CoA also inhibits mitochondrial oxidation of FA (Strable and Ntambi, 2010). Our analysis of liver gene expression revealed no diet differences in expression of genes associated with FA synthesis, namely SREBF-1, ACACA, and FASN (Table 5). This goes along with our histology findings.

Based on the relative expression of tested genes, those associated with β -oxidation (SCAD, MCAD, and LCAD) were more abundant at the time of biopsy in 42-d-old Jersey calves than those associated with FA synthesis (SREBF-1, ACACA, and FASN; Table 5). Data also suggest that, numerically, baseline animals had relatively higher expression of SREBF-1 and ACACA than 42-d-old Jersey calves; whereas the opposite was true of SCAD, MCAD, and LCAD (Table 5).

Potential downfalls were noted in this study with regard to determination of gene expression in the liver. First, liver biopsy collection time in relation to feeding may have been an issue. Biopsies were performed approximately 6 h after morning feeding, which may have been too long after a feeding to observe differences in liver gene expression. Second, a relatively small number of animals per treatment were biopsied, leading to low statistical power. Another potential downfall is that only gene expression was analyzed here, rather than protein abundance or enzyme activity related to these

Table 5. Relative mRNA abundance of selected genes in liver biopsies from 42-d-old Jersey calves fed 1 of 4 diets¹

Gene ²	Treatment ³				SEM (n = 3)	P-value ⁴
	pSWM (n = 4)	100:00 (n = 4)	80:20 (n = 3)	60:40 (n = 4)		
β -oxidation						
<i>SCAD</i>	2.906	2.756	3.646	5.771	1.660	0.457
<i>MCAD</i>	2.462	3.697	3.425	2.537	0.999	0.689
<i>LCAD</i>	3.253	3.542	2.998	5.008	1.463	0.705
FA synthesis						
<i>SREBF-1</i>	0.738	0.279	0.246	0.438	0.237	0.379
<i>ACACA</i>	0.649	0.214	0.891	0.311	0.225	0.143
<i>FASN</i>	1.517	0.426	1.534	0.575	0.811	0.588

¹Data are expressed as $2^{-\Delta\Delta C_t}$ values; numbers are fold differences in gene expression in relation to baseline samples obtained at 2 d of age (e.g., 2.906 = 2.906-fold more mRNA than baseline).

²*SCAD* = short-chain acyl-CoA dehydrogenase; *MCAD* = medium-chain acyl-CoA dehydrogenase; *LCAD* = long-chain acyl-CoA dehydrogenase; *SREBF-1* = sterol regulatory element binding transcription factor 1; *ACACA* = acetyl CoA carboxylase; *FASN* = fatty acid synthase.

³Treatments were pasteurized saleable whole milk (pSWM; 27.9% CP, 33.5% fat, DM basis); calf milk replacer (CMR; Cow's Match-Jersey Blend, Land O'Lakes Animal Milk Products, Shoreview, MN) containing 100% of fat as edible lard (100:00; 29.3% CP, 29.1% fat); CMR containing 20% of total fat as coconut oil (CO; 80:20; 28.2% CP, 28.0% fat); CMR containing 40% of total fat as CO (60:40; 28.2% CP, 28.3% fat).

⁴Significance declared when $P \leq 0.02$.

genes of interest. It is feasible that post-transcriptional changes in these genes occur that may ultimately affect either protein production or function or both.

Blood Metabolites

Fasting serum TP concentration was not affected by the interaction of diet and week, the main effect of diet, or the main effect of week, and averaged 5.04 g/dL across all diets and weeks (data not shown). This is in agreement with research done by Lee et al. (2009) where TP concentration in Holstein calves fed SWM was not different from those fed CMR (24% CP, 25% fat, DM basis) and averaged 5.36 g/dL.

Diet by Week Effects. Interactions of diet by week for plasma glucose ($P = 0.044$) and plasma TG ($P = 0.001$) concentrations were observed (Figure 2A and B). In general, glucose concentration decreased with age, as expected. At the beginning of the trial, fasting plasma glucose averaged 98 mg/dL for all diets; at the end of the trial, glucose concentrations averaged 76 mg/dL. In calves on pSWM, glucose changed over time in a complex pattern [Figure 2A; significant linear ($P = 0.001$) and quadratic ($P = 0.008$) contrast effects]. All calves fed CMR experienced linear decreases in glucose over time ($P = 0.001$ for 100:00, 80:20, and 60:40, respectively; Figure 2A).

As with glucose, the diet by week interaction was significant for serum TG ($P = 0.001$). In calves fed pSWM, fasting serum TG decreased in a linear manner ($P = 0.001$) from 0 to 9 wk (Figure 2B). In calves fed

100:00, TG changed over time in a complex pattern [Figure 2B; significant linear ($P = 0.001$) and quadratic ($P = 0.001$) contrast effects]. Calves fed 80:20 and 60:40 showed quadratic ($P = 0.001$ and $P = 0.001$, respectively) changes in TG from 0 to 9 wk (Figure 2B). Concentrations of TG in blood are indicative of LCFA absorbed from the diet that are incorporated into chylomicrons or from TG synthesized by and secreted from the liver. Here, calves fed pSWM most often had numerically higher fasting serum TG concentration than calves fed 80:20; this likely corresponds with the fact that calves fed pSWM ingested more total fat and, specifically, more total LCFA (Table 3). Another possibility is that clotting in the abomasum affects serum TG appearance in and clearance from the blood. To that point, Petit et al. (1988), fed Holstein calves either a clotting CMR or a nonclotting CMR (both 25% CP, 20% fat, DM basis), and noticed that calves fed clotting CMR had more uniform concentrations of plasma TG throughout a 24-h period, measured when calves were approximately 2-wk-old. Calves fed the nonclotting CMR showed plasma TG peaks 2 h postfeeding, with a decline thereafter (Petit et al., 1988).

Diet Effects. Fasting serum NEFA ($P = 0.007$) and PUN ($P = 0.003$) concentrations were affected by diet. Irrespective of week, calves fed 80:20 had higher serum NEFA concentrations than calves fed pSWM (0.28 vs. 0.21 mmol/L $\pm$ 0.02, respectively; $P = 0.022$; Figure 2C). The higher fasting NEFA concentrations in calves fed 80:20, as compared with those fed pSWM is in agreement with results from Bascom et al. (2007), where Jer-

Figure 2. (A) Weekly 12-h fasting glucose; (B) triglyceride (TG); (C) NEFA; (D) and plasma urea nitrogen (PUN) concentrations in Jersey calves fed 1 of 4 diets. Diets were pasteurized saleable whole milk (pSWM; 27.9% CP, 33.5% fat, DM basis); calf milk replacer (CMR; Cow's Match-Jersey Blend, Land O'Lakes Animal Milk Products, Shoreview, MN) containing 100% of fat as edible lard (100:00; 29.3% CP, 29.1% fat); CMR containing 20% of fat as coconut oil (CO; 80:20; 28.2% CP, 28.0% fat); and CMR containing 40% of fat as CO (60:40; 28.2% CP, 28.3% fat). Weaning began at 7 wk of age; calves averaged 8.2 wk of age at weaning (weaning period bracketed); weaning age did not differ by diet. A. The diet by week interaction was significant for glucose ($P = 0.044$); pSWM was linear ($P = 0.001$) and quadratic ($P = 0.008$) over time; and 100:00, 80:20, and 60:40 were linear over time ($P = 0.001$, 0.001 , and 0.001 , respectively). B. The diet by week interaction was significant for TG ($P = 0.001$); pSWM was linear ($P = 0.001$) over time; 100:00 was linear ($P = 0.001$) and quadratic ($P = 0.001$) over time; and 80:20 and 60:40 were quadratic over time ($P = 0.001$ and 0.001 , respectively). C. No interaction observed between diet and week for NEFA ($P = 0.225$). The main effect of diet was significant ($P = 0.007$); NEFA concentration differed between pSWM and 80:20 ($P = 0.022$). Coconut oil inclusion had a linear ($P = 0.010$) but not quadratic ($P = 0.344$) effect on NEFA concentrations. A main effect of week for NEFA ($P = 0.005$) was also observed. Regardless of diet, NEFA concentrations were linear ($P = 0.015$), but not quadratic ($P = 0.225$), over time. D. No interaction observed between diet and week for PUN ($P = 0.945$). The main effect of diet was significant ($P = 0.003$); PUN concentration differed between pSWM and 80:20 ($P = 0.001$). Coconut oil inclusion did not affect PUN concentration. A main effect of week for PUN ($P = 0.001$) was also observed. Regardless of diet, PUN concentrations were quadratic ($P = 0.001$), but not linear ($P = 0.075$), over time.

sey calves tended to have higher NEFA concentrations (2 h postfeeding) when fed a CMR (27% CP; 33% fat; DM basis) compared with those fed SWM. Although measured NEFA can be from the ingestion of dietary FA, the 0600-h blood samples obtained here were after a 12-h fast, making it highly unlikely that measured NEFA came directly from dietary FA. This may suggest that calves fed 80:20 were in mild negative energy balance at the time of sample collection, and mobilized FA from body stores to meet energy needs before the next feeding. However, it should be noted that calves fed 80:20 grew the same as those fed pSWM (Table 2).

Irrespective of week, CO inclusion in the diet caused a linear ($P = 0.010$) decrease in NEFA concentrations (Figure 2C). Calves fed 100:00 had the highest NEFA values (0.294 mmol/L), followed by 80:20 (0.279 mmol/L), and 60:40 (0.220 mmol/L; Figure 2C). We expected that NEFA concentrations would increase as CO inclusion increased due to SCFA and MCFA being directly absorbed into the circulatory system; however, this is not what we observed and is likely be related to time of sample collection (after an overnight fast) in calves sampled at 0600 h. It is interesting to speculate why this linear effect of CO inclusion was observed. Others have noted relative increases in serum NEFA with CMR feeding when edible lard is the primary fat source (Tikofsky et al., 2001; Bartlett et al., 2006; Bascom et al., 2007). Perhaps replacing edible lard with increasing amounts of CO alters fat metabolism to be more similar to the metabolism of milk fat, which contains more MCFA and less LCFA than edible lard. Though not one of our preplanned contrasts, the reader can see that the pattern of fasting NEFA concentration over 9 wk is similar in calves fed 60:40 and pSWM (Figure 2C).

Irrespective of week, calves fed pSWM had higher PUN concentration than calves fed 80:20 (6.09 ± 0.15 vs. 5.33 ± 0.15 mg/dL, respectively; $P = 0.001$; Figure 2D). This is likely due to the inherent MUN content of pSWM and the low amount of NPN in CMR. These findings are in agreement with research done by Górka et al. (2011), where it was shown that Holstein or Holstein $\times$ Limousine calves fed SWM had higher PUN concentrations before the morning feeding when compared with cohorts fed CMR (22% CP; 17.5% fat; DM basis). As expected, CO inclusion did not affect PUN (Figure 2D).

Week Effects. An effect of week was observed for both fasting serum NEFA ($P = 0.005$) and PUN concentrations ($P = 0.001$). Irrespective of diet, NEFA concentrations increased linearly ($P = 0.015$) by week, with the lowest concentration observed at 2 wk (0.18 mmol/L) and the highest concentration at 8 wk (0.34 mmol/L; Figure 2C). Irrespective of diet, PUN concentrations were quadratic ($P = 0.001$) over time, with the

highest concentrations observed at 0 and 9 wk (6.35 and 7.02 mg/dL, respectively) and lowest at 5 wk (4.97 mg/dL; Figure 2D).

The first hypothesis of this study was that feeding CMR diets containing 0, 20, or 40% of total fat as CO to Jersey calves would result in either linear or quadratic effects on lipid deposition in the liver, gene expression in the liver, and blood metabolites. No changes in lipid content of the liver or liver gene expression were noted, so we failed to reject the null hypothesis that fat content of the liver and liver gene expression will be unaffected by the FA profile of the liquid feed. As for blood metabolites, for the vast majority of response variables measured we failed to reject our null hypothesis that blood metabolites will be unaffected by the FA profile of the liquid feed. Noted exceptions were with 12-h fasting plasma glucose, serum TG, and NEFA. Diet by week effects were noted for 12-h fasting plasma glucose and serum TG. Irrespective of week, inclusion of CO in the diet caused a linear decrease in 12-h fasting NEFA concentrations. This is interesting because a linear increase in total MCFA consumption with CO inclusion in CMR has been noted (Bowen Yoho et al., 2013). In general, lower NEFA concentrations are desirable in growing calves; the implications here are not clear but may merit further research with CO for this reason.

The second hypothesis was that feeding a CMR diet containing 20% of total fat as CO to Jersey calves would result in similar lipid deposition in the liver, gene expression in the liver, and blood metabolites when compared with Jersey calves fed pSWM. Liver lipid deposition and gene expression were not different between calves fed pSWM and calves fed 80:20. Some differences in 12-h fasting blood metabolites were noted between calves fed pSWM and calves fed 80:20. Effects attributed exclusively to diet were observed for serum NEFA and PUN. Irrespective of week, calves fed 80:20 had higher serum NEFA concentrations than calves fed pSWM. Calves fed pSWM higher PUN than 80:20, which the authors attribute to MUN in pSWM. The authors concede that comparisons between pSWM and 80:20 should be interpreted lightly because DMI from the liquid portion of the diet, as well as total fat intake were higher in calves fed pSWM, as a consequence of the experimental design.

CONCLUSIONS

In conclusion, for the vast majority of response variables measured we failed to reject our null hypotheses that lipid metabolism will be unaffected by the FA profile of the liquid feed. Our findings here, coupled with those noted in the companion paper by Bowen Yoho et

al. (2013), seemingly provide evidence that MCFA in preruminant Jersey calf diets may not be as biologically important as we thought, with the possible exception being lower fasting serum NEFA concentrations with increased dietary MCFA.

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