Expression of key lipid metabolism genes in adipose tissue is not altered by once-daily milking during a feed restriction of grazing dairy cows

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ABSTRACT

The objective of this study was to investigate the effect of reduced milking frequency, at 2 feeding levels, on gene expression in adipose tissue of grazing dairy cows during early lactation. Multiparous Holstein-Friesian and Holstein-Friesian $\times$ Jersey cows ($n = 120$) were grazed on pasture and milked twice daily ($2\times$) from calving to 34 ± 6 d in milk (mean $\pm$ standard deviation). Cows were then allocated to 1 of 4 treatments in a 2×2 factorial arrangement. Treatments consisted of 2 milking frequencies ($2\times$ or once daily; $1\times$) and 2 feeding levels for 3 wk: adequately fed (AF), consuming 14.3 kg of dry matter/cow per day, or underfed (UF), consuming 8.3 kg of dry matter/cow per day. After the treatment period, all cows were fed to target grazing residuals $\geq 1,600$ kg of DM/cow per day and milked $2\times$ for 20 wk. Adipose tissue was collected from 12 cows per treatment by subcutaneous biopsy at -1 , 3, and 5 wk relative to treatment start, RNA was extracted, and transcript abundance of genes involved in lipid metabolism was quantified using a linear mixed model. At the end of the 3-wk treatment period, transcript abundance of genes involved in fatty acid (FA) uptake into adipose tissue (*LPL*), FA synthesis [FA synthase (*FASN*) and stearoyl-coenzyme A desaturase (*SCD*)], FA oxidation [acyl-coenzyme A synthetase long-chain family member 1 (*ACSL1*) and carnitine palmitoyltransferase 2 (*CPT2*)], glyceroneogenesis [glycerol-3-phosphate dehydrogenase 1 (*GPD1*) and pyruvate carboxylase (*PC*)], and triacylglyceride synthesis [diacylglycerol O-acyltransferase 2 (*DGAT2*)] were greater in AF1 $\times$ cows compared with all other treatments. However, when cows were underfed, no effects of milking frequency were observed on transcript abundance of genes involved in adipose lipid metabolism. Despite increases in plasma NEFA concentrations in UF cows, no effects of underfeeding were observed on the transcription of lipolytic

genes. At 5 wk, after cows were returned to $2\times$ milking and standard feed allowance, transcript abundances of genes involved in FA synthesis [acetyl-coenzyme A carboxylase α (*ACACA*) and *SCD*] were increased in cows previously UF. Expression of *ACSL1* was decreased in UF1 $\times$ cows relative to UF2 $\times$ cows and *CPT2* expression was greater in AF1 $\times$ cows compared with AF2 $\times$ cows. In conclusion, after 3 wk of reduced milking frequency during a feed restriction, transcription of genes involved in lipid metabolism in adipose tissue were not altered, possibly due to the reduced milk production in these animals. However, 3 wk of $1\times$ milking in AF cows increased transcription of genes involved in FA synthesis, oxidation, and triacylglyceride synthesis.

Key words: caloric restriction, adipose tissue, milking frequency, lipostatic theory

INTRODUCTION

Feed shortages can occur in pasture-based dairy systems when the quality or availability of pasture is negatively affected by weather conditions or poor grazing management. When DMI is limited in lactating dairy cows, homeorhetic changes occur to ensure that energy is available to sustain maintenance and milk production (Bauman and Currie, 1980). This can result in a state of negative energy balance and reductions in plasma glucose and insulin concentrations, leading to increased mobilization of adipose tissue reserves for maintenance and milk production (Chilliard et al., 2000).

Lipid mobilization and the increase in plasma NEFA concentrations are the net results of changes to the balance between esterification, lipolysis, and reesterification, as some of the FA released during lipolysis is reesterified to triacylglycerol (**TAG**; Chilliard et al., 2000). Increased NEFA release during a period of negative energy balance is proposed to be a result of decreased reesterification of TAG (Wilson, 1983; Dunshea et al., 1990), and increased lipolysis (Bauman and Currie, 1980) mediated by β -adrenergic receptors (Sumner and McNamara, 2007). Plasma NEFA concentrations plateau or decrease after 4 to 8 d, even with continued

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feed restriction (Vernon, 1992; Gross et al., 2011). This plateau is possibly due to negative feedback to minimize the toxic effects of high NEFA concentrations or to restrict the loss of adipose tissue reserves (Chilliard et al., 2000), as it has been suggested that mammals try to maintain a certain adipose content (Kennedy, 1953) and, in particular, lactating dairy cows (Roche et al., 2008). Managing a cow's energy balance in seasonal dairy systems is crucial during a feed restriction in early or mid-lactation, as negative energy balance can lower milk production and increase the incidence of metabolic disorders.

Reducing milking frequency (**MF**) from twice- (**2×**) to once-daily (**1×**) is one strategy proposed to manage the energy balance of dairy cows (Auldist and Prosser, 1998; Kay et al., 2013), as 1× milking reduces milk production (Davis et al., 1999) and, consequently, decreases energy demands by the mammary gland. Cows milked 1× have reduced BCS and BW loss, and improved energy balance compared with cows milked 2× (Rémond et al., 2002; Guinard-Flament et al., 2007). Milking cows 1× instead of 2× during a feed restriction limits both the increase in plasma NEFA concentrations and the decrease in plasma glucose and insulin concentrations (Guinard-Flament et al., 2007; Kay et al., 2013). However, Kay et al., (2013) reported that the improvements in energy status due to 1× milking were not sufficient to prevent BCS loss during 3 wk of underfeeding of grazing dairy cows during early lactation.

The mechanisms underlying the adipose tissue response to reduced MF during a feed restriction remain to be determined. In lactating goats, the increase in plasma NEFA during a nutrient deficit coincides with changes in adipose tissue gene expression (Faulconnier et al., 2011). Expression of genes involved in hydrolysis of TAG for uptake by adipose tissue [lipoprotein lipase (**LPL**)], FA synthesis [acetyl-CoA carboxylase α (**ACACA**) and stearoyl-CoA desaturase (**SCD**)], glycerol-3-phosphate production [glycerol-3-phosphate dehydrogenase 1 (**GPD1**)], TAG synthesis [glycerol-3-phosphate acyltransferase mitochondrial (**GPAM**)], and FA oxidation [acyl-CoA synthetase long-chain family member 1 (**ACSL1**)] were decreased, whereas the expression of genes involved in FA transport (FA-binding protein 4 (**FAPB4**)] and inhibition of TAG uptake [angiotensin-like 4 (**ANGPTL4**)] were increased (Faulconnier et al., 2011).

Adipose tissue metabolism during the transition period in cows indicates that lipogenesis is decreased in relation to intake, whereas lipolysis is correlated with milk production (McNamara, 1989); therefore, both aspects may be modified in cows milked 1× during a nutrient deficit. Lipolysis, however, is not consistently accompanied by changes in gene expression, as many of

the enzymes are regulated posttranslationally (Sumner and McNamara, 2007; Sumner-Thomson et al., 2011; Khan et al., 2013). These results indicate that transcript abundances of many aspects of lipid metabolism are altered during periods of nutrient deficit; however, it is unclear if 1× milking modifies gene transcription, or whether lipolytic gene expression changes will be measured in grazing cows.

The different effects of nutrition and milk production on lipid metabolism, combined with the lower plasma NEFA concentrations in cows milked 1× relative to 2× (Kay et al., 2013), led to the hypothesis that milking cows 1× during a feed restriction would minimize lipolysis and enhance lipogenesis in adipose tissue. Therefore, the objective of this experiment was to measure the expression of genes involved in lipid metabolism in adipose tissue, during and after a feed restriction in cows milked 1× or 2×, to determine the effect of reduced MF during an energy deficit.

MATERIALS AND METHODS

Experimental Design and Treatments

The study was conducted at the Westpac Taranaki Agriculture Research Station, (Hawera, New Zealand; 39°35' S 174°17' E) from July to October 2009. All treatments and measurements were approved by the Ruakura Animal Ethics Committee (Hamilton, New Zealand).

The experimental design, grazing management, milk production, BW, BCS, and plasma hormone and metabolite measurements were as described previously (Kay et al., 2013). Briefly, multiparous Holstein-Friesian and Holstein-Friesian $\times$ Jersey dairy cows ($n = 120$) were grazed on pasture as 1 herd (to target postgrazing residuals of $>1,600$ kg of DM/ha), and milked 2× for the first 34 ± 6 DIM (mean $\pm$ SD). Cows were then allocated to 1 of 4 treatments in a 2×2 factorial arrangement. Treatments consisted of 2 MF (1× or 2×) and 2 feeding levels [adequately fed (**AF**) or underfed (**UF**)] and were imposed for 3 wk. At the start of the treatment period, pasture allowances were incrementally increased or decreased over a 3-d period to target an average DMI of 15 or 9 kg of DM/cow per day in the AF and UF treatments, respectively. Daily milking times were 0700 for 1X (24 h milking interval) and 0700 and 1500 for 2X (16/8 h milking interval). To reduce variability due to DIM, cows were grouped into 2 experimental cohorts: cows that calved from July 17 to August 7 ($n = 66$) were included in cohort 1 and cows that calved from August 8 to September 1 ($n = 54$) were included in cohort 2. Both cohorts were managed identically. Following the treatment period, all animals

were milked 2× and grazed together, with pasture allowances targeting postgrazing residuals >1,600 kg of DM/ha for 20 wk.

Tissue Sampling

Subcutaneous adipose tissue was collected on 3 occasions (−1, 3, and 5 wk relative to treatment start) from 12 cows randomly selected within each treatment group. Controlled intravaginal drug-releasing devices containing progesterone (Zoetis, West Ryde, NSW, Australia; Macmillan and Peterson, 1993) were inserted 2 d prior, and removed on the day of all tissue biopsies. These were used to prevent estrous-associated behaviors, which could have potentially damaged the healing biopsy site. Subcutaneous adipose samples were collected posterior to the shoulder blade, and approximately 10 cm down the withers. The site was clipped and cleansed with iodine before local anesthetic (2% lignocaine) was administered. A 3-cm incision was made through the skin, and 30 to 80 mg of adipose tissue was removed using forceps and a scalpel. Biopsies were immediately placed in screw-capped, microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

Extraction of RNA and cDNA Synthesis

Adipose biopsies of approximately 80 mg were homogenized by adding 3 ball bearings of 1/8-inch (3-mm) diameter (Farrell Bearings Ltd., Hamilton, New Zealand) into a tube containing the biopsy and RLT lysis buffer (Qiagen GmbH, Hilden, Germany) and loading samples into a TissueLyser II (Qiagen GmbH) for 2 min. Chloroform was then added and samples were centrifuged at $12,000 \times g$ for 15 min at 4°C. Once the nucleic acids were separated from the protein and lipid globules, total RNA was extracted using a Qiagen RNeasy kit (Qiagen GmbH). Extracted RNA was treated with DNase (Ambion DNA-free kit; Ambion Inc., Austin, TX). The RNA quantity, purity, and integrity were determined using a NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE) and the Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip kit (Agilent Technologies Inc., Palo Alto, CA). Samples with a ratio of absorbance measured at 260 nm to absorbance measured at 280 nm (A260/A280) greater than 1.7 (2 = pure) and an RNA integrity number above 5.4 (mean 7.0 ± 0.8) were used for cDNA synthesis. All RNA samples were stored at −80°C.

Of the 144 adipose tissue biopsies, RNA was successfully extracted from 125; 19 samples were either too small to be used or the RNA was degraded. Final numbers were even across treatments: AF2×: n = 31

(−1 wk: n = 7, 3 wk: n = 12, and 5 wk: n = 12); UF2×: n = 31 (−1 wk: n = 8, 3 wk: n = 11, and 5 wk: n = 12); AF1×: n = 31 (−1 wk: n = 9, 3 wk: n = 10, and 5 wk: n = 12); and UF1×: n = 32 (−1 wk: n = 9, 3 wk: n = 11, and 5 wk: n = 12). For cDNA synthesis, 1.2 µg of each RNA sample and a final concentration of 27 µM of random pentadecamer primers were used. Total RNA was reverse transcribed using the Invitrogen SuperScript III SuperMix kit (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions (final volume = 60 µL). Reverse-transcriptase negative controls were generated by excluding the enzyme. The cDNA samples were stored at −20°C.

Quantitative PCR and mRNA Analyses

Using reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis, the expression of 24 genes involved in lipid metabolism was measured. For lipogenesis, *ACACA*, FA synthase (*FASN*), *SCD*, thyroid hormone responsive (*THRSP*), *GPAM*, lipin 1 (*LPIN1*), diacylglycerol O-acyltransferase 2 (*DGAT2*), *GPD1*, pyruvate carboxylase (*PC*), phosphoenolpyruvate carboxykinase 1 (*PCK1*), *LPL*, and peroxisome proliferator-activated receptor gamma (*PPARG*) were targeted. The transcript variant B of the insulin receptor (*INSR*) was measured to detect any changes to insulin sensitivity and 3-oxoacid CoA transferase 1 (*OXCT1*) was targeted to determine if ketone bodies were being broken down.

For lipolysis, *ANGPTL4*, hormone-sensitive lipase (*LIPE*), monoglyceride lipase (*MGLL*), perilipin 1 (*PLIN1*), and patatin-like phospholipase domain-containing 2 (*PNPLA2*) were targeted, whereas for FA oxidation *ACSL1* and carnitine palmitoyltransferase 2 (*CPT2*) were selected. An assay for *CPT1B* was designed and tested; however, expression levels were below detectable amounts in the current study. The remaining genes chosen are important in FA transport post lipolysis (*FABP4*), and the somatotrophic axis [growth hormone receptor 1b (*GHR1B*) and insulin-like growth factor 1 (*IGF1*)].

The RT-qPCR was conducted using Roche real-time PCR master mix (LightCycler 480 Probes Master; Roche Diagnostics GmbH, Mannheim, Germany) and Roche Universal Probe Library assays analyzed on the Roche LightCycler 480 instrument (Roche Diagnostics GmbH). Assays were designed to publicly available bovine gene sequences (NCBI; <http://www.ncbi.nlm.nih.gov/gene>) using Roche Universal Probe Library design software. Assays were designed to span an intron-exon boundary of genes and specificity of assays tested using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; Altschul et al., 1990). The details of the

assays are described in Table 1. Primers were manufactured by Sigma or Invitrogen (St. Louis, MO) at 25 or 50 nm respectively, and purified by desalting. The RT-qPCR reaction volume was 10 μ L, consisting of 4 μ L of cDNA and 6 μ L of master mix (5 μ L of Roche Probes Master, 0.4 μ L of each 5 μ M primer, 0.075 μ L of probe, and 0.125 μ L of H₂O). Solutions were pipetted out using an epMotion 5075 robot (Eppendorf South Pacific Pty. Ltd., North Ryde, NSW, Australia) before plates were transferred into the LightCycler 480. Standard cycling conditions were used [95°C for 10 min, (95°C for 10 s and 60°C for 30 s) $\times$ 45 cycles, and then 40°C for 30 s]. Each RT-qPCR run included 2 negative controls (i.e., a no-template control with water added instead of cDNA and a reverse-transcriptase negative control), and 3 inter-run calibrators. Triplicate measurements were undertaken for all samples and standard curves with standard deviations of less than 0.15 cycles were used for quantitation. Assays were tested for specificity by running an agarose gel (3%) of the PCR products to ensure amplicon specificity and length. The efficiency of each assay was between 1.80 and 2.0, except for *INSR*, isoform B (1.64).

The Roche LightCycler 480 software was used to analyze gene expression using relative standard curve second-derivative maximum analysis. A 5-point standard curve of serial cDNA dilutions was used, ranging from 3.33e-1 to 1.37e-3. Endogenous control genes were tested across all samples and their suitability determined using NormFinder and GeNorm software (Vandesompele et al., 2002; Andersen et al., 2004). The candidate endogenous control genes were β -actin, eukaryotic translation initiation factor 3, subunit K, phosphoglycerate kinase 1, and ribosomal protein S15a. The 2 most-stable genes were eukaryotic translation initiation factor 3, subunit K (stability value = 1.12) and ribosomal protein S15a (stability value = 0.98). The Roche LightCycler 480 software was then used to conduct advanced relative quantification analysis of gene expression using the normalization factor of the 2 endogenous control genes. The geometric mean of the inter-run calibrators was calculated for each plate and applied to each sample.

Calculations and Statistical Analysis

Data from each gene were log₁₀-transformed before analysis, due to heterogeneity of variance, and pretreatment measurements (−1 wk) were used for covariate analyses. Data were analyzed using mixed models fitted with REML in GenStat software (version 14.1, 2011; VSN International Ltd., Hemel Hempstead, UK), including cohort, feeding level, MF, and the interaction

of feeding level and MF as fixed effects, and cow as a random effect. Differences were considered significant at $P < 0.05$, and a trend declared at $P < 0.10$.

RESULTS

Pasture and Production Measures

Average DMI was 14.3 ± 3.6 kg and 8.3 ± 2.9 kg of DM/cow per day for the AF and UF treatments, respectively. Milk-production measures, plasma hormone and metabolite concentrations, BW, and BCS are described in Kay et al. (2013); however, relevant mean data are displayed in Table 2. In brief, at the end of the first week of the treatment period (wk 1), an interaction was observed between MF and feeding level for milk yield, such that milk yield was 20% lower in AF1 $\times$ cows compared with AF2 $\times$ cows and 18% less in UF2 $\times$ cows compared with AF2 $\times$ cows (Table 2). Milk yields from UF1 $\times$ cows were reduced by 30% compared with AF2 $\times$ cows, demonstrating the combined negative effect of reduced MF and feeding level. At wk 3, AF1 $\times$ cows continued to produce 20% less milk; whereas UF2 $\times$ cows had yield losses of 36% and UF1 $\times$ cows produced 44% less milk compared with AF2 $\times$ cows. Posttreatment (wk 5), when all cows were milked 2 $\times$ and on the AF treatment, no interactions were observed between MF or feeding level; cows previously milked 1 $\times$ produced less milk (5%) than those continually milked 2 $\times$ and cows previously UF produced less milk (13%) compared with those continually AF.

At wk 1, plasma NEFA concentrations were lower in 1 $\times$ and AF cows relative to 2 $\times$ and UF cows (Table 2). An interaction was observed between MF and feeding level in wk 3; NEFA concentrations were greater in all UF cows, but the increase was less when UF cows were milked 1 $\times$ relative to 2 $\times$. At wk 5, cows previously UF had lower NEFA concentrations compared with cows continually AF. No effect of MF was observed on NEFA concentrations at wk 5.

Gene Expression Results

The transcript abundance of genes involved in lipogenic and other lipid metabolism processes are presented in Tables 3 and 4, respectively. After 3 wk of altered MF and feeding level, MF by feeding level interactions were observed for the lipogenic genes *FASN*, *SCD*, *GPD1*, *PC*, *DGAT2*, and *LPL*, and a trend ($P = 0.07$) for an interaction of MF and feeding level on *GPAM* expression (Table 3). Expression of these genes was greater in AF1 $\times$ cows compared with all other treatment groups, which did not differ from each other.

Table 1. Characteristics of gene-specific quantitative PCR assays

Gene ¹	GenBank accession number	Primer direction	Primer sequence ²	UPL probe number and sequence ³	Amplicon size (bp) and location	Dilution of cDNA
Endogenous control gene						
<i>EIF3K</i>	NM_001034489	Forward	AAGTTGCTCAAGGGGATCG	1; CCTGGAGC	77; exon 1/2	1/100
		Reverse	TTGGCCTGTGTCTCCACATA			
<i>RPS15A</i>	NM_001037443	Forward	TCAGCCCTAGATTTGATGTGC	32; CTGCTCCC	104; exon 3/4	1/100
		Reverse	GCCAGCTGAGGTTGTCAGTA			
Gene of interest						
<i>ACACA</i>	NM_174224	Forward	AACATCCCCACGCTAAACAG	25; CTCCTCCA	61; exon 29/30	1/100
		Reverse	GAGTCATGCCGTAGTGGTTG			
<i>ACSL1</i>	NM_001076085	Forward	ACTGCTCCACAAGGGCTTC	50; GCTCCAGA	93; exon 4/5	1/100
		Reverse	CATCCCTGTTCAATGATCACC			
<i>ANGPTL4</i>	NM_001046043	Forward	CGCGGAGACAAGAACTGC	92; CAGGAGCC	76; exon 6/7	1/10
		Reverse	CGTTTCAGGTTGGAGTGACTG			
<i>CPT2</i>	NM_001045889	Forward	CACCATTAGAAGATACCTCAGTGC	110; AGCCTCTG	94; exon 2/3	1/10
		Reverse	TCCAGTTTCAAACCTCTTACACAAC			
<i>DGAT2</i>	NM_205793	Forward	ACCCTCATAGCCGCCTACTC	67; CTCCAGCA	88; exon 1/2	1/10
		Reverse	GCCAAGTGACAGAAAACAGGT			
<i>FABP4</i>	NM_174314	Forward	ACAAGTACAAAACCTGGGATGGAA	157; ACCACCAT	92; exon 3/4	1/100
		Reverse	TGACACCATTTCATGACACATTC			
<i>FASN</i>	NM_001012669	Forward	CCTATGGCCTACAGTGTACAGGA	89; CAGCATCC	78; exon 36/37	1/50
		Reverse	TGATGCACTCGATGTAGTAGGTG			
<i>GHR1B</i>	NM_176608	Forward	GCTCGCAGGTCTACAGGT	59; CAGTGGCA	109; exon 1B/2	1/10
		Reverse	GACAAGGAAAGCTGGTGTGG			
<i>GPAM</i>	NM_001012282	Forward	TGCCACCAACGTAACCTGAAA	150; GAACAGCA	65; exon 4/5	1/50
		Reverse	CCACTTCTGCAATTGCCTCT			
<i>GPD1</i>	NM_001035354	Forward	TGTAGGCTCTGGCAACTGG	26; CAGCCCAG	86; exon 1/2	1/100
		Reverse	TCACCCGTGGGTCAAAGT			
<i>IGF1</i>	NM_001077828	Forward	ATGTACTGCGCGCCTCTC	104; GTGCCCAG	114; exon 3/4	1/10
		Reverse	CCCTCTACTTGTGTTCTTCAAATG			
<i>INSR</i> , isoform B	AJ320235	Forward	TGCACAACGTGGTTTTTCATC	124; CTTCAGGC	75; exon 7/8	1/10
		Reverse	GTTTTCTCGAAGGCCTAGC			
<i>LIPE</i>	NM_001080220	Forward	CGAGATCCAGGTGCTATCGT	87; CTGCCACC	106; exon 4/5	1/100
		Reverse	CGAGGTCAGAGGCATTTC			
<i>LPIN1</i>	NM_001206156	Forward	TCGTGGTGAAGATCGGAAAT	68; CTGCTCCT	81; exon 9/10	1/50
		Reverse	GTTTTCTGGAAGGCCTGCAT			
<i>LPL</i>	NM_001075120	Forward	GACAGGATGTGGCCAAGTTT	158; TGGATGGC	61; exon 3/4	1/100
		Reverse	TTGCCAGGGGATAGTTAAA			
<i>MGLL</i>	NM_001206681	Forward	GTCGGCGACACTTTTCAAG	109; GACTCCAG	107; exon 4/5	1/100
		Reverse	CCGTCTTATTCCTCGAGAGC			
<i>OXCT1</i>	NM_001076070	Forward	GCAAAGCTGCAGAAACCAC	22; TGGTGGAG	61; exon 2/3	1/50
		Reverse	GCAAATGATCCAATATCGACAA			
<i>PC</i>	NM_177946	Forward	GAGGTGGTCCGCAAGATG	39; AGGTGGAG	107; exon 3/4	1/10
		Reverse	TCGTGCAGGGAAGTGATG			
<i>PCK1</i>	NM_174737	Forward	TGGATGAAATTTGACCAACAAG	147; GCCATCAA	94; exon 5/6	1/10
		Reverse	GATTTGTCCTCACAGAGGTTCC			
<i>PLIN1</i>	NM_001083699	Forward	CCCTCCAGTATCCTCCTGAAA	161; GCTGAAGG	90; exon 3/4	1/100
		Reverse	GCACACTGATGCTGTTCTCTG			
<i>PNPLA2</i>	NM_001046005	Forward	CGGTGTCCTTCACCATCC	27; GCTGCCTG	64; exon 7/8	1/50
		Reverse	ATCCACCGGATGTCCTCAG			

Continued

Table 1 (Continued). Characteristics of gene-specific quantitative PCR assays

Gene ¹	GenBank accession number	Primer direction	Primer sequence ²	UPL probe number and sequence ³	Amplicon size (bp) and location	Dilution of cDNA
<i>PPARG</i>	NM_181024	Forward	GCGACTTAGCAATATTTATAGCTGTC	165; GAAGCCCA	105; exon 6/7	1/10
		Reverse	AGGCTTGACGACAGATGTCT			
<i>SCD</i>	NM_173959	Forward	CCGACCTAAGAGCCGAGAA	9; TGGTGTATG	67; exon 4/5	1/100
		Reverse	AACAACAGGACACAGGTTTG			
<i>THRSP</i>	AY656814	Forward	ATGGGACAGGCCGTCTAG	8; GAAGGCAG	71; exon 1/2	1/100
		Reverse	TGCACCATGTCTTCAATCACA			

¹*EIF3K* = eukaryotic translation initiation factor 3, subunit K; *RPS15A* = ribosomal protein S15a; *ACACA* = acetyl-CoA carboxylase α ; *ACSL1* = acyl-CoA synthetase long-chain family member 1; *ANGPTL4* = angiopoietin-like 4; *CPT2* = carnitine palmitoyltransferase 2; *DGAT2* = diacylglycerol O-acyltransferase 2; *FABP4* = FA-binding protein 4; *FASN* = FA synthase; *GHR1B* = growth hormone receptor 1b; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *GPD1* = glycerol-3-phosphate dehydrogenase 1; *IGF1* = insulin-like growth factor; *INSR*, isoform B = insulin receptor b; *LIPE* = hormone-sensitive lipase; *LPIN1* = lipin 1; *LPL* = lipoprotein lipase; *MGLL* = monoglycerol lipase; *OXCT1* = 3-oxoacid CoA transferase 1; *PC* = pyruvate carboxylase; *PCK1* = phosphoenolpyruvate carboxykinase 1; *PLIN1* = perilipin; *PNPLA2* = patatin-like phospholipase domain containing 2; *PPARG* = peroxisome proliferator-activated receptor gamma; *SCD* = stearoyl-CoA desaturase; *THRSP* = thyroid hormone responsive.

²Primer sequences are shown 5' to 3'.

³UPL = Universal Probe Library.

Expression of *THRSP* was lower in cows milked 1× compared with 2×, and in UF compared with AF cows (Table 3).

Milking frequency by feeding level interactions were also observed at treatment wk 3 for the FA oxidation genes *ACSL1* and *CPT2*, with AF1× cows having greater *ACSL1* expression than all other treatments and greater *CPT2* expression than AF2× cows and UF1× cows (Table 4). Expression of *FABP4* tended ($P = 0.06$) to be lower in UF relative to AF cows, and a trend ($P = 0.06$) existed for a MF by feeding level interaction for *ANGPTL4*, as UF1× cows had lower expression compared with all other treatments, which did not differ from each other (Table 4). In addition, expression of *IGF1* was greater in cows milked 1× compared with those milked 2× (Table 4).

Two weeks after cows returned to 2× milking and AF (wk 5), transcription of some genes involved in FA metabolism were still altered. Expression of *ACACA* was greater in cows previously UF (Table 3) compared with AF cows. Cows milked 2× and previously UF had greater *SCD* expression than AF2× and UF1× cows, but not AF1× cows (Table 3). Furthermore, cows previously milked 1× had lower expression of *ACSL1* (Table 4) than cows milked 2× throughout the trial.

DISCUSSION

Adequately fed cows that were milked 1× had increased transcript abundance of genes involved in FA synthesis (*FASN* and *SCD*), glyceroneogenesis (*GPD1* and *PC*), and TAG synthesis (*GPAM* and *DGAT2*), which indicated that the reduction in milk production and energy output due to 1× milking enabled nutrients to be partitioned to adipose tissue. However, when cows were underfed, no difference was observed between cows milked 1× and 2× in the transcription of genes involved in lipid metabolism, indicating that 1× milking during a severe feed restriction does not have any beneficial effect on storage of nutrients in adipose tissue.

Provided Cows Were AF, 1× Milking For 3 wk Altered Lipid Metabolism

Milking cows 1× for 3 wk increased the expression of genes involved in anabolic lipid pathways when these cows were AF. Adequately fed cows milked 1× for 3 wk had increased transcript abundance of genes involved in TAG hydrolysis for uptake by adipose tissue (*LPL*), FA synthesis (*FASN* and *SCD*), glycerol-3-phosphate production (*GPD1*), and TAG synthesis (*GPAM* and *DGAT2*). Although no significant interaction was observed in *THRSP* expression, AF cows and cows milked

Table 2. Mean milk production and plasma NEFA concentrations ($\log_{10}$ -transformed with back-transformed values in parentheses) from grazing cows ($n = 30$ per treatment; 34 ± 6 DIM) adequately fed (AF; 14.3 ± 3.6 kg of DMI/cow per day) or underfed (UF; 8.3 ± 2.9 kg of DMI/cow per day) and milked once ($1\times$) or twice ($2\times$) daily for 3 wk¹

Item	Week	Treatment				SED ²	P-value		
		AF2 $\times$	AF1 $\times$	UF2 $\times$	UF1 $\times$		MF ³	FL ⁴	MF $\times$ FL
Milk (kg/cow per day)	1	1.79	1.43	1.47	1.26	0.04	<0.001	<0.001	<0.05
	3	1.74	1.40	1.11	0.98	0.04	<0.001	<0.001	<0.001
	5	1.66	1.52	1.39	1.37	0.05	<0.05	<0.001	0.09
NEFA ($\log_{10}$ mmol/L)	1	-0.28 (0.53)	-0.51 (0.31)	0.19 (1.55)	0.01 (1.03)	0.04	<0.001	<0.001	0.32
	3	-0.36 (0.43)	-0.60 (0.25)	0.05 (1.12)	-0.04 (0.92)	0.04	<0.001	<0.001	<0.01
	5	-0.40 (0.40)	-0.38 (0.41)	-0.48 (0.33)	-0.47 (0.34)	0.04	0.71	<0.001	0.93

¹Following the treatment period (wk 5), all cows were milked $2\times$ and offered a pasture allowance to target grazing residuals $>1,600$ kg of DM/ha.

²SED = SE of the difference between the means.

³MF = milking frequency.

⁴FL = feeding level.

$1\times$ had greater expression of this lipogenic gene, possibly indicating that milking frequency and nutrition do not have an additive effect on the regulation of this gene, or that with a bigger sample size the effect may have become significant.

The increased expression of these genes after 3wk of $1\times$ milking in AF cows is consistent with the lower circulating plasma NEFA and BHBA concentrations, indicating reduced net export of FA from adipose and reduced uptake by the liver for ketogenesis. The results indicate that AF1 $\times$ cows are in a greater energy balance, supported by the greater plasma glucose, insulin, and IGF1 concentrations reported by Kay et al. (2013). Although transcription of *GPAM* and *DGAT2* were upregulated, no concomitant upregulation of *LPIN1* or *PPARG* occurred; *LPIN1* catalyzes the synthesis of diacylglycerol from phosphatidate; however, unlike *GPAM* and *DGAT2* the enzyme is located in the cytosol and also acts as a transcriptional coactivator (Reue and Brindley, 2008). The extra roles of *LPIN1* may explain the discrepancies between mRNA expression of the 3 genes involved in TAG synthesis.

Although greater transcript abundance of genes involved in FA and TAG synthesis was observed after 3 wk of $1\times$ milking in AF cows, none of these effects were present at 5 wk (after cows had been milked $2\times$ for 2 wk), indicating that increased synthesis of FA and TAG was not maintained. This contrasts with the longer-term positive effect of $1\times$ milking on glucose and insulin concentrations and BW gain in AF cows, which continued beyond the 3-wk treatment period (Kay et al., 2013). Lipogenic gene transcription in cows previously milked $1\times$ may have been reduced to similar amounts as in cows milked $2\times$ due to the increase in milk production and limited substrate availability; however, this is not consistent with the greater insulin concentrations. Although the mechanism for increased

long-term BW gain in AF1 $\times$ cows (Kay et al., 2013) has not been established, increased FA and TAG synthesis during the treatment period, as indicated by the gene transcription results, may have contributed.

The mRNA expression of 2 genes involved in FA oxidation (*ACSL1* and *CPT2*) was also greater in AF1 $\times$ cows, indicative of an increase in the use of FA as energy in adipose tissue. Studies have indicated a strong relationship between FA uptake and oxidation, as overexpression of FA transport proteins, such as CD36, are associated with a corresponding increase in FA oxidation (Ibrahimi et al., 1999; Boden et al., 2005). Greater plasma insulin in cows milked $1\times$ (Kay et al., 2013) and the greater transcript abundance of *LPL* in AF1 $\times$ cows at wk 3 support greater FA uptake in cows milked $1\times$ and the subsequent increase in FA oxidation. Fatty acid oxidation is not historically thought to be important in white adipose tissue; however, a role may exist for FA oxidation in generating energy for FA-TAG cycling (Flachs et al., 2013), which is thought to be important in enabling flexible regulation of metabolism. Additionally, recent studies have reported that FA generated from lipolysis rather than synthesized de novo are important in regulating PPAR α in cardiac tissue (Haemmerle et al., 2011) and in adipose tissue (Mottillo et al., 2012) and PPAR alpha is a key transcription factor for oxidative genes. However, PPAR delta and gamma have twice the expression in bovine adipose tissue compared with PPAR alpha (Bionaz et al., 2013); therefore, more investigation is required to determine if this occurs in the ruminant. In summary, FA oxidation in adipose tissue may play a supporting role in FA-TAG cycling, or lipolysis may enhance oxidative gene transcription.

Increased expression of genes involved in FA uptake and oxidation may also be a control mechanism against excessive TAG accumulation and the maintenance of

Table 3. Lipogenic gene expression in adipose tissue from grazing cows (n = 30 per treatment; 34 ± 6 DIM) adequately fed (AF; 14.3 ± 3.6 kg of DMI/cow per day) or underfed (UF; 8.3 ± 2.9 kg of DMI/cow per day) and milked once (1×) or twice (2×) daily for 3 wk¹

Gene ²	Week	Treatment				SED ³	P-value		
		AF2×	AF1×	UF2×	UF1×		MF ⁴	FL ⁵	MF × FL
FA synthesis									
<i>ACACA</i>	3	1.30	1.39	1.30	1.11	0.12	0.50	0.14	0.10
	5	1.05	0.82	1.50	1.27	0.22	0.18	<0.05	0.99
<i>FASN</i>	3	0.66	1.58	0.52	0.48	0.16	<0.001	<0.001	<0.001
	5	0.63	0.63	0.99	0.44	0.24	0.10	0.71	0.11
<i>SCD</i>	3	0.88	1.94	0.98	0.93	0.27	<0.01	<0.05	<0.01
	5	1.01	1.11	1.62	0.84	0.28	0.06	0.519	<0.05
<i>THRSP</i>	3	1.81	2.41	1.51	1.62	0.22	<0.05	<0.01	0.12
	5	1.28	1.34	1.79	1.36	0.36	0.26	0.39	0.34
Glycerolipid synthesis									
<i>GPAM</i>	3	0.76	1.44	0.71	0.87	0.19	<0.01	<0.05	0.07
	5	0.87	0.85	1.19	0.79	0.22	0.16	0.47	0.25
<i>LPIN1</i>	3	1.49	1.27	1.46	1.34	0.21	0.26	0.83	0.72
	5	1.08	0.86	0.93	0.70	0.20	0.14	0.30	0.96
<i>DGAT2</i>	3	1.70	2.55	1.61	1.62	0.20	<0.01	<0.01	<0.01
	5	1.56	1.73	2.18	1.67	0.27	0.30	0.19	0.08
Glyceroneogenesis									
<i>GPD1</i>	3	2.47	3.34	2.09	2.27	0.24	<0.01	<0.001	<0.05
	5	2.35	2.18	2.43	2.07	0.37	0.30	0.94	0.71
<i>PC</i>	3	0.81	1.27	0.89	0.80	0.16	0.10	0.08	<0.05
	5	0.82	0.92	0.93	0.78	0.17	0.79	0.83	0.32
<i>PCK1</i>	3	1.52	1.95	1.43	1.35	0.32	0.32	0.16	0.30
	5	1.34	1.70	1.38	1.57	0.38	0.30	0.84	0.76
Insulin receptor									
<i>INSR</i> , isoform B	3	0.76	0.84	0.84	0.86	0.09	0.48	0.48	0.67
	5	0.63	0.54	0.48	0.45	0.13	0.55	0.19	0.77
Lipoprotein hydrolysis									
<i>LPL</i>	3	0.64	1.13	0.65	0.69	0.15	<0.05	<0.05	<0.05
	5	0.77	0.64	0.96	0.78	0.17	0.19	0.18	0.86
Ketolysis									
<i>OXCT1</i>	3	0.39	0.43	0.35	0.32	0.09	0.83	0.26	0.55
	5	0.51	0.30	0.34	0.30	0.09	0.10	0.24	0.21
Transcription factor									
<i>PPARG</i>	3	1.18	1.24	1.15	1.12	0.18	0.86	0.57	0.71
	5	0.97	0.83	1.05	0.89	0.19	0.25	0.62	0.93

¹Following the treatment period (wk 5), all cows were milked 2× and offered a pasture allowance to target grazing residuals >1,600 kg of DM/ha. Data are expressed as log₁₀-transformed arbitrary units normalized to the expression of ribosomal protein S15a and eukaryotic translation initiation factor 3, subunit K.

²*ACACA* = acetyl-CoA carboxylase α; *DGAT2* = diacylglycerol O-acyltransferase 2; *FASN* = FA synthase; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *GPD1* = glycerol-3-phosphate dehydrogenase 1 (soluble); *INSR*, isoform B = insulin receptor b; *LPIN* = lipin 1; *LPL* = lipoprotein lipase; *OXCT1* = 3-oxoacid CoA transferase 1; *PC* = pyruvate carboxylase; *PCK1* = phosphoenolpyruvate carboxykinase 1; *PPARG* = peroxisome proliferator-activated receptor gamma; *SCD* = stearoyl-CoA desaturase; *THRSP* = thyroid hormone responsive.

³SED = SE of the difference between the means.

⁴MF = milking frequency.

⁵FL = feeding level.

an adipose tissue storage set point. This phenomenon is referred to as lipostasis and was first proposed by Kennedy (1953), who reported that animals with free access to food regulate their stored reserves either through changes in energy intake or expenditure. Although most of the data supporting lipostasis in ruminants come from observations of reduced weight loss in thinner cows (Roche et al., 2005, 2008), studies exist that report decreased intake in sheep with sufficient adipose reserves (Holter et al., 1990; Tolcamp et al., 2006), and in cows with voluntary feed intake per liter of milk decreased as BCS was gained (Broster and

Broster, 1998). These studies indicate that behavioral adjustments may be invoked to maintain lipostasis, and metabolic adjustments also occur. Recent work investigating the role of FA oxidation in white adipose has indicated that adipose-specific *ACSL1* knockout mice have 50 to 80% lower FA oxidation rates and increased adiposity (Ellis et al., 2010). Therefore, the greater FA oxidation in adipose tissue of AF cows milked 1× may be a mechanism through which lipid reserves are regulated in animals with surplus energy available due to decreased energy output (via reduced milk production) and with no energy input restrictions. Overall, milking

Table 4. Lipolytic, FA oxidative and somatotrophic axis gene expression in adipose tissue from grazing cows (n = 30 per treatment; 34 ± 6 DIM) adequately fed (AF; 14.3 ± 3.6 kg of DMI/cow per day) or underfed (UF; 8.3 ± 2.9 kg of DMI/cow per day) and milked once (1×) or twice (2×) daily for 3 wk¹

Gene ²	Wk	Treatment				SED ³	P-value		
		AF2×	AF1×	UF2×	UF1×		MF ⁴	FL ⁵	MF × FL
Lipolysis									
<i>ANGPTL4</i>	3	1.67	1.68	1.75	1.43	0.12	0.10	0.30	0.06
	5	1.19	1.17	1.13	0.88	0.23	0.45	0.26	0.49
<i>LIPE</i>	3	1.63	1.71	1.68	1.52	0.16	0.77	0.52	0.31
	5	1.24	1.08	1.15	0.97	0.26	0.38	0.56	0.96
<i>MGLL</i>	3	1.22	1.31	1.16	1.15	0.13	0.61	0.24	0.62
	5	0.86	0.81	0.81	0.72	0.17	0.60	0.54	0.88
<i>PLIN1</i>	3	1.64	1.59	1.61	1.41	0.14	0.26	0.30	0.45
	5	1.12	1.03	1.21	0.96	0.23	0.30	0.98	0.63
<i>PNPLA2</i>	3	0.89	1.09	0.88	0.84	0.15	0.43	0.23	0.28
	5	0.72	0.61	0.68	0.52	0.17	0.28	0.64	0.84
FA oxidation									
<i>ACSL1</i>	3	0.71	1.15	0.74	0.74	0.14	<0.05	0.06	<0.05
	5	0.67	0.55	0.90	0.48	0.20	<0.05	0.69	0.28
<i>CPT2</i>	3	0.26	0.53	0.38	0.29	0.12	0.19	0.50	<0.05
	5	0.34	0.50	0.41	0.40	0.11	0.31	0.75	0.24
FA transport									
<i>FABP4</i>	3	2.59	2.41	2.34	1.85	0.31	0.19	0.06	0.49
	5	1.99	1.83	2.14	1.61	0.30	0.10	0.75	0.39
Somatotrophic axis									
<i>GHR1B</i>	3	0.90	1.14	0.91	0.89	0.14	0.23	0.24	0.22
	5	0.85	0.67	0.75	0.65	0.19	0.29	0.66	0.79
<i>IGF1</i>	3	0.42	0.70	0.47	0.54	0.12	<0.05	0.57	0.26
	5	0.71	0.75	0.83	0.73	0.15	0.83	0.68	0.54

¹Following the treatment period (wk 5), all cows were milked 2× and offered a pasture allowance to target grazing residuals >1,600 kg of DM/ha. Data are expressed as log₁₀-transformed arbitrary units normalized to the expression of ribosomal protein S15a and eukaryotic translation initiation factor 3, subunit K.

²*ANGPTL4* = angiopoietin-like 4; *LIPE* = hormone-sensitive lipase; *MGLL* = monoglyceride lipase; *PLIN1* = perilipin; *PNPLA2* = patatin-like phospholipase domain containing 2; *ACSL1* = acyl-CoA synthetase long-chain family member 1; *CPT2* = carnitine palmitoyltransferase 2; *FABP4* = FA-binding protein 4; *GHR1B* = growth hormone receptor 1b; *IGF1* = insulin-like growth factor 1.

³SED = SE of the difference between the means.

⁴MF = milking frequency.

⁵FL = feeding level.

adequately fed cows 1× for 3 wk is associated with greater FA uptake, synthesis, and oxidation and may have lactation-long effects on BW.

Milking UF Cows 1× Did Not Alter Expression of Genes Involved in Lipid Metabolism

At the end of the 3-wk feed restriction, no transcriptional differences were observed between UF1× and UF2× animals in all but 1 gene measured. The transcript abundance of *ANGPTL4* tended to be lower in UF cows milked 1× compared with 2×, consistent with the reported effects of *ANGPTL4* adipose mRNA increasing with negative energy balance (Koltes and Spurlock, 2012). The *ANGPTL4* protein binds to LPL, converting the enzyme from an active to an inactive state, and is reported to be a fasting-induced regulator of LPL activity (Wang and Eckel, 2009). Therefore, decreased transcription of *ANGPTL4* in UF cows milked 1× compared with 2× may result in less inhibition of

LPL enzyme activity, and a subsequent increase in TAG lysis and FA uptake by adipose tissue. The LPL protein is regulated posttranscriptionally; during fasting conditions it has been reported that LPL activity is reduced, with no change reported in mRNA or protein concentrations (Doolittle et al., 1990). Indeed, *ANGPTL4* mRNA transcript abundance is reported to be negatively correlated with LPL activity in rats (Doolittle et al., 1990). As no difference was observed in *LPL* transcription between UF cows that were milked 1× or 2×, further research is required to determine if LPL activity may have been altered.

The lack of an effect of 1× milking in UF cows on the expression of genes involved in FA synthesis, re-esterification, lysis, and transport indicates that at 3 wk, milking UF cows 1× did not alter lipid metabolism. Although this premise is supported by the lack of an effect on BCS loss with 1× milking, the profile of circulating plasma hormones and metabolites indicate that 1× milking supports a greater positive energy balance

than 2× milking (Kay et al., 2013). Focusing only on UF animals, cows milked 1× had lower NEFA, lower BHBA, greater glucose, and greater IGF1 concentrations (Kay et al., 2013). Many of the plasma measures displayed a pattern similar to that of NEFA. At the end of wk 1, plasma NEFA concentrations from UF cows milked 1× were 30% lower than in UF cows milked 2× (Kay et al., 2013). Although an effect of reduced MF on NEFA concentrations was still observed at wk 3 (when gene expression was measured), the difference in NEFA concentrations between 1× and 2× milking was only 12% (Kay et al., 2013). The smaller difference in NEFA concentration between UF cows milked 1× and 2× at 3 wk is most probably due to the reduction in energy output via reduced milk production and lipostatic regulation to prevent excessive tissue mobilization in UF cows milked 2×. These plasma data indicate an immediate lipolytic response to underfeeding at wk 1 that is initially alleviated by 1× milking (via reduced lipolysis and FA export or increased lipogenesis); however, by wk 3, adaptive mechanisms such as reduced milk production play a role in regulating energy balance. These adaptations to the energy restriction may be the cause of the similar expression of genes involved in lipid metabolism in UF cows milked 1× and 2× at wk 3.

Lipolytic Gene Expression Was Not Increased by 3 wk of Severe Feed Restriction

To supply nutrients for milk production, we hypothesized that greater lipolysis would occur in UF cows; however, no effect of feed restriction was observed on transcription of the 4 lipolytic genes measured (*LIPE*, *MGLL*, *PLIN1*, and *PNPLA2*). Either lipolysis was not enhanced to provide nutrients for milk production or gene transcription was not the best measure of lipolysis in the current study. The lack of a lipolytic effect was hypothesized to be a protective mechanism to limit plasma NEFA concentrations due to excessive tissue mobilization, which can lead to fatty liver and contribute to ketosis (Bobe et al., 2004). Peak NEFA concentrations were reported in UF2× cows in wk 1 of the treatment period (Kay et al., 2013); therefore, it is possible that lipolytic gene transcription was greater in the first week of the feed restriction, resulting in NEFA release. However, this may have been downregulated by wk 3 when tissues were collected. Limiting lipolytic gene expression may have occurred through negative feedback due to high plasma NEFA concentration (Dunshea et al., 1990); however, the mechanism for this is not clear. Studies in fasted rats indicate that in the third phase of starvation (characterized by protein breakdown), NEFA concentrations decrease and *LIPE* expression (both protein and mRNA) also decrease

relative to early starvation as nutrient use shifts from fat to protein reserves (Bertile and Raclot, 2011). Additionally, after 3 wk of feed restriction, milk production in UF cows had decreased by 34% (Kay et al., 2013), indicating that lipostatic mechanisms to maintain adipose tissue were activated, potentially reducing the requirement for lipolysis.

Alternatively, due to the posttranslational regulation of most of the lipolytic genes, gene expression may not provide the most accurate measure of lipolysis. Although one study did indicate an increase in transcription of genes coding for lipolytic enzymes (Sumner-Thomson et al., 2011), these did not occur until later in lactation. It is more likely, in response to a feed restriction, that posttranscriptional regulation exerts the most control on lipolysis. Limited effects of UF on lipolytic gene transcription is consistent with the increased NEFA concentrations; therefore, after 3 wk of feed restriction, it appears that cows limit energy output and increase lipolysis through posttranscriptional mechanisms to cope with the feed deficit.

Returning to 2× or AF Affects Transcription of Genes Involved in FA Cycling

Several genes had altered transcript abundance following the return to 2× milking and AF. Transcription of *ACACA*, *FASN*, *SCD*, and *DGAT2* was increased in cows previously UF and milked 2× once returned to adequate intakes compared with AF2× or UF1× cows, indicating an effect of refeeding when not mitigated by the increase in MF. Although milk production and NEFA concentrations were reduced in UF cows at wk 5, indicating reduced energy demand, insulin concentrations were similar between treatment groups. As the increase in feed had a positive effect on lipogenic gene transcription, it is possible that these previously UF cows may have increased sensitivity to insulin during this recovery period. Reduced NEFA concentrations may enhance insulin sensitivity (Chilliard et al., 2000), leading to an increase in FA precursors, such as acetate, being absorbed by the adipose tissue, and a subsequent increase in lipogenesis. Increased lipogenesis in cows previously UF is another example of the lipostatic theory. Due to the feed restriction, BCS has been lost; therefore, additional adipose tissue reserves are required to build up BCS to prerestriction amounts. This theory may explain not only why genes involved in FA synthesis are upregulated, but also why milk production does not immediately return to amounts achieved prerestriction.

At wk 5, the expression of *ACSL1* was reduced in cows previously milked 1× compared with cows milked 2× continually. This indicates that the switch from 1×

back to 2× milking, and subsequent increase in milk production enhanced partitioning of FA to the mammary gland for use in milk fat production. The increase in energy demand due to increased MF and milk production, or the increased energy supply through increased intake, altered the transcription of genes involved in FA synthesis or breakdown.

CONCLUSIONS

Milking cows 1× when AF promoted the expression of genes involved in TAG storage; however, when cows were UF, 1× milking did not alter expression of genes involved in lipid metabolism compared with 2× milking. It is possible that transcriptional effects may have been present during wk 1 of the feed restriction; however, by 3 wk, milk production and blood metabolite measures indicated that the cows were reducing their energy output in response to the nutrient deficit, which may have limited the mobilization of adipose tissue reserves at this time point, to maintain a minimum adipose storage level. Future work investigating protein expression at wk 1 and 3 would further clarify the temporal effects of 1× milking. In summary, after 3 wk of nutrient deficit, 1× milking does not alter transcription of genes involved in lipid metabolism compared with 2× milking.

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