J. Dairy Sci. 96:3825–3834
http://dx.doi.org/10.3168/jds.2013-6576

Conjugated linoleic acid-induced milk fat depression in lactating ewes is accompanied by reduced expression of mammary genes involved in lipid synthesis[1]

M. Hussein,* K. H. Harvatine,† W. M. P. B. Weerasinghe,‡[2] L. A. Sinclair,‡ and D. E. Bauman*[3]
*Department of Animal Science, Cornell University, Ithaca, NY 14853
†Department of Animal Science, Pennsylvania State University, University Park 16802
‡Department of Animal Production, Welfare, and Veterinary Sciences, Harper Adams University, Newport, Shropshire, TF10 8NB, UK

ABSTRACT

Conjugated linoleic acids (CLA) are produced during rumen biohydrogenation and exert a range of biological effects. The *trans*-10,*cis*-12 CLA isomer is a potent inhibitor of milk fat synthesis in lactating dairy cows and some aspects of the mechanism have been established. Conjugated linoleic acid-induced milk fat depression has also been observed in small ruminants and our objective was to examine the molecular mechanism in lactating ewes. Multiparous lactating ewes were fed a basal ration (0.55:0.45 concentrate-to-forage ratio; dry matter basis) and randomly allocated to 2 dietary CLA levels (n = 8 ewes/treatment). Treatments were zero CLA (control) or 15 g/d of lipid-encapsulated CLA supplement containing *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers in equal proportions. Treatments were fed for 10 wk and the CLA supplement provided 1.5 g of *trans*-10,*cis*-12/d. No treatment effects were observed on milk yield or milk composition for protein or lactose at wk 10 of the study. In contrast, CLA treatment significantly decreased both milk fat percentage and milk fat yield (g/d) by about 23%. The de novo synthesized fatty acids (FA; <C16) were significantly decreased in proportion (15%) and daily yield (27%), and the proportion of preformed FA (>C16) was increased (10%) for the CLA treatment. In agreement with the reduced de novo FA synthesis, mRNA abundance of acetyl-coenzyme A carboxylase α, FA synthase, stearoyl-CoA desaturase 1, and glycerol-3-phosphate acyltransferase 6 decreased by 25 to 40% in the CLA-treated group. Conjugated linoleic acid treatment did not significantly reduce the mRNA abundance of enzymes involved in NADPH production, but the mRNA abundance for sterol regulatory element-binding factor 1 and insulin-induced gene 1, genes involved in regulation of transcription of lipogenic enzymes, was decreased by almost 30 and 55%, respectively, with CLA treatment. Furthermore, mRNA abundance of lipoprotein lipase decreased by almost 40% due to CLA treatment. In conclusion, the mechanism for CLA-induced milk fat depression in lactating ewes involved the sterol regulatory element-binding protein transcription factor family and a coordinated downregulation in transcript abundance for lipogenic enzymes involved in mammary lipid synthesis.
Key words: conjugated linoleic acid, milk fat, lipogenesis, mammary

INTRODUCTION

Conjugated linoleic acid (**CLA**) is a generic term used to describe positional and geometric isomers of linoleic acid. Several CLA isomers are naturally produced by rumen bacteria as intermediates in the biohydrogenation of dietary PUFA, with *cis*-9,*trans*-11 CLA being the predominant isomer found in ruminant-sourced foods (Bauman and Lock, 2006). Conjugated linoleic acid isomers also originate from industrial hydrogenation and *cis*-9,*trans*-11 and *trans*-10,*cis*-12 are the 2 isomers that have been most extensively studied. Research over the last decade has established CLA as unusual bioactive FA that exert a range of biological effects in different tissues and species, including antiobesity, anticarcinogenic, antidiabetogenic, and antiatherogenic effects (Belury, 2002; Bhattacharya et al., 2006). Baumgard et al. (2000) were the first to demonstrate that *trans*-10,*cis*-12 CLA resulted in a reduction in milk fat synthesis in lactating dairy cows, and this discovery provided a basis to explain the cause of diet-induced milk fat depression (**MFD**), a syndrome in lactating cows that had perplexed dairy producers and scientists

Received January 14, 2013.
Accepted February 28, 2013.
[1]Supported in part by National Research Initiative Competitive Grants no. 2006-35206-16643 from the US Department of Agriculture (USDA) Cooperative State Research, Education, and Extension Service, Agriculture and Food Research Initiative Competitive Grant no. 2010-65206-20723 from the USDA National Institute of Food and Agriculture, and Cornell Agricultural Experiment Station.
[2]Present address: Veterinary Research Institute, Gannoruwa, Peradeniya, Sri Lanka.
[3]Corresponding author: deb6@cornell.edu

for over a century (Bauman and Griinari, 2003; Bauman et al., 2011).

The molecular mechanism behind CLA-induced MFD is not completely resolved; however, the phenotypic characterization provides insight into the functional mechanism. In CLA-induced MFD in dairy cows, fat is the only milk component inhibited with *trans*-10,*cis*-12 CLA treatment. Furthermore, the reduction in milk fat secretion involves FA of all chain lengths, but effects are particularly pronounced for de novo synthesized FA (Bauman and Griinari, 2003). Cellular level investigations have clearly shown a coordinated downregulation in transcript abundance and (or) enzymatic activity for lipogenic enzymes involved in milk fat synthesis in the mammary gland of lactating cows and rodent models (Bauman et al., 2011). Molecular mechanisms mediating the inhibitory effect of *trans*-10,*cis*-12 CLA on mammary lipogenesis have not been extensively investigated, but results support a central role for sterol regulatory element-binding transcription factor family (Peterson et al., 2004; Harvatine and Bauman, 2006; Gervais et al., 2009).

Conjugated linoleic acid-induced MFD has also been observed in small ruminants including sheep (Lock et al., 2006; Sinclair et al., 2007; Weerasinghe et al., 2012) and goats (Lock et al., 2008; Shingfield et al., 2009). Although ruminants share similarities in many aspects, distinct differences exist related to ruminal lipid metabolism and the relative sensitivity of mammary lipogenic processes (Chilliard et al., 2003; Shingfield et al. 2010), and this might modify the mammary response to CLA treatment. For example, in lactating goats, plant oil supplements result in a reduction in milk fat secretion of de novo FA that is independent of mammary expression or activity of acetyl-CoA carboxylase α (**ACACA**) and FA synthase (**FASN**; Bernard et al., 2009).

Dairy ewes may represent a good model to examine the mechanism of CLA-induced MFD. They are relatively available, cost effective, manageable in size, and daily milking allows a quantitative evaluation of treatment effects on milk fat yield and FA composition. Furthermore, the relationship between *trans*-10,*cis*-12 CLA dose and the reduction in milk fat output is similar to cows when dose is expressed on a metabolic BW basis (Lock et al., 2006; Sinclair et al., 2007). To date, the molecular basis for MFD, whether induced by diet or CLA supplements, has not been investigated in lactating ewes. Therefore, the objective of the current study was to investigate the molecular mechanism mediating MFD in lactating ewes fed a CLA supplement containing *trans*-10,*cis*-12 CLA. For this purpose, we used tissue samples obtained from lactating ewes that were fed a rumen-protected CLA supplement for 10 wk. Results for CLA effects on performance, organ weight, and carcass composition are reported in a companion paper (Sinclair et al., 2010).

MATERIALS AND METHODS

Animals and Treatments

All experimental procedures involving lactating ewes were conducted at Harper Adams University (Newport, Shropshire, UK) in accordance with the UK Animals (Scientific Procedures) Act 1986, with details reported in the companion publication (Sinclair et al., 2010). Briefly, at d 16 ± 1.6 (mean ± SE) postpartum, 16 multiparous Friesland and British Milk Sheep ewes were randomly allocated to 2 treatments (randomized block design) based on breed, milk yield and milk fat yield as measured in the previous 7 d, BW, and BCS. Ewes were milked twice daily and fed a basal ration (0.55:0.45 concentrate-to-forage ratio, DM basis) that was composed mainly of hay, rolled barley, and dried molassed sugar beet feed (Sinclair et al., 2010). Dietary ME and CP averaged (per kilogram of DM) 10.9 MJ and 156 g, respectively, with fresh feed offered once per day at 1.05 × ad libitum intake.

Treatments for the present study involved diets that were supplemented with CLA at 2 levels: no CLA (control, **CON**) or 15 g of CLA supplement/d (**+CLA**). The supplement, a lipid-encapsulated CLA that contained 2 CLA isomers in equal proportions, provided 1.5 g of *trans*-10,*cis*-12/d and an equal amount of *cis*-9,*trans*-11 CLA (Lutrell; BASF SE, Ludwigshafen, Germany). Ewes received the 2 experimental treatments throughout a 10-wk period. With the exception of milk fat, the phenotype between the CON and +CLA treatments was comparable after 10 wk of treatment. A third treatment (40 g of CLA supplement/d) reported in the companion paper (Sinclair et al., 2010) was not included in the present study. The 40 g/d CLA group differed in milk yield, milk protein yield, and BW change. Similar off-target effects of CLA have been previously reported in lactating cows receiving high doses of CLA (Bell and Kennelly, 2003).

At the end of the experimental period, ewes were slaughtered over a 72-h period by stunning and exsanguination. Subsamples of mammary secretory tissues from the left side of the mammary gland were immediately dissected and cubes were prepared. The cubes (≤0.5 cm) were immediately placed in a 15-mL disposable sample tube and immersed in 5 mL of RNAlater solution (RNAlater tissue collection: RNA stabilization solution; Ambion Inc., Austin, TX). After sample tubes

were stored at 4°C for 24 h, the RNAlater solution was decanted and tissue samples were blotted, transferred into a 2-mL Eppendorf tube, and stored at −80°C.

Milk was analyzed for fat and protein as previously described (Sinclair et al., 2007), with lipids extracted and FA methylated and analyzed by a gas chromatograph using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25-mm i.d.; Varian Inc., Palo Alto, CA; Lock et al., 2006). In the calculation of yield for milk FA, the glycerol in milk fat was accounted for according to Schauff et al. (1992).

RNA Extraction and Real-Time PCR

Total RNA was extracted and purified from approximately 30 mg of mammary tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen Inc., Valencia, CA). Potential contamination from genomic DNA was excluded through on-column DNase treatment (RNase-Free DNase Set; Qiagen Inc.). The RNA purity was assessed spectrophotometrically and RNA concentration and quality were verified using an Agilent 2100 Bioanalyzer (RNA 6000 Nano Chip; Agilent Technologies Inc., Santa Clara, CA). Two micrograms of total RNA were reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Carlsbad, CA) with random primers. Quantitative real-time PCR assays were performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems Inc.) with a 2-step program (95°C for 15 s and 60°C for 60 s). Quantitative real-time PCR reactions included Power SYBR Green PCR Master Mix (Applied Biosystems Inc.), 400 nmol/L of gene-specific forward and reverse primers (Invitrogen Corp., Carlsbad, CA), and 25 ng of cDNA. Postamplification dissociation curves were performed to verify the presence of a single amplification product. Relative expression levels were calculated following the relative standard curve method (part no. 4371095 Rev B; Applied Biosystems Inc.) using a 7-point standard curve constructed from pooled cDNA using 2-fold serial dilutions that were included in each plate.

Primer3Plus (Untergasser et al., 2007) was used to design primer pairs of genes of interest (Table 1). Primers were designed from nucleotide sequences specific for sheep or other mammalian species (cow, horse, mouse, and human) when sheep information was unavailable (http://www.ncbi.nlm.nih.gov/nucleotide/).

Statistical Analysis

Data were analyzed using Student's or unequal variance unpaired *t*-test implemented in the R statistical environment (R Development Core Team, 2011). A normalization factor was generated based on recommendations of Vandesompele et al. (2002) from 5 house-keeping genes [18S ribosomal RNA (*18S*), actin β (*ACTB*), ribosomal protein S13 (*RPS13*), ribosomal protein S20 (*RPS20*), and ribosomal protein L13a (*RPL13A*); Table 1] and used as a covariate in gene expression analysis. From the studentized residuals versus fitted values plot, observations >3 or < −3 were considered outliers and excluded from analysis; these were rarely more than 1 per response variable.

RESULTS

Performance-Related Variables

Effects of dietary treatment on performance parameters of lactating ewes at wk 10 of treatment are presented in Table 2. No treatment effects were observed on feed intake or BW between animals in the CON group and those receiving the +CLA treatment. Similarly, no treatment effects were observed on milk yield or milk composition for protein or lactose. In contrast, the +CLA treatment decreased milk fat concentration and milk fat yield. Examination of the temporal pattern revealed that decreases were relatively constant over the treatment period (Sinclair et al., 2010), and at wk 10, the decreases averaged 22 and 24% for milk fat concentration ($P < 0.01$) and milk fat yield ($P < 0.05$), respectively (Table 2).

Comparison of effects of CLA supplement on the FA composition of milk fat demonstrated that the proportions of several FA were altered (Table 3). Specifically, C6:0, C8:0, C10:0, and C12:0 were significantly reduced for the +CLA treatment, whereas C18:0, *cis*-9,*trans*-11 CLA, and *trans*-10,*cis*-12 CLA were increased. Effects of +CLA treatment were further examined by grouping milk FA according to carbon source and comparing the profile and yield (Figure 1). The +CLA treatment had the greatest effect on de novo synthesized FA (<16 carbons), with a 15% decrease in proportion ($P < 0.05$) and a 27% decrease in daily yield ($P < 0.05$). The +CLA treatment also increased the proportion of FA >16 carbons (11%; $P < 0.05$).

Gene Expression

The effects of dietary treatment on the expression of genes involved in mammary lipid synthesis are presented in Figures 2, 3, and 4. The expression of several lipogenic genes were strongly reduced by +CLA treatment. The mRNA abundances for *ACACA*, *FASN*, stearoyl-CoA desaturase 1 (***SCD1***), and glycerol-3-phosphate acyltransferase 6 (*AGPT6*) were reduced by 25 to 40% in the +CLA group compared with CON. The mRNA

Table 1. Ovine primers used in real-time PCR analysis

Gene[1]	Forward primer[2]	Reverse primer
18S	GATCCATTGGAGGGCAAGTCT	GCAGCAATTTAATATACGCTATTGG
ABCG1	CCAGTTCTGCATCCTCTTC	TAGGCCTTCAGGCTGTACCA
ACTB	GCGTGGCTACAGCTTCACC	CTTGATGTCACGGACGATTTC
ACAC	TTTCCTAAATTTTTCACGTTCC	AGGCTCCAGGTGACGATACA
ACSS1	TGAGCCTGGAACTGAAGTGA	GACTCCATACCTCTTGAGTGTGTT
AGPAT6	ACTTCCAGTACATCAGCCTGCGGC	CGTGAAAGCGAGAGCTATCCTG
CD36	TGTGTTTGGAGGGATTCT	CCTTGGCTAGATAACGAACTCTG
DGAT1	CACTGGGACCTGAGGTGTCA	AACCGTGCGTTGCTTAAGATC
FABP3	GGACAGCAAGAATTTCGATGA	CGATGATTGTGGTAGGCTTG
FASN	TGGTGACAGATGATGACAGG	GAAGAAGGAAGCGTCAAACC
G6PD	GGGATCCTGCGAGAAGAG	CCGGAACAGCCACCAGAT
GPAM	GCATTGGTCGGTGTAAGCAT	TTCTTTCCACTTCAAGGTTGC
IDH1	GTGTGCCACCATCACCCCCG	CCAGGCCCCGGCACAACAAA
INSIG1	GTCATCGCCACCATCTTCTC	GACTGTCGATGCAGGGGTA
INSIG2	TCCAGTGTGATGCGGTGTGTA	TGGATAGTGCAGCCAGTGTGA
LPL	ACCTGAAGACTCGTTCTCAGATG	GGCCTGGTTGGTGTATGTATT
MUC1	TCAGGTTCAGGCCAGGATCTG	ATACTGGCTGCGTGTGCTTC
PGD	ATGGCCCAAGCTGACATT	GTGCCCTTCGCCTCATTA
PPARG	AAGAGCCTTCCAACTCCCTCA	CCGGAAGAAACCCTTGCAT
RPL13A	AGCCACCCTGGAGGAGAAGCG	TTTCGGCCTGCTTCCGTAGCC
RPS13	GACGACGTGAAGGAGCAGAT	CTTGTGCAACACCATGAGAG
RPS20	TTCACCGGATTAGGATCACC	CTTTTCCTTCGCGCCTCT
S14	CCTCACCCATCTTACCCTGA	TTGCAGGTCCAGGTCTTTCT
SCAP	CCATGTGCACTTCAAGGAGGA	ATGTCGATCTTGCGTGTGGAG
SCD1	CATCAACCCCCGAGAGAATA	GGTGTGGTGGTAGTTGTGGA
SREBF1	CCAGCTGACAGCTCCATTGA	TGCGCGCCACAAGGA

[1]*ABCG1* = ATP-binding cassette subfamily G member 1; *ACACA* = acetyl-CoA carboxylase α; *ACSS1* = acyl-CoA synthetase short-chain family member 1; *ACTB* = actin β; *AGPAT6* = 1-acylglycerol-3-phosphate O-acyltransferase 6; *CD36* = FA translocase; *DGAT1* = diacylglycerol O-acyltransferase 1; *FABP3* = FA-binding protein 3; *FASN* = FA synthase; *G6PD* = glucose-6-phosphate dehydrogenase; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *IDH1* = NADP-isocitrate dehydrogenase 1; *INSIG1* = insulin-induced gene 1; *INSIG2* = insulin-induced gene 2; *LPL* = lipoprotein lipase; *MUC1* = mucin 1; *PGD* = phosphogluconate dehydrogenase; *PPARG* = peroxisome proliferator-activated receptor gamma; *RPS13* = ribosomal protein S13; *RPS20* = ribosomal protein S20; *RPL13A* = ribosomal protein L13a; *S14* = thyroid hormone responsive spot 14; *SCAP* = SREBF cleavage-activating protein; *SCD1* = stearoyl-CoA desaturase 1; *SREBF1* = sterol regulatory element-binding factor 1; *18S* = ribosomal RNA.

[2]Primers are reported as 5′ to 3′ sequence.

abundance for enzymes involved in NADPH production [glucose-6-phosphate dehydrogenase (***G6PD***), phosphogluconate dehydrogenase (***PGD***), and NADP-isocitrate dehydrogenase (***IDH1***)] were not different between treatments, although G6PD transcript abundance was 30% lower for the +CLA treatment ($P = 0.14$). Among proteins related to transcriptional regulation of lipogenic genes, +CLA treatment decreased

Table 2. Treatment effect on performance variables in dairy ewes

	Treatment[1]			
Variable	CON	+CLA	SEM	*P*-value
BW (kg)	63.1	60.0	3.0	0.49
DMI[2] (kg/d)	2.18	2.09	0.07	0.40
Milk yield (g/d)	1,028	1,016	78	0.91
Milk fat				
g/100 g	6.15	4.83	0.29	0.01
g/d	92.5	70.6	6.0	0.05
Milk protein				
g/100 g	4.93	4.82	0.14	0.56
g/d	49.8	48.1	3.6	0.74
Milk lactose				
g/100 g	4.70	4.77	0.06	0.46
g/d	48.8	48.6	3.8	0.96

[1]Lactating ewes received either control (CON; no supplement) or conjugated linoleic acid supplement (+CLA; 1.5 g of *trans*-10,*cis*-12 CLA/d) for 10 wk starting on d 16 postpartum. During treatment, wk-10 BW and milk yield were determined and milk samples were collected and analyzed. Values represent means and SEM for 8 ewes/treatment.

[2]Average daily DMI during the 10-wk experimental period.

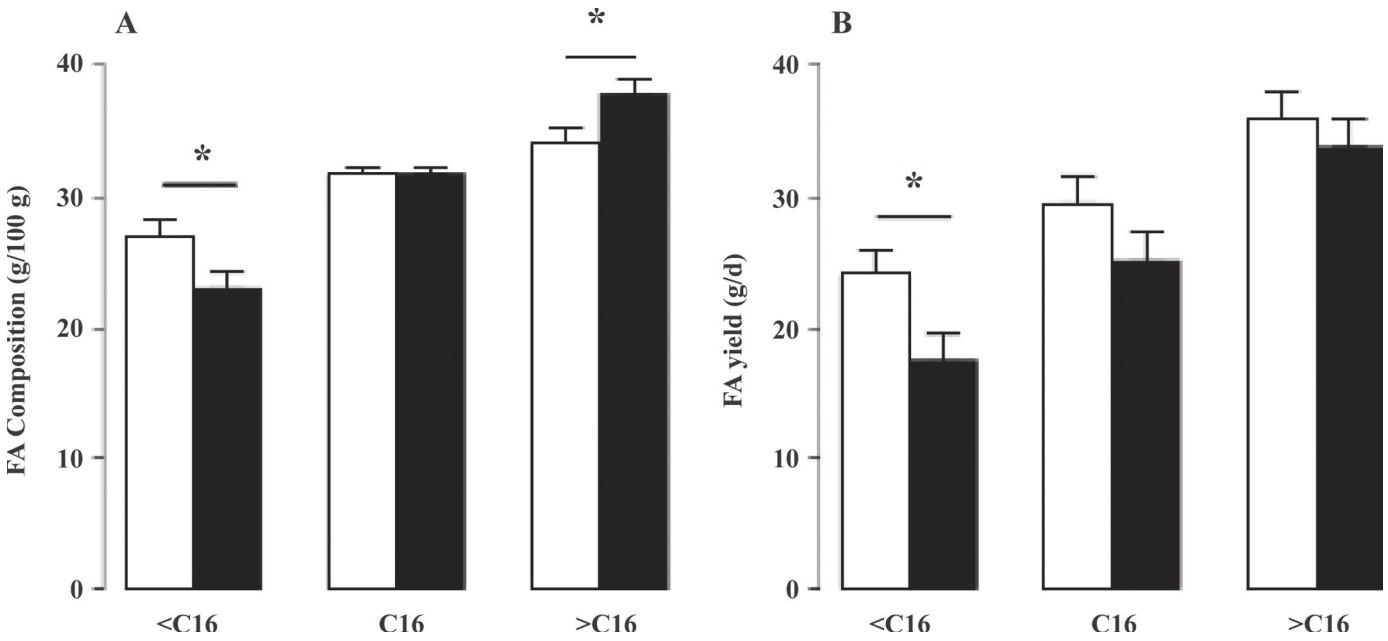

Figure 1. Treatment effects on the composition (panel A) and yield (panel B) of milk FA. Lactating ewes received either control (CON; no supplement; open bars) or conjugated linoleic acid supplement (+CLA; black bars) for 10 wk starting on d 16 postpartum. Milk samples were collected during the last week of treatment and analyzed for FA profile and yield. FA are grouped according to source, with <16 carbons representing de novo-synthesized FA, >16 carbons representing preformed FA taken up from circulation, and 16-carbon FA being derived from both sources. Values represent means for 8 ewes/treatment, with SE bars as indicated. Significant differences are indicated by an asterisk (*). For FA profile, treatment differences were <16 carbons: $P < 0.05$, 16 carbons: $P = 0.88$, and >16 carbons: $P < 0.05$. For milk FA yield, treatment differences were <16 carbons: $P < 0.05$, 16 carbons: $P = 0.18$, and >16 carbons: $P = 0.51$.

Table 3. Treatment effect on milk FA profile for dairy ewes[1]

	Treatment			
Variable	CON	+CLA	SEM	*P*-value
FA				
4:0	2.65	2.88	0.04	<0.01
6:0	1.97	1.51	0.08	<0.01
8:0	1.82	1.23	0.10	<0.01
10:0	5.93	3.94	0.36	<0.01
12:0	3.51	2.76	0.25	<0.05
14:0	9.60	9.32	0.45	0.66
16:0	31.77	31.89	0.52	0.88
cis-9 16:1	1.17	0.93	0.06	<0.01
18:0	8.62	10.63	0.56	<0.05
trans-9 18:1	0.18	0.15	0.01	0.11
trans-10 18:1	0.17	0.19	0.01	0.11
trans-11 18:1	1.05	1.13	0.05	0.28
trans-12 18:1	0.24	0.23	0.02	0.65
cis-9 18:1	19.62	20.96	0.56	0.14
cis-9,*cis*-12 18:2	2.08	2.16	0.11	0.61
cis-9,*cis*-12,*cis*-15 18:3	0.39	0.37	0.01	0.26
cis-9,*trans*-11 CLA	0.48	0.62	0.03	<0.01
trans-10,*cis*-12 CLA	<0.01	0.08	0.00	<0.001
Desaturation index				
cis-9 16:1/(*cis*-9 16:1 + 16:0)	0.04	0.03	0.00	<0.05
cis-9 18:1/(*cis*-9 18:1 + 18:0)	0.70	0.66	0.01	0.06

[1]Lactating ewes received either control (CON; no supplement) or conjugated linoleic acid (+CLA; 1.5 g of *trans*-10,*cis*-12 CLA/d) supplement for 10 wk starting on d 16 postpartum. During treatment, wk-10 milk samples were collected and analyzed for FA profile. Values represent means ± SE for 8 ewes/treatment.

the mRNA abundance of the sterol regulatory element-binding transcription factor 1 (***SREBF1***), insulin-induced gene 1 (***INSIG1***), and ATP-binding cassette subfamily G member 1 [*ABCG1;* target gene of liver X receptor α (***LXRα***)], by 30 to 55%. However, no effect was observed on the mRNA abundance of insulin-induced gene 2 (*INSIG2*), SREBF cleavage-activating protein (***SCAP***), thyroid hormone responsive spot 14 (***S14***), or peroxisome proliferator-activated receptor gamma. Also, CLA decreased mRNA abundance of lipoprotein lipase (***LPL***) by almost 40%, but did not affect mRNA abundance of other proteins involved in FA uptake or transport, FA translocase (***CD36***) and FA-binding protein 3 (***FABP3***).

DISCUSSION

Fat is the most variable component of milk and is especially responsive to nutrition, thereby offering a practical tool to alter its yield and composition (Chilliard et al., 2000). One nutritional situation of practical and biological interest in ruminants is diet-induced MFD. Recent work has established that diet-induced MFD is caused by biohydrogenation intermediates produced during rumen fermentation, and the most extensively investigated of these is *trans*-10,*cis*-12 CLA (Bauman et al., 2008). In lactating cows, the downregulation of mammary lipid synthesis is the most obvious biomarker in CLA-induced MFD, and fat is the only milk component inhibited with *trans*-10,*cis*-12 CLA treatment. Although the mechanism for CLA-induced MFD is not fully understood in dairy cows (Harvatine et al., 2009; Bauman et al., 2011), the ability of CLA to induce MFD has also been observed in lactating ewes (Lock et al., 2006; Sinclair et al., 2007). Further, when extrapolated to a metabolic liveweight basis, the dose response relationship of daily CLA intake and MFD in sheep was similar to that reported for dairy cows. Therefore, the objective of the present investigation was to con-

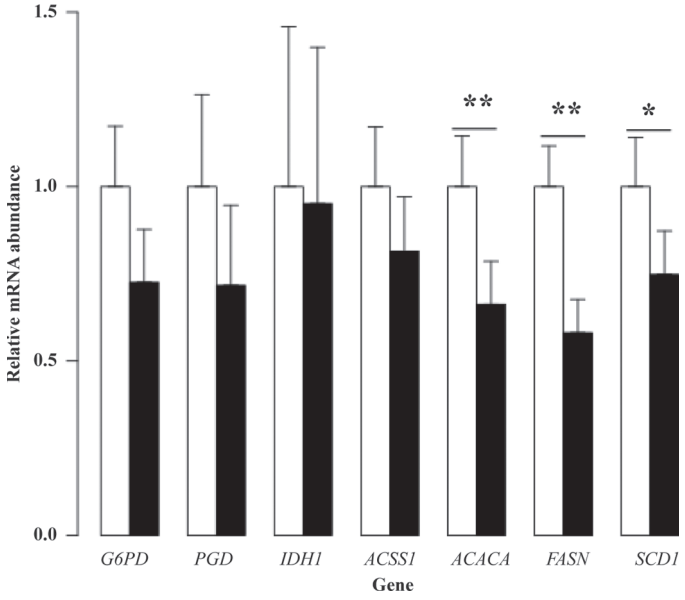

Figure 2. Treatment effects on mammary expression of genes involved in de novo synthesis of FA. Lactating ewes received either control (CON; open bars; no supplement) or conjugated linoleic acid supplement (+CLA; black bars) for 10 wk starting on d 16 postpartum. Mammary gland samples were collected at the end of the treatment and total RNA was analyzed by quantitative real-time PCR for the mRNA abundance of indicated genes. *G6PD* = glucose-6-phosphate dehydrogenase; *PGD* = phosphogluconate dehydrogenase; *IDH1* = isocitrate dehydrogenase 1 (NADP+); *ACSS1* = acyl-CoA synthetase short-chain family member 1; *ACACA* = acetyl-CoA carboxylase α; *FASN* = FA synthase; *SCD1* = stearoyl-CoA desaturase 1. Values represent means for 8 ewes/treatment, with SE bars as indicated. Significant differences are indicated by asterisks for $P < 0.05$ (**) and $P < 0.10$ (*). Treatment *P*-values for individual genes were *G6PD*: $P = 0.14$, *PGD*: $P = 0.25$, *IDH1*: $P = 0.92$, *ACSS1*: $P = 0.30$, *ACACA*: $P < 0.05$, *FASN*: $P < 0.01$, and *SCD1*: $P < 0.10$.

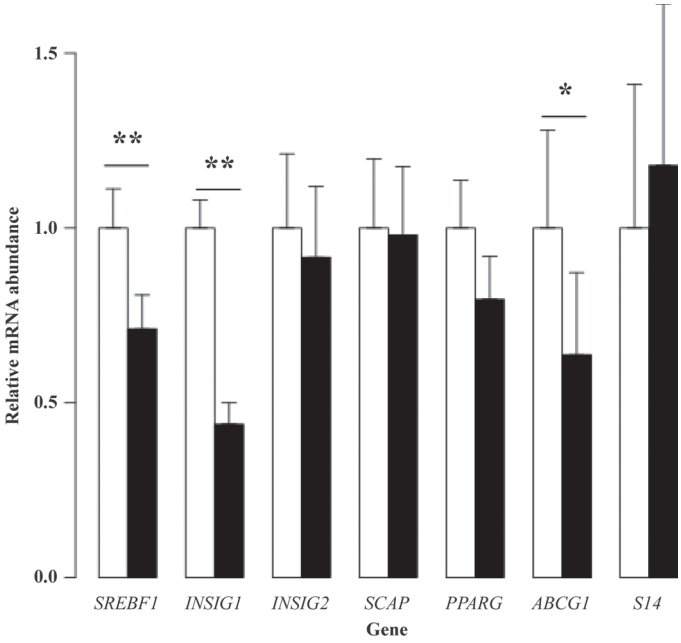

Figure 3. Treatment effects on mammary expression of genes involved in transcription regulation. Lactating ewes received either control (CON; no supplement; open bars) or conjugated linoleic acid supplement (+CLA; black bars) for 10 wk starting on d 16 postpartum. Mammary tissue samples were collected at the end of the treatment and total RNA was analyzed by quantitative real-time PCR for the mRNA abundance of indicated genes. *SREBF1* = sterol regulatory element-binding factor 1; *INSIG1* = insulin-induced gene 1; *INSIG1* = insulin-induced gene 2; *SCAP* = SREBF cleavage-activating protein; *PPARG* = peroxisome proliferator-activated receptor gamma; *ABCG1* = ATP-binding cassette subfamily G member 1; *S14* = thyroid hormone responsive spot 14. Values represent means for 8 ewes/treatment, with SE bars as indicated. Significant differences are indicated by asterisks for $P < 0.05$ (**) and for $P < 0.10$ (*). Treatment *P*-values for individual genes were *SREBF1*: $P < 0.05$, *INSIG1*: $P < 0.001$, *INSIG2*: $P = 0.70$, *SCAP*: $P = 0.92$, *PPARG*: $P = 0.16$, *ABCG1*: $P = 0.07$, and *S14*: $P = 0.68$.

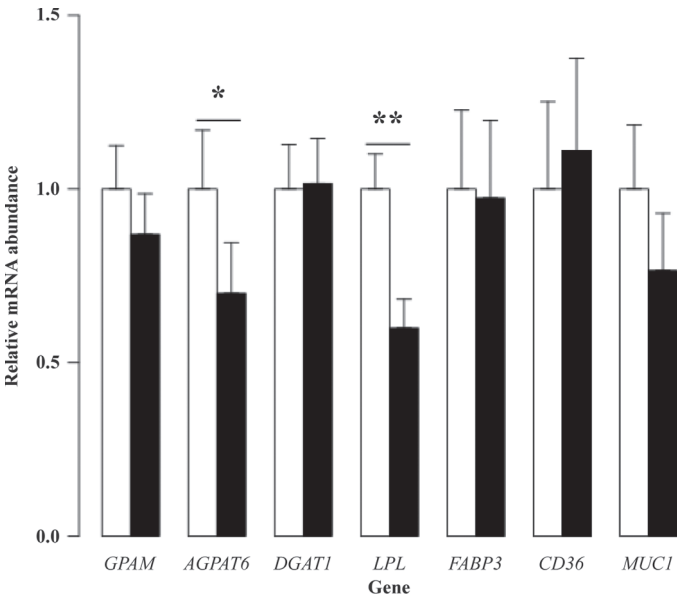

Figure 4. Treatment effects on mammary expression of genes involved in the uptake and esterification of FA. Lactating ewes received either control (CON; no supplement; open bars) or conjugated linoleic acid supplement (+CLA; black bars) for 10 wk starting on d 16 postpartum. Mammary tissue samples were collected at the end of the treatment and total RNA was analyzed by quantitative real-time PCR for the mRNA abundance of indicated genes. *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *AGPAT6* = 1-acylglycerol-3-phosphate O-acyltransferase 6; *DGAT1* = diacylglycerol O-acyltransferase 1; *LPL* = lipoprotein lipase; *FABP3* = FA-binding protein 3; *CD36* = FA translocase; *MUC1* = mucin 1. Values represent means for 8 ewes/treatment, with SE bars as indicated. Significant differences are indicated by asterisks for $P < 0.05$ (**) and for $P < 0.10$ (*). Treatment *P*-values for individual genes were *GPAM*: $P = 0.32$, *AGPAT6*: $P = 0.10$, *DGAT1*: $P = 0.91$, *LPL*: $P < 0.01$, *FABP3*: $P = 0.91$, *CD36*: $P = 0.67$, and *MUC1*: $P = 0.23$.

sider dairy ewes as a model of CLA-induced MFD and conduct an initial characterization of the molecular mechanism of CLA-induced MFD in ewes.

Consistent with previous investigations using dairy cows, CLA treatment in the current study decreased both milk fat concentration and milk fat yield by approximately 23%. This reduction is similar to the 17% decrease that is predicted based on the relationship of milk fat concentration of *trans*-10,*cis*-12 CLA and the percentage reduction in milk fat yield developed for dairy cows abomasally infused with *trans*-10,*cis*-12 CLA (de Veth et al., 2004). The slight difference may be accounted for by the ruminal formation of other bioactive isomers from the CLA supplement. Furthermore, the CLA effect in dairy ewes was specific for mammary lipid synthesis for the +CLA treatment dose, as no treatment effects occurred on milk yield or milk composition of protein or lactose.

The FA in milk fat arise from either uptake of preformed FA from circulation or de novo synthesis within the mammary epithelial cells. Short- and medium-chain FA (4 to 14 carbons) arise almost exclusively from de novo synthesis in mammary tissue, with acetate and to a lesser extent β-hydroxybutyrate serving as carbon sources (Bauman and Davis, 1974). On the other hand, long-chain FA (>16 carbons) are derived predominately from the uptake of FA from circulating lipoproteins (major) and nonesterified FA (minor), whereas FA of 16 carbons in length originate from both de novo synthesis and uptake of preformed FA (Bauman and Griinari, 2003). In ruminants, about one-half of the FA in milk fat are derived from each source on a molar basis (Bauman and Davis, 1974). During CLA-induced MFD in dairy cows, the reduction in milk fat involves FA of all chain lengths but the decrease is more substantial for de novo-synthesized FA (Bauman et al., 2008). A more pronounced reduction in de novo-synthesized FA has also been reported for CLA-induced MFD in dairy ewes (Lock et al., 2006; Sinclair et al., 2007). In the present study, CLA treatment significantly decreased the yield of short- and medium-chain FA (<16 carbons) by 27% ($P < 0.05$).

The response of lactating mammals to physiological doses of *trans*-10,*cis*-12 CLA demonstrates that CLA effects are highly specific for mammary lipid synthesis and involves the biochemical pathways of milk fat synthesis (Bauman et al., 2008). Briefly, CLA induces a coordinated downregulation in mRNA abundance and (or) enzymatic activity for genes involved in the uptake, de novo synthesis, desaturation, and esterification of FA in mammary tissue of both dairy cows (Baumgard et al., 2002; Peterson et al., 2003; Harvatine and Bauman, 2006; Gervais et al., 2009) and lactating rodents (Lin et al., 2004; Ringseis et al., 2004; Hayashi et al., 2007; Kadegowda et al. 2010).

The present study extends the investigation of CLA-induced MFD to dairy ewes by characterizing the effects on mRNA abundance of genes relating to the lipogenic pathways for mammary synthesis of milk fat. Three enzymes involved in de novo lipogenesis were examined: (1) acyl-CoA synthetase short-chain 1 (*ACSS1*), the cytosolic enzyme that catalyzes the activation of acetate, (2) *ACACA*, the biotin-containing enzyme that catalyzes the formation of malonyl-CoA, and (3) *FASN,* the multifunctional protein that catalyzes the use of malonyl-CoA to form SFA. The mRNA abundances for *ACACA* and *FASN* were decreased by 35 to 40% in the mammary tissue from ewes receiving the CLA supplement. In the biochemical pathway for the use of preformed FA we examined *LPL*, whose primary function is the hydrolysis of triglycerides in circulating chylomicra and very low-density lipoproteins, and *FABP3* and *CD36* that are involved in the uptake and intracellular transport of FA. Mammary mRNA abundance of *LPL* was reduced by 40% in the CLA treat-

ment group, whereas mRNA abundance for *FABP3* and *CD36* was unchanged. Most of the oleic acid in milk fat is synthesized in the mammary gland from stearic acid via the action of SCD1 (Harvatine et al., 2009); in the present study, mammary mRNA abundance of *SCD1* was reduced over 25% in the CLA-treated group. Milk FA are mainly secreted as triglycerides (Bauman and Davis, 1974; Bernard et al., 2008). In the present study, CLA treatment decreased the mRNA abundance of *AGPAT6* by almost 30% compared with CON. Overall, the present study examined key enzymes involved in milk fat synthesis and observed that mRNA abundance for most was reduced after 10 wk of treatment with *trans*-10,*cis*-12 CLA. The changes in mRNA abundance for an individual enzyme may represent a primary mechanistic response or a secondary adaptation to the reduction in milk fat synthesis. However, wherever values for the same enzymes have been reported, similar decreases in mammary mRNA abundance have been reported for *trans*-10,*cis*-12 CLA treatment of dairy cows (Baumgard et al., 2002; Peterson et al., 2003; Harvatine and Bauman, 2006; Gervais et al., 2009) and lactating rodents (Lin et al., 2004; Ringseis et al., 2004; Hayashi et al., 2007; Kadegowda et al., 2010).

The biosynthesis of FA also requires considerable quantities of reducing equivalents with 2 NADPH required for each 2-carbon elongation of the FA (Bauman and Davis, 1974). In rodent models, the first 2 enzymes of the pentose phosphate pathway (G6PD and PGD) contribute about one-half of the reducing power for FA synthesis, with the remainder coming from the malate transhydrogenation cycle via NADP-malate dehydrogenase. In ruminant mammary tissue, NADP-malate dehydrogenase is negligible and its contribution of NADPH is replaced by cytosolic IDH1 (Bauman and Davis, 1974). In the present study, CLA treatment had no significant effect on the mRNA abundance of *IDH1*, *G6PD*, or *PGD*.

The molecular mechanisms mediating the CLA-induced inhibition of milk fat synthesis are not well understood, but a role for the SREBF family of transcription factors was proposed (Baumgard et al., 2002) based on their function as global regulators of expression for many genes involved in lipid synthesis (Eberlé et al., 2004; Shimano, 2009). The SREBF are synthesized in the endoplasmic reticulum where they are anchored by INSIG protein. To effect transcription, SREBF must first be escorted to the Golgi by SCAP, where the active N-terminal fragment is released by proteolytic cleavage. This active fragment is translocated to the nucleus where it affects transcription rates of specific genes by binding to a sterol regulatory element DNA sequence in the gene promoter region. Of the genes quantified in the present study, those with a sterol regulatory element DNA sequence include *ACACA*, *ACSS1*, *FABP3*, *FASN*, *G6PD*, *GPAM*, *IDH1*, *INSIG1*, *PGD*, *S14*, *SCD1* and *SREBPF1* (Ikeda et al., 2001; Horton et al., 2003; Eberlé et al., 2004; Ma and Corl, 2012). A role for SREBF1 was supported in studies with a bovine mammary epithelial cell line, where *trans*-10,*cis*-12 CLA decreased abundance of the nuclear active SREBF1 protein (Peterson et al., 2004). Subsequent studies showed a downregulation of *SREBF1* and *INSIG1* in milk fat-depressed cows that were receiving *trans*-10,*cis*-12 CLA supplements (Harvatine and Bauman, 2006; Gervais et al., 2009). Consistent with these results, CLA treatment of lactating ewes decreased the mammary mRNA abundance of the *SREBF1* and *INSIG1* by almost 30 and 55%, respectively, in the present study. However, no treatment effect was observed on mRNA abundance of *SCAP* or *INSIG2*. Sterol regulatory element-binding transcription factor 1 is also regulated by LXRα in mammary epithelial cells and LXR activation increased de novo FA synthesis and *FASN* mRNA abundance associated with upregulation of transcription, translation, and proteolytic cleavage of SREBF1 (McFadden and Corl, 2010; Oppi-Williams et al., 2013). In the current study, CLA treatment tended to decrease mRNA abundance of *ABCG1*, an LXR target gene, by almost 36% ($P = 0.07$), which suggests a downregulation in the activity or abundance of LXRα due to CLA treatment.

Conjugated linoleic acid treatment has also caused a reduction in mammary mRNA for *S14* in lactating cows (Harvatine and Bauman, 2006) and mice (Kadegowda et al., 2010), but no effect of CLA treatment was observed on mRNA abundance of *S14* in the present study. The exact role of S14 in the regulation of lipogenesis is unknown, but several reports have demonstrated that mRNA expression of *S14* is highly responsive to changes in lipogenesis in adipose tissue and liver (summarized by Cunningham et al., 1998), as well as the aforementioned studies with mammary tissue. Expression of *S14* is very dynamic and is expected to be responsive to short-term treatment. Expression may have been affected by the handling of animals before sample collection, as the ewes were without feed for 2 to 6 h before slaughter.

CONCLUSIONS

The present study demonstrated that CLA supplementation to lactating ewes decreased milk fat secretion with de novo FA synthesis being most markedly affected. Consistent with this and similar to other species, a coordinated downregulation in the mRNA expression of key enzymes involved in de novo lipogenesis occurred. These reductions were associated with the

SREBF transcription system based on the corresponding reduction in the mRNA expression of *SREBF1* and *INSIG1*, and the fact that the genes for the affected enzymes contain a sterol response element in their promoter region. Lastly, NADPH synthesis does not appear to be a primary driver of the reduced mammary lipogenic capacity, although a modest downregulation of expression of NADPH-generating enzymes in the pentose phosphate pathway may occur. Overall, the ewe represents a good model to examine fat synthesis in the mammary gland, and the CLA-induced milk fat depression in the lactating ewe appears to occur by mechanisms similar to those in other species.

REFERENCES

Bauman, D. E., and C. L. Davis. 1974. Biosynthesis of milk fat. Pages 31–75 in Lactation: A Comprehensive Treatise, Vol. 2. B. L. Larson and V. R. Smith, ed. Academic Press, New York, NY.

Bauman, D. E., and J. M. Griinari. 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23:203–227.

Bauman, D. E., K. J. Harvatine, and A. L. Lock. 2011. Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Annu. Rev. Nutr. 31:299–319.

Bauman, D. E., and A. L. Lock. 2006. Conjugated linoleic acid: Biosynthesis and nutritional significance. Pages 93–136 in Advanced Dairy Chemistry. Vol. 2: Lipids. 3rd ed. P. F. Fox and P. L. H. McSweeney, ed. Springer, New York, NY.

Bauman, D. E., J. W. Perfield, K. J. Harvatine, and L. H. Baumgard. 2008. Regulation of fat synthesis by conjugated linoleic acid: Lactation and the ruminant model. J. Nutr. 138:403–409.

Baumgard, L. H., B. A. Corl, D. A. Dwyer, A. Saebø, and D. E. Bauman. 2000. Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 278:R179–R184.

Baumgard, L. H., E. Matitashvili, B. A. Corl, D. A. Dwyer, and D. E. Bauman. 2002. *trans*-10, *cis*-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. J. Dairy Sci. 85:2155–2163.

Bell, J. A., and J. J. Kennelly. 2003. Short communication: Postruminal infusion of conjugated linoleic acids negatively impacts milk synthesis in Holstein cows. J. Dairy Sci. 86:1321–1324.

Belury, M. A. 2002. Dietary conjugated linoleic acid in health: Physiological effects and mechanisms of action. Annu. Rev. Nutr. 22:505–531.

Bernard, L., M. Bonnet, C. Leroux, K. J. Shingfield, and Y. Chilliard. 2009. Effect of sunflower-seed oil and linseed oil on tissue lipid metabolism, gene expression, and milk fatty acid secretion in Alpine goats fed maize silage-based diets. J. Dairy Sci. 92:6083–6094.

Bernard, L., C. Leroux, and Y. Chilliard. 2008. Expression and nutritional regulation of lipogenic genes in the ruminant lactating mammary gland. Adv. Exp. Med. Biol. 606:67–108.

Bhattacharya, A., J. Banu, M. Rahman, J. Causey, and G. Fernandes. 2006. Biological effects of conjugated linoleic acids in health and disease. J. Nutr. Biochem. 17:789–810.

Chilliard, Y., A. Ferlay, R. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity: Nutritional control of saturated, polyunsaturated, *trans* and conjugated fatty acids. Ann. Zootech. 49:181–205.

Chilliard, Y., A. Ferlay, J. Rouel, and G. Lamberet. 2003. A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. J. Dairy Sci. 86:1751–1770.

Cunningham, B. A., J. T. Moncur, J. T. Huntington, and W. B. Kinlaw. 1998. "Spot 14" protein: A metabolic integrator in normal and neoplastic cells. Thyroid 8:815–825.

de Veth, M. J., J. M. Griinari, A.-M. Pfeiffer, and D. E. Bauman. 2004. Effect of CLA on milk fat synthesis in dairy cows: comparison of inhibition by methyl esters and free fatty acids, and relationships among studies. Lipids 39:365–372.

Eberlé, D., B. Hegarty, P. Bossard, P. Ferré, and F. Foufelle. 2004. SREBP transcription factors: Master regulators of lipid homeostasis. Biochimie 86:839–848.

Gervais, R., J. W. McFadden, A. J. Lengi, B. A. Corl, and P. Y. Chouinard. 2009. Effects of intravenous infusion of *trans*-10, *cis*-12 18:2 on mammary lipid metabolism in lactating dairy cows. J. Dairy Sci. 92:5167–5177.

Harvatine, K. J., Y. R. Boisclair, and D. E. Bauman. 2009. Recent advances in the regulation of milk fat synthesis. Animal 3:40–54.

Harvatine, K. J., and D. E. Bauman. 2006. SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA. J. Nutr. 136:2468–2474.

Hayashi, A. A., S. R. de Medeiros, M. H. Carvalho, and D. P. D. Lanna. 2007. Conjugated linoleic acid (CLA) effects on pups growth, milk composition and lipogenic enzymes in lactating rats. J. Dairy Res. 74:160–166.

Horton, J. D., N. A. Shah, J. A. Warrington, N. N. Anderson, S. W. Park, M. S. Brown, and J. L. Goldstein. 2003. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc. Natl. Acad. Sci. USA 100:12027–12032.

Ikeda, Y., J. Yamamoto, M. Okamura, T. Fujino, S. Takahashi, K. Takeuchi, T. F. Osborne, T. T. Yamamoto, S. Ito, and J. Sakai. 2001. Transcriptional regulation of the murine acetyl-CoA synthetase 1 gene through multiple clustered binding sites for sterol regulatory element-binding proteins and a single neighboring site for Sp1. J. Biol. Chem. 276:34259–34269.

Kadegowda, A. K. G., E. E. Connor, B. B. Teter, J. Sampugna, P. Delmonte, L. S. Piperova, and R. A. Erdman. 2010. Dietary *trans* fatty acid isomers differ in their effects on mammary lipid metabolism as well as lipogenic gene expression in lactating mice. J. Nutr. 140:919–924.

Lin, X., J. J. Loor, and J. H. Herbein. 2004. *Trans*10,*cis*12-18:2 is a more potent inhibitor of de novo fatty acid synthesis and desaturation than *cis*9,*trans*11-18:2 in the mammary gland of lactating mice. J. Nutr. 134:1362–1368.

Lock, A. L., M. Rovai, T. A. Gipson, M. J. de Veth, and D. E. Bauman. 2008. A conjugated linoleic acid supplement containing *trans*-10, *cis*-12 conjugated linoleic acid reduces milk fat synthesis in lactating goats. J. Dairy Sci. 91:3291–3299.

Lock, A. L., B. M. Teles, J. W. Perfield, D. E. Bauman, and L. A. Sinclair. 2006. A conjugated linoleic acid supplement containing *trans*-10, *cis*-12 reduces milk fat synthesis in lactating sheep. J. Dairy Sci. 89:1525–1532.

Ma, L., and B. A. Corl. 2012. Transcriptional regulation of lipid synthesis in bovine mammary epithelial cells by sterol regulatory element binding protein-1. J. Dairy Sci. 95:3743–3755.

McFadden, J. W., and B. A. Corl. 2010. Activation of liver X receptor (LXR) enhances de novo fatty acid synthesis in bovine mammary epithelial cells. J. Dairy Sci. 93:4651–4658.

Oppi-Williams, C., J. K. Suagee, and B. A. Corl. 2013. Regulation of lipid synthesis by liver X receptor α and sterol regulatory element-binding protein 1 in mammary epithelial cells. J. Dairy Sci. 96:112–121.

Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2003. Diet-induced milk fat depression in dairy cows results in increased *trans*-10, *cis*-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. J. Nutr. 133:3098–3102.

Peterson, D. G., E. A. Matitashvili, and D. E. Bauman. 2004. The inhibitory effect of *trans*-10, *cis*-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1. J. Nutr. 134:2523–2527.

R Development Core Team. 2011. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Ringseis, R., D. Saal, A. Müller, H. Steinhart, and K. Eder. 2004. Dietary conjugated linoleic acids lower the triacylglycerol concentration in the milk of lactating rats and impair the growth and increase the mortality of their suckling pups. J. Nutr. 134:3327–3334.

Schauff, D. J., J. H. Clark, and J. K. Drackley. 1992. Effects of feeding lactating dairy cows diets containing extruded soybeans and calcium salts of long-chain fatty acids. J. Dairy Sci. 75:3003–3019.

Shimano, H. 2009. SREBPs: Novel aspects of SREBPs in the regulation of lipid synthesis. FEBS J. 276:615–621.

Shingfield, K. J., L. Bernard, C. Leroux, and Y. Chilliard. 2010. Role of *trans* fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. Animal 4:1140–1166.

Shingfield, K. J., J. Rouel, and Y. Chilliard. 2009. Effect of calcium salts of a mixture of conjugated linoleic acids containing *trans*-10, *cis*-12 in the diet on milk fat synthesis in goats. Br. J. Nutr. 101:1006–1019.

Sinclair, L. A., A. L. Lock, R. Early, and D. E. Bauman. 2007. Effects of *trans*-10, *cis*-12 conjugated linoleic acid on ovine milk fat synthesis and cheese properties. J. Dairy Sci. 90:3326–3335.

Sinclair, L. A., W. M. P. B. Weerasinghe, R. G. Wilkinson, M. J. de Veth, and D. E. Bauman. 2010. A supplement containing *trans*-10, *cis*-12 conjugated linoleic acid reduces milk fat yield but does not alter organ weight or body fat deposition in lactating ewes. J. Nutr. 140:1949–1955.

Untergasser, A., H. Nijveen, X. Rao, T. Bisseling, R. Geurts, and J. A. M. Leunissen. 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35(Suppl. 2):W71–W74.

Vandesompele, J., K. D. Preter, F. Pattyn, B. Poppe, N. V. Roy, A. D. Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:research0034–research0034.11.

Weerasinghe, W. M. P. B., R. G. Wilkinson, A. L. Lock, M. J. de Veth, D. E. Bauman, and L. A. Sinclair. 2012. Effect of a supplement containing *trans*-10,*cis*-12 conjugated linoleic acid on the performance of dairy ewes fed 2 levels of metabolizable protein and at a restricted energy intake. J. Dairy Sci. 95:109–116.