Effects of pasture versus confinement and marine oil supplementation on the expression of genes involved in lipid metabolism in mammary, liver, and adipose tissues of lactating dairy cows

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ABSTRACT

Research was conducted to evaluate the effects of management system (MS), marine lipid supplementation (LS), and their interaction on the relative mRNA abundance of 11 genes involved in lipid synthesis in mammary, liver, and subcutaneous adipose tissues in lactating dairy cows. These genes included those involved in FA uptake (*LPL*), de novo FA synthesis (*ACACA*, *FASN*), FA desaturation (*SCD1*, *FADS1*, *FADS2*), and transcriptional regulation of lipogenesis (*SREBF1*, *SCAP*, *INSIG1*, *THRSP*, and *PPARG*). Forty-eight periparturient Holstein cows were blocked by parity and predicted calving date and assigned to either a pasture ($n = 23$) or confinement ($n = 25$) system. Within each system, cows were allocated randomly (7–9 cows per treatment) to a control (no oil supplement) or 1 of 2 isolipidic (200 g/d) supplements, fish oil (FO) or microalgae (MA), for 125 ± 5 d starting 30 d pre-calving. The experiment was conducted as a split-plot design, with MS being the whole plot treatment and LS as the subplot treatment. At 100 ± 2 DIM, 4 cows from each treatment combination (24 cows in total) were euthanized and tissue samples were collected for gene expression analysis. No interactions between MS and LS were observed regarding any of the variables measured in this study. Milk production (34.0 vs. 40.1 kg/d), milk fat (1.10 vs. 1.41 kg/d), protein (0.95 vs. 1.22 kg/d), and lactose (1.56 vs. 1.86 kg/d) were lower for pasture compared with confinement. The effect of LS on milk production and milk composition (yields and contents) was significant only for milk fat content that was reduced with MA compared with FO (3.00 vs. 3.40%) and the control (3.56%). The mammary mRNA abundance of *PPARG* (–32%) and *FASN* (–29%) was lower in grazing compared with confined cows, which was accompanied by reduced (–43%) secretion of de

novo synthesized fatty acids in milk. Grazing was associated with reduced expression of *ACACA* (–48%), *FASN* (–48%), and *THRSP* (–53%) in subcutaneous adipose tissues, which was consistent with the lower body condition score (i.e., lower net adipose tissue deposition) in grazing compared with confined cows. Feeding either FO or MA downregulated hepatic expression of *FASN*, *SCD1*, *FADS2*, and *THRSP*. The reduced secretion of de novo synthesized fatty acids in milk of grazing cows compared with confined cows might be related in part to the downregulation of genes involved in lipid synthesis, and that LS have tissue-specific effects on expression of genes involved in lipid metabolism, with liver being the most responsive tissue. **Key words:** grazing, total mixed ration, fish oil, microalgae

INTRODUCTION

Feeding plant or marine oils to dairy cows has been shown to improve the milk fat content of beneficial unsaturated FA, including *cis*-9 18:1 (oleic acid; **OA**), n-3 PUFA, and *cis*-9, *trans*-11 CLA (common name rumenic acid; **RA**), and reduce the concentration of detrimental SFA, including 12:0, 14:0, and 16:0 (Chilliard et al., 2007). The latter effect has been attributed mainly to the downregulation of genes involved in milk fat synthesis by specific ruminal biohydrogenation (**RBH**) intermediates such as *trans*-10, *cis*-12 CLA (Harvatine et al., 2009a; Shingfield et al., 2010).

Previous studies (Chilliard et al., 2007; Vahmani et al., 2013) have shown that, compared with cows fed conserved forages and more grain in confinement, grazing cows produce milk fat with a higher content of beneficial unsaturated FA and lower content of detrimental SFA. However, the effect of dairy farm management system (**MS**; pasture vs. confinement) or its interaction with PUFA supplementation on milk fat synthesis has not been studied.

Research in rodents has shown that PUFA supplementation downregulates the expression of genes involved

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in lipid synthesis in liver and adipose tissue, resulting in decreased lipogenesis in these tissues (Jump, 2002; Wang and Jones, 2004). However, studies investigating effects of PUFA supplementation on gene expression in extra-mammary tissues in dairy cattle are scarce. Harvatine et al. (2009b) reported that intravenous infusion of *trans*-10, *cis*-12 CLA reduced mammary expression of lipoprotein lipase (*LPL*), fatty acid synthase (*FASN*), sterol regulatory element-binding transcription factor 1 (*SREBF1*), and thyroid hormone responsive spot 14 (*THRSP*) in dairy cows; however, the opposite effect (i.e., increased expression of these genes) was seen in adipose tissue. A recent study in dairy goats showed that feeding fish oil reduced the expression of *FASN*, stearoyl-CoA desaturase 1 (*SCD1*), and fatty acid desaturase 2 (*FADS2*) in liver, whereas the expression of these genes was not altered in mammary tissue and omental adipose tissue (Toral et al., 2013).

We hypothesized that MS, marine lipid supplementation (**LS**) or their interaction would affect the expression of genes involved in lipid synthesis in mammary, liver, and subcutaneous adipose (**SUBQ**), and that the effects would be tissue-specific. The objective of the current study was to determine the effect of MS, LS, and their interaction on mRNA abundance of genes encoding proteins required for FA uptake, de novo FA synthesis, desaturation, and transcriptional regulation of lipid synthesis in mammary, liver, and SUBQ in lactating dairy cows.

MATERIALS AND METHODS

Experimental Design, Treatments, and Animal Measurements

All procedures performed on cows in the current study were carried out according to the Canadian Council for Animal Care guidelines and were approved by the Animal Care and Use Committee at the Faculty of Agriculture of Dalhousie University. Details of the experimental design and animal management have been reported elsewhere (Vahmani et al., 2013). Briefly, 48 periparturient Holstein cows were blocked by parity and predicted calving date and assigned within block to either a pasture ($n = 23$) or confinement ($n = 25$). Within the system, cows were allocated randomly to a control (no oil supplement) or to 1 of 2 isolipidic (200 g/d) marine oil supplements, fish oil (**FO**) or microalgae (**MA**), for 125 ± 5 d starting at 30 d precalving. The number of cows per treatment within each MS varied between 7 and 9. Both supplements provided similar amounts of long-chain (C20–C22) PUFA (~65 g/d), but contained different proportions of eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**).

The predominant long-chain PUFA in MA was DHA (24% of total FA), whereas EPA and DHA were equally predominant and comprised about 27% of total FA in FO. Fatty acid composition of lipid supplements is presented in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2013-7290>). The experiment was conducted as a split-plot design, with MS as the whole plot treatment and LS as the subplot treatment. Both pasture and confinement groups were housed in a tie-stall barn from -30 until 28 ± 10 DIM and were fed a TMR with similar formulations. The pasture group was then adapted to a pasture and grazed rotationally on a perennial sward until the end of the experiment (95 ± 5 DIM). The confinement group remained in the tie-stall barn and was fed a TMR (forage-to-concentrate ratio of 56:44, DM basis) ad libitum twice daily (0700 and 1500 h). Grazing cows were fed 8.0 kg of concentrate/d (as-fed basis) in equal portions at milking (0600 and 1600 h). The concentrate was similar to that used in the TMR for confined cows. The nutrient and FA composition of feeds are available in Supplemental Tables S1 and S2 (<http://dx.doi.org/10.3168/jds.2013-7290>). Cows had continuous access to water.

Tissue Collection

Four cows were randomly selected from each treatment within each MS (24 cows in total) and transferred to a commercial abattoir at 100 ± 2 DIM, immediately after morning milking, for tissue sampling. Mammary, liver, and SUBQ (between the hook and pin bones) were sampled immediately after exsanguination, snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from 200 mg of mammary and liver tissues using the RNeasy Midi Kit and total RNA in SUBQ was extracted from 400 mg of tissue using the RNeasy Lipid Tissue Midi Kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Concentration of RNA was determined by absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and absorbance was measured to ensure the absorbance ratios of 260:280 and 260:230 were between 1.8 and 2.1 for all RNA samples. Integrity of RNA was confirmed by visualization of the 28S and 18S ribosomal RNA bands after electrophoresis of 500 ng of each RNA sample on a 1% agarose gel stained with ethidium bromide. One microgram of total RNA from each sample was reverse-transcribed in a final volume of 20 μL using the Quantitect Reverse Transcription kit

with genomic DNA Wipeout (Qiagen) according to the manufacturer's instructions. The cDNA samples were diluted 1:50 in DNase/RNase free water and stored at -30°C until analysis by quantitative real-time PCR (qRT-PCR). Serial dilutions (5 point, 5-fold dilution) of pooled cDNA (made from undiluted cDNA samples) were prepared for each tissue type for generation of standard curves for qRT-PCR.

Primer Design and Evaluation, and qRT-PCR

Primers were either designed using Primer 3.0 software (<http://frodo.wi.mit.edu/primer3/input.htm>) or drawn from previous publications. Sequences and other details of primers used in this study are presented in Supplemental Table S3 (<http://dx.doi.org/10.3168/jds.2013-7290>). The primers were subjected to a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure specificity of the primer and lack of homology with nontarget sequence. Primer specificity was verified by agarose gel electrophoresis of amplified cDNA (PCR product) and by melt-curve analysis following qRT-PCR. Each primer pair produced a single PCR product of expected size when visualized on agarose gel and yielded a single melt-curve peak.

Quantitative real-time PCR was performed in a MicroAmp Optical 96-well reaction plate (Applied Biosystems, Grand Island, NY) in a final volume of 20 μL per well containing 8.4 μL of diluted cDNA, 10 μL of FastStart Universal SYBR Green Master Mix (ROX; Roche Applied Science, Indianapolis, IN), and 0.8 μL each of 10 μM forward and reverse primers. Each plate contained all 24 samples within each tissue type in duplicate for the gene being evaluated on a 5-point standard curve of pooled cDNA in triplicate, as well as nontemplate and reverse transcription negative controls. The reactions were performed in an ABI Step-One-Plus real-time PCR system (Applied Biosystems) with initial denaturing for 10 min at 95°C , followed by 40 cycles of 15 s at 95°C (denaturation), and 1 min at 57.4°C (annealing + extension). This was followed by a melt-curve analysis to ensure specific amplification. Amplification efficiency (E) was calculated using the slope of the standard curve according to $E = 10^{(-1/\text{slope})} - 1$ (Step-One Plus software version 2.1, Applied Biosystems). Real-time PCR runs with efficiencies between 90 and 110% were considered acceptable and used for data analysis. Relative mRNA levels were calculated from the standard curve on each PCR plate using the Step-One Plus Software (version 2.1, Applied Biosystems).

Expression of genes involved in FA uptake (*LPL*), de novo FA synthesis [*FASN* and acetyl-coenzyme A carboxylase (*ACACA*)], FA desaturation [*SCD1*, FA

desaturase 1 and 2 (*FADS1* and *FADS2*)], and transcriptional regulation of lipogenesis [*SREBF1*, *THRSP*, SREBF chaperone (*SCAP*), insulin-induced gene 1 (*INSIG1*), and peroxisome proliferator-activated receptor- γ (*PPARG*)] were measured in mammary, liver, and SUBQ tissues (Supplemental Table S4; <http://dx.doi.org/10.3168/jds.2013-7290>). In addition to the target genes, the relative mRNA levels for 6 candidate internal control genes were determined in all samples for each tissue. These genes included mitochondrial ribosomal protein L39 (*MRPL39*), ubiquitously expressed transcript (*UXT*), eukaryotic translation initiation factor 3, subunit K (*EIF3K*), β -actin (*ACTB*), peptidylprolyl isomerase A (*PPIA*/cyclophilin A), and ribosomal proteins 9 (*RPS9*). Results were analyzed using geNorm software (<http://medgen.ugent.be/~jvdesomp/ge-norm/>) to determine gene expression stability (Vandesompele et al., 2002). For liver, *UXT*, *EIF3K*, and *RPS9* were the most stably expressed, whereas *UXT*, *EIF3K*, and *MRPL39* were the most stable in mammary and SUBQ tissues. The relative mRNA levels of the target genes were normalized using the geometric mean of the 3 selected internal control genes.

FA Analysis

Milk FA were analyzed by gas liquid chromatography according to the modified method of Kramer et al. (2008) as described by Vahmani et al. (2013). Yield of FA (g/d) were calculated according to Glasser et al. (2007) and were subsequently converted to molar yield (mol/d).

Statistical Analysis

Data were analyzed as a split-plot design using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) as described by Vahmani et al. (2013). Normalized mRNA abundance data were square root-transformed before statistical analysis. Least squares means were separated using the PDIF statement of SAS. Statistical significance was declared at $P \leq 0.05$ and tendencies at $0.06 \leq P \leq 0.10$.

RESULTS

Animal Performance, Energy Balance, and Blood Metabolites

Dry matter intake, milk yield, and milk composition were determined on 60 and 90 DIM and data have been described by Vahmani et al. (2013). Briefly, DMI (19.26 vs. 23.40 kg/d), milk production (34.0 vs. 40.1 kg/d), milk fat (1.10 vs. 1.41 kg/d), protein (0.95 vs.

1.22 kg/d), and lactose (1.56 vs. 1.86 kg/d) were lower ($P \leq 0.05$) for pasture compared with confinement. The effect of LS on milk production and milk composition (yields and contents) was significant ($P \leq 0.05$) only for milk fat content, which was reduced with MA compared with FO (3.00 vs. 3.40%) and the control (3.56%).

No interactions between MS and LS ($MS \times LS$) were observed regarding energy balance variables ($P \geq 0.11$; Table 1). Energy intake and plasma content of glucose were lower ($P \leq 0.03$) for grazing cows than for confined cows. Body condition score and calculated net energy balance (**EBAL**) tended ($P = 0.10$) to be lower for grazing cows compared with those in confinement. Concurrently, grazing cows also tended ($P = 0.06$) to have higher plasma concentrations of NEFA and BHBA. None of the energy balance variables was affected by LS ($P \geq 0.13$). Blood urea nitrogen was unchanged by either MS or LS ($P \geq 0.18$).

Milk FA Secretion

No significant $MS \times LS$ were observed ($P \geq 0.21$) for molar yields of any of the individual FA or the summations (Table 2). Secretion of 12:0, 14:0, 16:0, and 18:2 n-6 in milk were lower ($P \leq 0.01$) for grazing cows than for confined cows. Concurrently, the secretion of *trans*-11 18:1 (vaccenic acid) and RA were higher ($P \leq 0.05$), and the secretion of total CLA tended ($P = 0.07$) to be higher for grazing cows. The output of de novo synthesized FA in milk was lower ($P = 0.02$) for grazing cows compared with those in confinement. However, MS did not affect ($P = 0.87$) the output of preformed FA (i.e., FA taken up from blood). The molar and mass

yield of total FA tended ($P = 0.07$) to be lower for pasture than that for confinement.

Supplementation with MA increased ($P \leq 0.02$) the molar yield of vaccenic acid and RA compared with the control (Table 2), whereas feeding FO tended ($P = 0.07$) to increase the yield of these FA relative to the control. Both FO and MA increased ($P \leq 0.01$) the yield of *trans*-10 18:1 relative to the control, and the effect was greater with MA (93%) than with FO (47%). The yield of *trans*-10, *cis*-12 CLA, however, was not affected by LS. Supplementation with either FO or MA increased ($P \leq 0.02$) the secretion of total *trans* 18:1 and total CLA compared with the control. Lipid supplementation did not affect ($P \geq 0.13$) the output of any of the de novo synthesized FA in milk. The de-saturation indexes were not significantly affected ($P \geq 0.12$) by FS, LS, or their interaction (Table 2).

Gene Expression

No significant $MS \times LS$ were observed ($P \geq 0.12$) for the expression of any of the genes in mammary, liver, and SUBQ (Table 3). Tissues responded differently to the treatments in terms of relative mRNA abundance. In mammary tissue, the expression of *LPL* ($P = 0.08$), *FASN* ($P = 0.06$), *FADS1* ($P = 0.08$), and *SCAP* ($P = 0.09$) tended to be lower for pasture than that for confinement. The expression of *PPARG* and *FADS2* was significantly ($P \leq 0.04$) lower for cows on pasture compared with those in confinement. Supplementation with either FO or MA reduced ($P = 0.02$) the expression of mammary *SREBF1*. Concurrently, MA increased ($P \leq 0.04$) the abundance of *SCAP* mRNA compared with FO and the control.

Table 1. Effect of management system and lipid supplement on energy intake, energy balance, BW, BCS, and blood metabolites¹

Item	Management system ²			Lipid supplement ³				Effect (P -value) ⁴		
	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	$MS \times LS$
NE _L intake,* Mcal/d	34.20	42.03	3.30	38.52	37.47	38.35	3.34	0.02	0.65	0.97
EBAL, ⁵ Mcal/d	2.25	4.73	0.88	3.44	2.78	3.64	0.99	0.10	0.56	0.11
BW, kg	610	665	33.2	630	634	648	29.7	0.27	0.59	0.29
BCS ⁶	2.51	2.83	0.07	2.63	2.70	2.68	0.08	0.10	0.75	0.98
Glucose,* mmol/L	2.63	3.10	0.06	2.81	2.91	2.87	0.08	0.03	0.65	0.20
BHBA,* μ mol/L	898	520	99	721	644	735	93	0.06	0.16	0.84
NEFA,** mmol/L	0.16	0.12	0.01	0.15	0.13	0.14	0.01	0.06	0.13	0.36
BUN, mmol/L	5.48	5.17	0.24	5.58	5.47	4.91	0.28	0.47	0.18	0.81

¹Least squares means are from combined 60 and 90 DIM.

²PAS = pasture system; CON = confinement system.

³Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA), or control (CO; no marine oil supplement) for 120 $\pm$ 5 d starting at -30 DIM.

⁴ P -values for the effects of management system (MS), lipid supplement (LS), and their interaction ($MS \times LS$).

⁵Calculated net energy balance = net energy intake - (net energy for maintenance + net energy for lactation).

⁶Body condition score (1 = thin to 5 = fat; Wildman et al., 1982).

*Significant DIM $\times$ MS ($P \leq 0.05$); **Significant $MS \times LS \times DIM$ ($P \leq 0.05$).

Table 2. Effect of management system and lipid supplement on milk FA yield¹

Item	Management system ²			Lipid supplement ³				Effect (<i>P</i> -value) ⁴		
	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS × LS
FA ⁵										
10:0	0.135	0.196	0.050	0.164	0.174	0.159	0.052	0.21	0.94	0.33
12:0	0.133	0.260	0.034	0.216	0.202	0.170	0.035	0.02	0.13	0.40
14:0	0.379	0.662	0.070	0.570	0.518	0.474	0.073	0.02	0.17	0.46
14:1 <i>cis</i> -9	0.031	0.055	0.006	0.048	0.041	0.041	0.006	0.05	0.55	0.85
15:0	0.040	0.065	0.006	0.059	0.051	0.048	0.007	0.04	0.19	0.35
16:0	1.002	1.523	0.135	1.408	1.200	1.180	0.144	0.03	0.11	0.35
16:1 <i>cis</i> -9	0.048	0.065	0.007	0.060	0.051	0.056	0.007	0.10	0.40	0.38
17:0	0.029	0.036	0.003	0.033	0.032	0.031	0.003	0.10	0.73	0.54
18:0	0.420	0.412	0.032	0.434	0.428	0.385	0.036	0.82	0.40	0.97
Total 18:1 <i>trans</i>	0.218	0.205	0.024	0.157 ^a	0.218 ^b	0.259 ^b	0.026	0.60	0.00	0.89
Total 18:1 <i>cis</i>	0.728	0.738	0.064	0.711	0.734	0.754	0.069	0.87	0.78	0.78
18:1 <i>trans</i> -10	0.020	0.024	0.002	0.015 ^a	0.022 ^b	0.029 ^c	0.003	0.14	<0.01	0.47
18:1 <i>trans</i> -11	0.101	0.058	0.011	0.061 ^a	0.081 ^{ab}	0.098 ^b	0.012	0.04	0.01	0.97
18:1 <i>cis</i> -9	0.726	0.714	0.063	0.690	0.719	0.752	0.068	0.83	0.60	0.71
18:2n-6	0.072	0.095	0.011	0.084	0.083	0.083	0.011	0.07	0.97	0.95
18:3n-3	0.021	0.019	0.003	0.020	0.021	0.020	0.003	0.57	0.91	0.93
Total CLA	0.049	0.037	0.005	0.034 ^a	0.045 ^b	0.050 ^b	0.005	0.07	0.01	0.96
<i>cis</i> -9, <i>trans</i> -11 CLA	0.038	0.024	0.004	0.024 ^a	0.031 ^{ab}	0.036 ^b	0.004	0.05	0.02	0.97
<i>trans</i> -10, <i>cis</i> -12 CLA	0.002	0.002	0.000	0.002	0.002	0.002	0.000	0.30	0.45	0.43
20:5n-3	0.003	0.004	0.000	0.002 ^a	0.005 ^b	0.002 ^a	0.000	0.11	<0.01	0.31
22:5n-3	0.003	0.003	0.000	0.002	0.004	0.002	0.000	0.13	<0.01	0.89
22:6n-3	0.003	0.004	0.000	0.000 ^a	0.005 ^b	0.008 ^c	0.001	0.47	<0.01	0.69
Summation ⁶										
De novo FA	0.739	1.299	0.153	1.111	1.023	0.922	0.159	0.02	0.22	0.54
Preformed FA	1.565	1.584	0.156	1.456	1.637	1.631	0.165	0.87	0.29	0.69
Total FA, mol/d	3.607	4.676	0.473	4.318	4.109	3.997	0.495	0.07	0.66	0.67
Total FA, g/d	1,058.2	1,316.0	128.1	1,188.8	1,217.7	1,154.9	130.7	0.07	0.80	0.21
Desaturation index ⁷										
C14 index	0.108	0.109	0.006	0.110	0.102	0.112	0.007	0.89	0.56	0.92
C16 index	0.046	0.040	0.003	0.042	0.041	0.046	0.003	0.28	0.13	0.13
C18 index	0.639	0.621	0.012	0.627	0.615	0.649	0.013	0.33	0.12	0.59

^{a-c}Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).

¹Least squares means from 90 DIM.

²PAS = pasture system; CON = confinement system.

³Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA), or control (CO; no marine oil supplement) for 125 ± 5 d starting at −30 DIM.

⁴*P*-values for the effects of management system (MS), lipid supplement (LS), and their interaction (MS × LS).

⁵*trans* 18:1 = total *trans* 18:1 isomers (*trans*-4 to *trans*-16 18:1); Total CLA = total CLA isomers

⁶De novo = sum of FA with chain lengths from 6 to 14; Preformed = sum of FA with chain lengths from 18 to 24.

⁷C14 Desaturation index = *cis*-9 14:1/(14:0 + *cis*-9 14:1); C16 Desaturation index = *cis*-9 16:1/(16:0 + *cis*-9 16:1); C18 Desaturation index = *cis*-9 18:1/(18:0 + *cis*-9 18:1).

In SUBQ, expression of *ACACA*, *FASN*, *INSIG1*, and *THRSP* tended to be lower ($P \leq 0.07$) for pasture than for confinement (Table 3). Lipid supplementation did not affect ($P \geq 0.14$) the mRNA expression of any of the genes evaluated in SUBQ.

Management system did not affect ($P \geq 0.20$) the expression of any of the genes evaluated in liver except *ACACA*, whose expression tended ($P = 0.10$) to be higher in grazing cows (Table 3). However, compared with mammary and SUBQ, more genes were significantly affected by LS in liver. The expression of *FASN*, *SCD1*, *FADS2*, and *THRSP* were reduced ($P \leq 0.02$) by both FO and MA. The expression of *FADS1* was reduced ($P = 0.01$) with MA compared with the control, and was not significantly ($P = 0.13$) affected with

FO. The abundance of *SREBF1* mRNA tended ($P = 0.07$) to be reduced with either FO or MA, whereas the *SCAP* mRNA levels tended ($P = 0.08$) to be increased by LS.

DISCUSSION

EBAL and Blood Metabolites

The lower EBAL of cows on pasture compared with those in confinement was expected due to lower energy intake and higher activity associated with grazing (Bargo et al., 2002). The lower blood glucose level in grazing cows is consistent with previous studies and is mainly related to the lower intake of NSC compared with confined cows (Kay et al., 2005). The increased

Table 3. Effect of management system and lipid supplement on relative mRNA abundance of genes coding for lipogenic enzymes, desaturation enzymes, and transcription regulators in mammary, subcutaneous adipose (SUBQ), and liver of lactating Holstein cows¹

Variable ²	Management system ³			Lipid supplement ⁴				Effect (<i>P</i> -value) ⁵		
	PAS	CON	SEM	CO	FO	MA	SEM	MS	LS	MS × LS
Mammary										
<i>LPL</i>	1.540	2.069	0.131	1.870	1.739	1.805	0.142	0.08	0.75	0.92
<i>ACACA</i>	1.594	1.846	0.099	1.669	1.660	1.830	0.111	0.15	0.36	0.49
<i>FASN</i>	1.536	2.156	0.140	1.763	1.807	1.968	0.158	0.06	0.52	0.31
<i>SCD1</i>	1.538	1.938	0.125	1.785	1.714	1.715	0.130	0.15	0.89	0.82
<i>FADS1</i>	1.552	1.918	0.131	1.677	1.705	1.823	0.141	0.08	0.52	0.12
<i>FADS2</i>	1.421	2.177	0.131	1.857	1.749	1.791	0.144	0.03	0.78	0.13
<i>SREBF1</i>	1.687	1.874	0.063	1.980 ^a	1.671 ^b	1.691 ^b	0.077	0.17	0.02	0.34
<i>SCAP</i>	1.442	1.898	0.130	1.518 ^a	1.644 ^a	1.948 ^b	0.146	0.09	0.04	0.24
<i>INSIG1</i>	1.237	1.743	0.207	1.483	1.439	1.548	0.246	0.20	0.95	0.18
<i>THRSP</i>	1.510	1.971	0.114	1.929	1.729	1.564	0.138	0.11	0.21	0.20
<i>PPARG</i>	1.589	2.348	0.193	1.847	1.841	2.216	0.200	0.04	0.09	0.12
SUBQ										
<i>LPL</i>	1.201	1.550	0.224	1.182	1.400	1.544	0.228	0.27	0.28	0.94
<i>ACACA</i>	0.975	1.887	0.183	1.164	1.528	1.601	0.216	0.06	0.29	0.85
<i>FASN</i>	0.890	1.707	0.223	1.052	1.389	1.455	0.245	0.06	0.25	0.92
<i>SCD1</i>	0.900	1.802	0.239	1.077	1.400	1.575	0.235	0.12	0.24	0.52
<i>FADS1</i>	1.648	1.470	0.239	1.884	1.516	1.278	0.265	0.53	0.14	0.14
<i>FADS2</i>	1.700	1.449	0.341	1.894	1.431	1.399	0.366	0.47	0.29	0.17
<i>SREBF1</i>	1.486	1.430	0.175	1.625	1.535	1.399	0.263	0.84	0.38	0.82
<i>SCAP</i>	1.436	1.549	0.143	1.433	1.507	1.537	0.160	0.59	0.86	0.77
<i>INSIG1</i>	0.818	1.579	0.146	1.210	1.033	1.353	0.178	0.07	0.47	0.51
<i>THRSP</i>	0.750	1.605	0.159	0.955	1.251	1.327	0.178	0.06	0.29	0.46
<i>PPARG</i>	1.608	1.339	0.134	1.488	1.317	1.616	0.149	0.18	0.22	0.96
Liver										
<i>LPL</i>	1.142	1.209	0.116	1.193	1.214	1.119	0.110	0.73	0.77	0.14
<i>ACACA</i>	1.378	1.012	0.094	1.299	1.189	1.096	0.115	0.10	0.48	0.82
<i>FASN</i>	1.354	1.263	0.090	1.612 ^a	1.189 ^b	1.124 ^b	0.111	0.55	0.02	0.80
<i>SCD1</i>	1.186	1.152	0.101	1.684 ^a	1.145 ^b	0.678 ^c	0.124	0.83	<0.01	0.31
<i>FADS1</i>	1.230	1.198	0.124	1.578 ^a	1.237 ^{ab}	0.828 ^b	0.152	0.87	0.01	0.21
<i>FADS2</i>	1.196	1.233	0.099	1.667 ^a	1.055 ^b	0.922 ^b	0.122	0.82	<0.01	0.73
<i>SREBF1</i>	1.179	1.302	0.061	1.369	1.220	1.094	0.075	0.29	0.07	0.85
<i>SCAP</i>	1.273	1.196	0.087	1.123	1.307	1.211	0.081	0.60	0.08	0.40
<i>INSIG1</i>	1.196	1.122	0.177	1.310	1.089	1.079	0.191	0.73	0.46	0.19
<i>THRSP</i>	1.034	1.205	0.094	1.479 ^a	1.087 ^b	0.792 ^b	0.115	0.33	<0.01	0.73
<i>PPARG</i>	1.786	2.122	0.158	1.633	2.241	1.987	0.195	0.27	0.12	0.85

^{a-c}Values within lipid supplement and in the same row not sharing a common superscript are significantly different ($P \leq 0.05$).

¹Least squares means for mRNA levels (expressed in arbitrary units) at 100 DIM.

²*LPL* = lipoprotein lipase; *ACACA* = acetyl-CoA carboxylase; *FASN* = FA synthase; *SCD1* = stearoyl-CoA desaturase 1 (Δ^9 -desaturase); *FADS1* = FA desaturase 1 (Δ^5 -desaturase); *FADS2* = FA desaturase 2 (Δ^6 -desaturase); *SREBF1* = sterol regulatory element-binding transcription factor-1; *SCAP* = *SREBF* chaperone; *INSIG1* = insulin-induced gene 1; *THRSP* = thyroid hormone responsive spot 14; *PPARG* = peroxisome proliferator-activated receptor- γ .

³PAS = pasture system; CON = confinement system.

⁴Supplementation with rumen-protected fish oil (FO), rumen-protected microalgae (MA), or control (CO; no marine oil supplement) from -30 to 100 DIM.

⁵*P*-values for the effects of management system (MS), lipid supplement (LS), and MS by LS interaction (MS × LS).

levels of plasma NEFA and BHBA in grazing cows are consistent with lower EBAL, which is associated with greater mobilization of adipose FA compared with confined cows (Agenas et al., 2002; Kay et al., 2005). Consistent with the increased catabolism in adipose tissue, we observed a tendency ($P = 0.10$) for lower BCS in grazing compared with confined cows (Table 1).

Milk FA Secretion and Mammary Gene Expression

The reduced mammary levels of *FASN* mRNA (-29%; $P = 0.06$) in grazing compared with confined

cows was accompanied by reduced (-43%; $P = 0.02$) secretion of de novo synthesized FA in milk. This observation is consistent with the role of *FASN* in milk fat synthesis, which is to produce even-chain FA with 4 to 16 carbons (Bernard et al., 2008). We are unaware of comparable studies in which effects of MS on mammary gene expression in dairy cows were measured. Studies where cows were fed milk fat-depressing diets (i.e., low-forage and high-oil diets) show downregulation of genes involved in mammary lipid synthesis (e.g., *ACACA*, *FASN*, and *SCD1*), which was associated with increased milk *trans*-10 18:1 and *trans*-10,*cis*-12 CLA (Ahnadi et

al., 2002; Harvatine and Bauman, 2006; Angulo et al., 2012). Contrary to these studies, we did not observe greater concentrations of either *trans*-10,*cis*-12 CLA or *trans*-10 18:1 in milk from grazing cows, despite lower mammary *FASN* gene expression and depressed secretion of de novo synthesized FA. This discrepancy could be due to other differences between pasture and confinement treatments, including energy intake, ruminal VFA production, PUFA intake, and production of other RBH intermediates. Further investigation is needed to elucidate the role of these factors in regulating milk fat synthesis in dairy cows.

Despite the lower *LPL* expression (-26% ; $P = 0.06$) in the mammary tissue of grazing cows, which is responsible for the uptake of preformed FA from circulating chylomicrons and low-density lipoproteins, the yield of preformed FA in milk was similar ($P = 0.87$) between treatments. Consistent with the current study, Angulo et al. (2012) observed that reduced mammary expression of *LPL* in cows fed milk fat-depressing diets was not accompanied by lower secretion of preformed FA in milk, whereas, in the same study, the reduction in milk de novo synthesized FA was accompanied by downregulation of *FASN* in mammary tissue. Conversely, increased secretion of C18 FA in the milk of goats supplemented with plant oils was not accompanied by increased mammary *LPL* mRNA abundance or activity (Bernard et al., 2009). The authors related this observation to the fact that *LPL* may not be a rate-limiting enzyme in the mammary secretion of milk FA in ruminants and that other factors, such as substrate availability (i.e., availability of circulating preformed FA or triacylglycerol) or mammary *LPL* partitioning, might play a more important role in regulating the secretion of preformed FA in milk.

The relatively lower mammary expression of *FADS1* and *FADS2* in grazing cows could be due to the higher intake of 18:3 n-3 (α -linolenic acid; **ALA**) on pasture, which could directly or indirectly (i.e., through increased ruminal production of RBH intermediates) reduce the expression or activity of these enzymes (Chuang et al., 2004; Zhu et al., 2010). The extent of downregulation was more pronounced for *FADS2* (-35% , $P = 0.03$) than for *FADS1* (-19% , $P = 0.08$). Other information on this effect in the cow was not found; however, flaxseed oil, a rich source of ALA, fed to mice also reduced hepatic expression of *FADS2* mRNA but did not affect that of *FADS1* (Zhu et al., 2010). Fatty acid desaturase 1 and 2 are expressed in many tissues (e.g., brain, liver, heart, and placenta) and are essential for the synthesis of highly unsaturated FA (e.g., 20:4 n-6, 20:5 n-3, and 22:6 n-3) from 18:2 n-6 and 18:3 n-3 (Nakamura and Nara, 2003). In the current study, despite the reduced mammary expression of *FADS1* and *FADS2* in grazing

cows, the secretion of 20:5 n-3 and 22:6 n-3 in milk was not different between pasture and confinement. This could be due to the fact that the majority of dietary PUFA, including ALA, the precursor for synthesis of EPA and DHA, is biohydrogenated in the rumen and that the conversion of ALA of to 20:5 n-3 and 22:6 n-3 is very limited in mammals (Burdge, 2006), including dairy cows (Lor et al., 2005; Ponter et al., 2006).

In grazing cows, as compared with confined cows, a 32% reduction ($P = 0.04$) was observed in mammary expression of *PPARG*, a ligand-activated nuclear receptor that plays a key role in transcriptional regulation of various proteins involved in lipogenesis and adipogenesis (Kersten, 2001). Bionaz and Lor (2008) suggested that this transcription factor may be involved in the regulation of milk fat synthesis in dairy cows. These authors observed an upregulation of *PPARG* transcription at the onset of lactation (~ 3 -fold relative to -15 DIM), which was sustained throughout lactation. Furthermore, ligand activation of *PPARG* in bovine mammary cells (Kadegowda et al., 2009) and ligand activation or overexpression of *PPARG* in goat mammary cells (Shi et al., 2013a,b) resulted in upregulation of lipogenic genes, such as *ACACA*, *FASN*, *LPL*, *SREBF1*, and *SCD1*. In our study, the lower mammary expression of *FASN* and the ensuing reduced secretion of de novo synthesized FA in the milk of grazing cows compared with confined cows might be related in part to the downregulation of mammary *PPARG* gene expression. However, it is noteworthy that mammary expression of *PPARG* or its co-activators was not altered when cows were fed milk fat-depressing diets (Harvatine and Bauman 2006; Invernizzi et al., 2010; Vyas et al., 2013). Therefore, more studies are needed to elucidate the role of *PPARG* in regulating milk fat synthesis particularly in the etiology of milk fat depression.

Feeding FO or MA reduced ($P = 0.02$) the expression of *SREBF1* in mammary by 15% compared with the control. Sterol regulatory element binding factor 1 is a transcription factor that activates the transcription of key enzymes involved in milk fat synthesis (Harvatine et al., 2009a). Downregulation of genes involved in mammary lipid synthesis (e.g., *ACACA*, *FASN*, and *SCD1*) during diet-induced milk fat depression has been related to reductions in the transcription and activation of *SREBF1* by specific RBH intermediates, particularly *trans*-10,*cis*-12 CLA (Peterson, et al., 2004; Harvatine and Bauman, 2006; Angulo et al., 2012). In the current study, milk *trans*-10,*cis*-12 CLA content was not affected by LS; however, both FO and MA increased milk fat content of *trans*-10 18:1 (the RBH product of *trans*-10,*cis*-12 CLA), which has also been associated with diet-induced milk fat depression in dairy cows (Shingfield et al., 2010). Contrary to previous

findings, the reduced mammary expression of *SREBF1* in cows fed marine oils in the current study was not accompanied by either reduced gene expression of genes involved in milk fat synthesis or reduced milk fat yield. This discrepancy could be due to the higher doses of supplemental lipids used in previous studies (3.1–4.5 vs. 1.0% DM) and the subsequent higher degree of downregulation of *SREBF1* (–15 vs. –27–35%) in the current study. Thus, it is possible that the downregulation of *SREBF1* by FO or MA in the current study was insufficient to alter the gene expression of enzymes involved in milk fat synthesis.

Supplementation with MA resulted in an increase in the expression (28%; $P = 0.04$) of *SCAP*, a *SREBF1* regulatory protein required for the proteolytic activation of *SREBF1*. Although not statistically significant, feeding FO resulted in a numerically greater mRNA expression of *SCAP* than that of control. This result is in agreement with Invernizzi et al. (2010), who reported a sustained upregulation of *SCAP* expression in mammary tissue of cows fed a milk fat-depressing diet containing a blend of fish and soybean oil for 21 d. Bionaz and Loores (2008) observed a gradual increase in the mammary mRNA abundance of *SCAP* of dairy cows throughout the lactation cycle. The increased mammary expression of *SCAP* in cows fed oil supplements may be an adaptive response to maintain milk fat synthesis (Invernizzi et al., 2010). Further studies are needed to determine the role of *SCAP* in milk fat synthesis.

mRNA Expression of Genes Involved in Lipid Synthesis in SUBQ

The greater expression of *ACACA* (94%; $P = 0.06$) and *FASN* (92%; $P = 0.06$) in SUBQ of confined cows compared with that of grazing cows (Table 3) is suggestive of increased adipose de novo lipogenesis and is consistent with the greater BCS in confined cows (Table 1). Dietary energy intake above the amounts needed for maintenance, growth, reproduction, and activity is the major determinant of the rate of adipogenesis in animals. Rocco and McNamara (2013) observed that energy restriction in early lactation reduced the rate of lipogenesis in SUBQ. Conversely, energy overfeeding resulted in upregulation of most of the genes involved in lipid synthesis in SUBQ, including *ACACA*, *FASN*, *LPL*, *SCD1*, *INSIG1*, *SCAP*, and *PPARG* in periparturient dairy cows (Ji et al., 2012) and *ACACA*, *FASN*, and *SCD1* in steers (Duckett et al., 2009). Therefore, the reduced expression of *ACACA* and *FASN* in SUBQ of grazing cows could be due to the lower energy intake and subsequent lower substrate availability for lipogenesis (Tables 1 and 3). Among the transcriptional regu-

lators evaluated in SUBQ, the only significant effect was for *THRSP*, whose expression was greater (114%; $P = 0.06$) in confined cows than in grazing cows. Thyroid hormone responsive spot 14 is a nuclear protein, and expression of this protein in the rodent liver and adipose tissue was induced by lipogenic stimuli, such as thyroid hormone and dietary carbohydrates, which in turn increased the rate of lipogenesis (Kinlaw et al., 1995; Obregon, 2008). Harvatine and Bauman (2006) observed a downregulation of *THRSP* in the mammary tissue of dairy cows fed milk fat-depressing diets and suggested a role for this nuclear protein in regulation of milk fat synthesis. Wang et al. (2009) reported that *THRSP* mRNA abundance in longissimus muscle from high-marbling beef heifers was highly correlated with intramuscular fat content. In another beef study (Grauagnard et al., 2009), *THRSP* expression in the longissimus lumborum muscle of growing steers was positively correlated with blood levels of both glucose and insulin, as well as with gene expression of lipogenic enzymes, including *ACACA* and *FASN*. Furthermore, feeding high-starch diets resulted in upregulation of *THRSP* mRNA in subcutaneous (Duckett et al., 2009) and intramuscular (Grauagnard et al., 2009) adipose tissues of steers. Grauagnard et al. (2009) suggested that *THRSP* might play an important role in transcriptional regulation of adipogenesis in cattle. Similarly, in the current study, the increased expression of *ACACA* and *FASN* in SUBQ of confined cows, which relates positively to their higher energy intake, may be mediated by *THRSP*. More studies will have to be conducted to determine the role of this transcription factor in the regulation of lipogenesis and adipogenesis in dairy cows.

Feeding milk fat-depressing diets containing a blend of fish and soybean oil (Thering et al., 2009) or intravenous infusion of *trans*-10, *cis*-12 CLA (Harvatine et al., 2009b) upregulated the SUBQ expression of *LPL*, *SCD1*, and *THRSP* in dairy cows. Those results suggested that the greater expression of these genes in SUBQ is related to the repartitioning of energy from milk fat production to adipogenesis. In the present study, we did not observe any effect of LS on gene expression in SUBQ, which is probably related to the lack of an LS treatment effect on milk fat yield.

Hepatic Expression of Genes Involved in Lipid Synthesis

The response of hepatic gene expression to LS was similar to what has previously been seen in rodents where dietary PUFA suppressed the transcription of several genes involved in lipid synthesis in liver, including *SREBF1*, *THRSP*, *FASN*, *ACACA*, *SCD1*, *FADS1*, and *FADS2* (Jump and Clarke, 1999; Jump, 2002; Matsu-

zaka et al., 2002). In contrast to humans and rodents, where the liver is the primary site of lipogenesis, ruminant liver has a very limited lipogenic capacity, particularly for FA synthesis (Bergen and Mersmann, 2005). The mRNA abundance of *ACACA*, *FASN*, and *LPL* was low in the liver of dairy goats compared with that of adipose and mammary tissues (Bernard et al., 2009). Conversely, *SCD1* was found to be highly expressed in the liver of lactating ewes (Ward et al., 1998) and goats (Bernard et al., 2009), implying that liver might play a role in Δ^9 -desaturation of absorbed FA. Herdmann et al. (2010) observed that the liver of beef cattle had a higher content of long-chain n-3 PUFA compared with erythrocytes, muscle, and adipose tissues, which was further increased by feeding ALA, suggesting that elongase and desaturase (i.e., *FADS1* and *FADS2*) enzymes are active in the liver of ruminants.

In rodents and humans, PUFA can reduce the accumulation of triacylglycerol (**TAG**) in the liver by enhancing hepatic FA oxidation and suppressing de novo FA and TAG synthesis (Jump and Clarke, 1999; Jump, 2002). The effect of PUFA on both FA oxidation and lipogenesis is mediated mainly via transcriptional regulatory mechanisms involving peroxisome proliferator-activated receptor- α (*PPAR α*) and *SREBF1*. Polyunsaturated FA are ligand activators of *PPAR α* , which upregulates the expression of genes involved in FA oxidation. In contrast, PUFA suppress both transcription and activation of *SREBF1* resulting in downregulation of genes involved in lipid synthesis. Adding flaxseed to diets of peripartur dairy cows reduced hepatic TAG concentrations after calving. The authors suggested that feeding a source of n-3 PUFA to peripartur cows might reduce the risk of developing fatty liver during the transition period (Petit et al., 2007). However, in a comparable study in which prepartur cows were supplemented with fish oil (0.9% of dietary DM), hepatic TAG content was not affected after calving despite an increased concentration of long-chain n-3 PUFA in the liver (Ballou et al., 2009). Unfortunately, we did not measure the hepatic content of TAG or expression of genes involved in TAG synthesis in the current study. More detailed studies are needed to confirm whether feeding long-chain n-3 PUFA can help prevent fatty liver in dairy cows.

CONCLUSIONS

The reduced secretion of de novo synthesized FA in milk from grazing cows compared with confined cows was associated with lower mammary expression of *FASN* and *PPARG*, suggesting that at least part of the difference in milk FA secretion between pasture and confinement systems could be mediated at the level

of gene transcription. Reduced expression of *ACACA*, *FASN*, and *THRSP* in the SUBQ of grazing cows in comparison to confined cows was consistent with a lower BCS (i.e., lower adipose tissue deposition) and was related to the lower energy intake of grazing cows. The effect of LS on gene expression was tissue-specific, with the greatest response observed in liver despite the minor role of liver in lipid synthesis in ruminants. Our results confirm previous research that gene transcription is an important regulatory mechanism for lipogenesis and that MS and LS have tissue-specific effects on expression of genes involved in lipid synthesis in dairy cattle, which have important influences on cow performance and healthfulness of the milk FA profile.

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