Gene expression in liver and adipose tissue is altered during and after temporary changes to postpartum milking frequency

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ABSTRACT

Short-term changes to milking frequency can alter the metabolic status of dairy cows depending on the duration, magnitude, and stage of lactation at which the milking frequency changes occur. Additionally, effects of altered milking frequency that are subsequent to cows returning to a normal twice-daily (2×) milking regimen are not well established. This study tested the hypothesis that plasma concentrations of key hormones and metabolites and transcription of genes involved in the somatotrophic axis and lipid metabolism would be altered in liver and subcutaneous adipose tissue from cows milked with different frequencies. Multiparous Holstein-Friesian dairy cows were allocated to 2× milking for the whole lactation, or once-(1×) or 3 times-(3×) daily milking for 3 or 6 wk, immediately postpartum, and then 2× milking for the remainder of the lactation. Liver and subcutaneous fat were biopsied at wk 1 (liver only), 3, 6, and 9 postpartum, and transcription of genes involved in the somatotrophic axis and lipid metabolism were measured. At wk 3, cows milked 3× had lower hepatic expression of growth hormone receptor (*GHR1A*) compared with cows milked 2× or 1×, and lower *IGF1* expression compared with cows milked 1×, indicating greater uncoupling of the somatotrophic axis. At wk 6, reduced transcription of total *GHR* and *GHR1B* occurred in the adipose tissue of cows milked 3×. Cows milked 1× had greater transcription in adipose tissue of lipogenesis genes at wk 3 and 6, and lipolysis genes at wk 6, compared with cows milked 2×, indicating a period of increased fatty acid storage, followed by increased fatty acid reesterification. At wk 9, cows previously milked 3× for 6 wk maintained lower transcription of genes involved in lipogenesis, lipolysis, and ketolysis in adipose tissue compared with cows milked 2×, indicat-

ing that the effects of 3× milking persist for at least 3 wk after switching to 2× milking. Results indicate that alterations to milking frequency affect the transcription of genes involved in lipid mobilization and storage, enabling the animal to manage the energy demands associated with the change in milk production. Some of these gene transcription changes were maintained in cows previously milked 3×, indicating that the adipose tissue gene expression changes were still required even after 3 wk of the less-demanding 2× milking regimen.

Key words: energy balance, somatotrophic axis, early lactation, lipid metabolism

INTRODUCTION

During early lactation, homeorhetic changes ensure that nutrients are partitioned toward milk production at the expense of tissue reserves (Bauman and Currie, 1980). These changes include a period of insulin resistance (Bell and Bauman, 1997; Vernon and Pond, 1997) and the uncoupling of the somatotrophic axis, which is reflected by decreased growth hormone (**GH**) receptor (**GHR**) expression in the liver (Kobayashi et al., 1999). The reduced GHR expression limits the hepatic production of IGF-1 (Lucy et al., 2009), and decreases the negative feedback of IGF-1 on GH production (Rhoads et al., 2004). The insulin resistance, high plasma GH, and low IGF-1 concentrations act in coordination to minimize glucose uptake into muscle and adipose tissue (Chagas et al., 2009; Lucy et al., 2009), and enhance lipolysis in adipose tissue (McNamara and Hillers, 1986; Khan et al., 2013), which results in FA efflux and an increase in nutrient provision for milk production from body stores.

Altering milking frequency during early lactation may modify these homeorhetic mechanisms to support the corresponding change in milk production. Increasing the frequency of milking may exacerbate the negative energy balance, due to the expected increase in milk production and the limitations of nutrient intake, especially in pasture-based dairy systems. The severity

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of the negative energy balance may also depend on the duration of the change in milking frequency. Increasing the milking frequency from twice daily ($2\times$) to 3 times daily ($3\times$) for 8 wk reportedly increased milk production, but also increased metabolic stress for the duration of the study (Andersen et al., 2004b). However, increasing milking frequency from $2\times$ to either $3\times$ or 4 times daily for 3 or 4 wk immediately postpartum, does not evoke major metabolic effects (McNamara et al., 2008; Soberon et al., 2010).

Increasing milking frequency increases the plasma concentrations of NEFA and BHBA, which indicate increased lipolysis and ketogenesis (Loiselle et al., 2009; Soberon et al., 2010). Furthermore, Eslamizad et al. (2010) reported decreased BCS (a tool for assessing body fat reserves; Roche et al., 2004) and plasma glucose, but no change in hepatic triacylglyceride (**TAG**) concentration, in cows milked 6 times daily compared with cows milked $3\times$. Additionally, limited data exist on any persistent or carryover effects of increased milking frequency on metabolic indices.

In comparison, decreasing milking frequency may improve energy balance. Cows milked once daily ($1\times$) have improved energy balance, reduced BCS loss, and reduced BW loss during early lactation (Patton et al., 2006; McNamara et al., 2008). The metabolic benefits of milking $1\times$ during early lactation are consistent across studies; however, less information is available on metabolic effects of $1\times$ milking once cows are switched to $2\times$. In a recent study (Kay et al., 2013), decreasing milking frequency from $2\times$ to $1\times$ did not alter plasma GH concentrations; however, glucose, insulin, and IGF-1 concentrations were greater, and BHBA and NEFA concentrations lower, in cows milked $1\times$ compared with $2\times$. Furthermore, after cows milked $1\times$ were switched to $2\times$ milking, glucose, insulin and IGF-1 concentrations tended to remain greater.

The milk production and BW measures from previous work (Phyn et al., 2011) indicate that metabolic changes occur in response to altered milking frequency and persist even when milked $2\times$. Therefore, to increase understanding of the homeorhetic mechanisms responsive to milking frequency, plasma concentrations of key hormones and metabolites and transcription of genes involved in the somatotrophic axis and lipid metabolism were measured in liver and subcutaneous adipose tissue from cows milked with different frequencies during the period immediately postpartum.

MATERIALS AND METHODS

Experimental Design and Treatments

The study was conducted at Lye Farm, DairyNZ (Hamilton, New Zealand; 37°46'S, 175°18'E) from June

to November 2009. All treatments and measurements were approved by the Ruakura Animal Ethics Committee (Hamilton, New Zealand).

The experimental design, grazing management, and supplementary feeding of cows are described in Phyn et al. (2011). Briefly, multiparous Holstein-Friesian and Holstein-Friesian $\times$ Jersey dairy cows ($n = 150$) were randomly allocated to 1 of 5 treatments ($n = 30$ /treatment) immediately postpartum (mean $\pm$ SD; July 15, 2009 $\pm$ 10.6 d). Treatments were (1) cows milked $2\times$ for the entire lactation (control), (2) cows milked $1\times$ for 3 wk postpartum and $2\times$ thereafter, (3) cows milked $1\times$ for 6 wk postpartum and $2\times$ thereafter, (4) cows milked $3\times$ for 3 wk postpartum and $2\times$ thereafter, and (5) cows milked $3\times$ for 6 wk postpartum and $2\times$ thereafter. Daily milking times were 0700 h for $1\times$ (24-h interval), 0700 and 1500 h for $2\times$ (16/8-h interval), and 0700, 1500, and 2200 h for $3\times$ (9/8/7-h interval).

All cows were offered a generous allowance of perennial ryegrass/white clover pasture (~ 30 to 45 kg of DM/cow per day measured to ground level with target postgrazing residuals of 1,800 kg of DM/ha). Cows grazed in $1\times$, $2\times$, and $3\times$ milking mobs in the same paddock separated by a wire so that they could be drafted independently to the farm dairy for milking. Due to the inherent difficulties of measuring individual DMI under grazing conditions, energy intakes per cow could not be measured, but high postgrazing residuals were targeted to ensure that DMI was not limited by pasture allocation. Furthermore, pasture silage was offered at, on average, 3.6 kg of DM/cow per day for 40 d during July and August to maintain pasture residuals when pasture availability was limited, and pasture quality was maintained by strategic mechanical cutting of high residuals or an immediate regrowth with a nontreatment group of cows. In addition, cows were offered 2 kg of DM/cow per day of pelleted maize/barley/molasses-based concentrate in 1 feed at 0900 h for 2 wk before their expected calving date. Following calving, the same concentrate was individually offered to cows at 4 kg of DM/cow per day during the a.m. milking until November 1 (i.e., ~ 109 DIM) and at 2 kg of DM/cow per day thereafter, until November 24.

Milk and Blood Sampling and Measurements

Milk production and BCS measurements were as described elsewhere (Phyn et al., 2011). Briefly, individual milk yields and milk composition (fat, CP, and lactose) were determined daily and weekly, respectively. Body condition score (1–10 scale, where 1 = emaciated and 10 = obese; Roche et al., 2004) were measured once per week until September and once every 2 wk thereafter. Blood was sampled on the same 1 d each week for the

first 12 wk postpartum. A 10-mL evacuated blood tube (Becton Dickinson, Franklin Lakes, NJ) containing 140 IU of sodium heparin was collected from each cow by coccygeal venipuncture immediately before the morning milking (approximately 0700 h). Samples were placed on ice and then centrifuged at $1,120 \times g$ for 12 min at 4°C. Plasma was harvested and stored at -20°C while awaiting subsequent analyses for metabolites (NEFA and glucose), and hormones (growth hormone, GH, insulin, and IGF-1) as described in Grala et al. (2014).

Briefly, analyses for plasma concentrations of NEFA [acyl Co-A synthetase, acyl-CoA oxidase (ACS-ACOD) colorimetric method using the NEFA C Kit from Wako, Osaka, Japan] and glucose [glucose oxidase/4-aminophenazone/phenol (GOD-PAP) method determined photometrically] were performed on a Modular P800 analyzer (Roche, Basel, Switzerland) at 37°C by Gribbles Veterinary Pathology Ltd. (Hamilton, New Zealand). The inter- and intraassay coefficients of variation were <2% for all assays.

Plasma GH (Downing et al., 1995), IGF-1 (Gluckman et al., 1983), and insulin (Hales and Randle, 1963) were measured in duplicate by double-antibody RIA by the University of Western Australia (Perth, Australia) with inter- and intraassay coefficients of variation <6%. Validation for bovine plasma was as previously reported (Chagas et al., 2007).

Plasma GH measurements used ovine GH [NIDDK-I-5; National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (**NHPP-NIDDK**), Bethesda, MD] and anti-ovine GH antiserum (NIDDK-anti-oGH-3; NHPP-NIDDK; final dilution 1:300,000). Plasma IGF-1 was assayed with human recombinant IGF-1 (ARM4050; Amersham-Pharmacia Biotech, Buckinghamshire, UK) and anti-human IGF-1 antiserum (AFP4892898; NHPP-NIDDK; final dilution 1:360,000) and insulin antiserum (GP2, 21/7/80), donated by Peter Wynn (Commonwealth Scientific and Industrial Research Organisation, Division of Animal Production, Blacktown, New South Wales, Australia), was raised in guinea pigs by using bovine insulin (BI 4499; Ely Lilly Pty. Ltd., West Ryde, Sydney, Australia).

Tissue Sampling

Liver and adipose tissue were collected at wk 1 (liver only), 3, 6, and 9 postpartum from a subset of cows ($n = 12/\text{treatment}$). The wk-1 liver sample was only collected from cows in 2 $\times$, 1 $\times$ for 3 wk, and 3 $\times$ for 3-wk treatment groups to minimize the number of cows subjected to 4 consecutive biopsies, as required for animal ethics approval. This encompassed all the treatments at this time point, as the effect of the dura-

tion of altered milking frequency was not a factor until the wk-6 biopsy. Subsequent liver and adipose samples were collected from all treatments. Intravaginal controlled internal drug release (CIDR) devices (Zoetis, NSW, Australia) containing 1.38 g of progesterone were inserted 2 d before and removed at the wk-3 and -9 biopsies to prevent estrous behaviors around the time of the biopsy.

Liver sample collection was performed from an area in the eleventh intercostal space as described by Lucy et al. (1998), resulting in samples of approximately 200 mg. 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; Loppaine; Ethical Agents Ltd., Auckland, New Zealand) 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 and snap-frozen in liquid nitrogen. Samples were stored at -80°C until RNA extraction (6 to 9 mo later).

Extraction of RNA and cDNA Synthesis

Total cellular RNA was isolated from the liver and adipose biopsies using a Qiagen RNeasy kit (Qiagen GmbH, Hilden, Germany). Liver biopsies of approximately 30 mg were homogenized by adding 3 ball bearings of 3-mm diameter (Farrell Bearings Ltd., Hamilton, New Zealand) into a tube containing the biopsy and RLT lysis buffer (Qiagen GmbH). Tubes were loaded into a TissueLyser II (Qiagen GmbH) and homogenized for 2 min. Total RNA was then extracted and treated with DNase (Ambion DNA-free kit; Ambion Inc., Austin, TX). Adipose biopsies of approximately 80 mg were homogenized as described above before adding 200 μL of chloroform and centrifuging 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 as above. The RNA quantity, purity, and integrity were determined by using a NanoDrop ND-1000 (NanoDrop Technologies LLC, Wilmington, DE) and the Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip kit (Agilent Technologies Inc., Palo Alto, CA). All RNA samples were stored at -80°C.

Of the 216 planned liver biopsies, 200 yielded usable tissue samples, with RNA successfully extracted from 196 of these. Final numbers for each treatment group were as follows: 1 $\times$ for 3 wk: total $n = 45$, 1 wk: $n = 10$, 3 wk: $n = 12$, 6 wk: $n = 12$, and 9 wk: $n = 11$; 1 $\times$ for 6 wk: total $n = 35$, 3 wk: $n = 11$, 6 wk: $n = 12$, 9 wk: $n = 12$; 2 $\times$: total $n = 45$, 1 wk: $n = 11$, 3 wk: $n = 12$, 6 wk: $n = 11$, and 9 wk: $n = 11$; 3 $\times$ for 3 wk: total

n = 35, 1 wk: n = 11, 3 wk: n = 10, 6 wk: n = 7, and 9 wk: n = 7; and 3× for 6 wk: total n = 36, 3 wk: n = 12, 6 wk: n = 12, and 9 wk: n = 12.

Of the 180 planned adipose biopsies, 176 yielded usable tissue samples, with RNA successfully extracted from 173 of these. Final numbers for each treatment group were as follows: 1× for 3 wk: total n = 33, 3 wk: n = 11, 6 wk: n = 12, and 9 wk: n = 10; 1× for 6 wk: total n = 36, 3 wk: n = 12, 6 wk: n = 12, and 9 wk: n = 12; 2×: total n = 36, 3 wk: n = 12, 6 wk: n = 12, and 9 wk: n = 12; 3× for 3 wk: total n = 32, 3 wk: n = 10, 6 wk: n = 11, and 9 wk: n = 11; and 3× for 6 wk: total n = 36, 3 wk: n = 12, 6 wk: n = 12, and 9 wk: n = 12.

Two micrograms of each liver RNA sample (final volume = 40 µL) and 0.25 µg of each adipose RNA sample (final volume = 40 µL) were used for cDNA synthesis using the Invitrogen SuperScript III SuperMix kit (Invitrogen Corp., Carlsbad, CA). Total RNA was reverse transcribed according to the manufacturer's instructions, using a final concentration of 27 µM of random pentadecamer primers. Reverse-transcriptase negative controls were generated by excluding the enzyme. Resultant cDNA samples were stored at -20°C.

Quantitative PCR and mRNA Analyses

Using reverse-transcription quantitative PCR (RT-qPCR) analysis, the expression of 25 target genes was determined in liver and adipose tissue (Table 1). Genes were targeted for their involvement in the somatotrophic axis in liver (n = 6 genes) and lipogenesis, lipolysis, and nutrient utilization in adipose (n = 21 genes) tissue. The RT-qPCR was performed as described in Grala et al. (2011) using Roche real-time PCR master mix (LightCycler 480 Probes Master; Roche Diagnostics Deutschland GmbH, Mannheim, Germany) and Roche Universal Probe Library assays analyzed on the Roche LightCycler 480 system (Roche Diagnostics Deutschland GmbH). Assays were designed to publicly available bovine gene sequences [National Center for Biotechnology Information (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 to prevent amplification of genomic DNA, and the specificity of PCR primers tested using the BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/>; Altschul et al. 1990). The primer and probe sequences and location of the assays are presented in Table 1. Primers were manufactured at 50 nmol (Invitrogen Corp.) and purified by desalting. The RT-qPCR reaction volume was 10 µL, consisting of 4 µL of cDNA and 6 µL of master mix (5 µL of Roche Probes Master, 0.4 µL of each 5 µM primer, 0.075 µL of probe, and 0.125 µL of H₂O). Reactions were set up using an

epMotion 5075 robot (Eppendorf, North Ryde, NSW, Australia) before plates of samples were transferred into the LightCycler 480 instrument. Standard cycling conditions were used [95°C for 10 min, (95°C for 10 s, 60°C for 30 s) × 45 cycles, and 40°C for 40 s].

To quantify gene expression, cDNA was diluted as per Table 1. Each RT-qPCR run included 2 negative controls (i.e., a no-template control with water added instead of cDNA and a reverse transcription negative control), and 3 interrune calibrators. Interrune calibrators were necessary, as the number of samples required 2 plates to be used for each gene (Hellemans et al. 2007). Triplicate measurements were performed 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 visualizing PCR products on an agarose gel (3%) with expected amplicon specificity and length. The per-cycle amplification efficiency of the PCR assays ranged between 1.88 and 1.97.

The Roche LightCycler 480 software was used to analyze gene expression using relative standard curve second-derivative maximum analysis. A 6-point standard curve of serial cDNA dilutions was used, ranging from 3.33×10^{-1} to 1.37×10^{-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., 2004a). In liver tissue, *RPS15A* and *YWHAZ* were the 2 most stably expressed genes (0.086 and 0.090 stability values, respectively), and provided the best combination (0.074 stability value). In adipose tissue, the 2 most stably expressed genes were *MRPL39* and *EIF3K*; however, NormFinder indicated that the best combination for elimination of treatment group bias was *MRPL39* and *ACTB* (stability value of 0.087). The Roche LightCycler 480 software was then used to perform advanced relative quantification analysis of gene expression using the normalization factor of the 2 endogenous control genes. The geometric mean of the interrune calibrators was calculated for each plate and applied to each sample.

Calculations and Statistical Analysis

Production, BCS, Hormone, and Metabolite Data. Repeated measurements through time were modeled for milk production, BCS, and plasma hormone and metabolite data using cubic splines within the linear mixed model framework in GenStat software (version 13.2; VSN International Ltd., UK) as described by Verbyla et al. (1999; results not presented). Milking frequency (1×, 2×, or 3×), duration (3 or 6 wk), the linear trend of time, and their interaction were

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	Target tissue	Dilution of cDNA
Endogenous control gene							
<i>RPS15A</i>	NM_001037443	Forward	TCAGCCCTAGATTTGATGTGC	32	104	Liver	1/100
		Reverse	GCCAGCTGAGGTTGTCTAGTA	CTGCTCCC	Exon 3/4		
<i>YWAHZ</i>	BC102382	Forward	TGAAAATGAAAGGAGACTACTACCG	30	84	Liver	1/50
		Reverse	GCTGTGACTGGTCCACAATC	GGCTGAGG	Exon 3/4		
<i>ACTB</i>	NM_173979	Forward	GCGGACAGGATGCAGAAA	37	89	Fat	1/100
		Reverse	ACGGAGTACTTGCGCTCAG	TGCCCTGG	Exon 3/4		
<i>MRPL39</i>	NM_001080730	Forward	CTCCTTATAGCTGTGCCATGC	4	70	Fat	1/50
		Reverse	CCGTCCACAAGAGCCAGA	GCAGGAAG	Exon 2/3		
Gene of interest							
<i>ACACA</i>	NM_174224	Forward	AACATCCCCACGCTAAACAG	25	61	Fat	1/50
		Reverse	GAGTCATGCCGTAGTGGTTG	CTCCTCCA	Exon 29/30		
<i>ACSL1</i>	NM_001076085	Forward	ACTGCTCCACAAGGGCTTC	50	93	Fat	1/100
		Reverse	CATCCCTGTTCAATGATCACC	GCTCCAGA	Exon 4/5		
<i>ANGPTL4</i>	NM_001046043	Forward	CGCGGAGACAAGAACTGC	92	76	Fat	1/10
		Reverse	CGTTCAGGTTGGAGTGACTG	CAGGAGCC	Exon 6/7		
<i>CPT2</i>	NM_001045889	Forward	CACCATAGAAAGATACCTCAGTGC	110	94	Fat	1/10
		Reverse	TCCAGTTTCAAACTCTTACACAACT	AGCCTCTG	Exon 2/3		
<i>ELOVL5</i>	NM_001046597	Forward	TCTCTGATCACCTCTTCACAA	91	95	Fat	1/50
		Reverse	CCGTTCTGGTGGTCCTTTT	GAGGAGAG	Exon 6/7		
<i>FASN</i>	NM_001012669	Forward	CCTATGGCCTACAGTGTACAGGA	89	78	Fat	1/10
		Reverse	TGATGCACTCGATGTAGTAGGTG	CAGCATCC	Exon 36/37		
<i>FABP4</i>	NM_174314	Forward	ACAAGTACAAAACCTGGGATGGAA	157	92	Fat	1/100
		Reverse	TGACACCATTTCATGACACATTC	ACCACCAT	Exon 3/4		
<i>GHRT</i>	NM_176608	Forward	CCATGGTTCTTAATTATTATCTTTGGA	117	105	Fat	1/50
		Reverse	TGGGGGTAGAATCAGCATCT	CTTGGGCT	Exon 7/8	Liver	1/100
<i>GHR1A</i>	AY748827	Forward	TCCAGCCTCTGTTTCAGGAG	120	64	Liver	1/10
		Reverse	GCTGCCAGAGATCCATACCT	CTGTGGGA	Exon 1A/2		
<i>GHR1B</i>	NM_176608	Forward	GCTCGCAGGTCCACAGGT	59	109	Fat	1/10
		Reverse	GACAAGGAAAGCTGGTGTGG	CAGTGGCA	Exon 1B/2		
<i>GPAM</i>	NM_001012282	Forward	TGCCACCAACGTAACGTAAA	150	65	Fat	1/100
		Reverse	CCACTTCTGCAATTGCCTCT	GAACAGCA	Exon 4/5		
<i>GPD1</i>	NM_001035354	Forward	TGTAGGCTCTGGCAACTGG	26	86	Fat	1/100
		Reverse	TCACCCGTGGGTCAAAGT	CAGCCCAG	Exon 1/2		
<i>IGFALS</i>	NM_001075963.1	Forward	GCGCAGGTGATGTTGTTGTA	28	60	Liver	1/50
		Reverse	GGCAGAGGGTAACGACGAC	GCGGCTGG	bp 68–127 Exon 1		
<i>IGFBP3</i>	NM_174556	Forward	GCGCCCTTACCTGCTACC	114	86	Liver	1/50
		Reverse	CAGCCTGGTTCTCTGTGCT	GACCACAG	Exon 1/2		
<i>IGF1</i>	NM_001077828	Forward	ATGTACTGCGCGCCTCTC	104	114	Fat	1/10
		Reverse	CCCTCTACTTGTGTTCTTCAAATG	GTGCCCAG	Exon 3/4	Liver	1/100
<i>INSRB</i>	AJ320235	Forward	TGCACAACGTGGTTTTCATC	124	75	Liver	1/100
		Reverse	GTTTCCTCGAAGGCCTAGC	CTTCAGGC	Exon 7/8		
<i>LIPE</i>	NM_001080220	Forward	CGAGATCCAGGTGCTATCGT	87	106	Fat	1/50
		Reverse	CGAGGTCAGAGGCATTTCA	CTGCCACC	Exon 4/5		
<i>LPL</i>	NM_001075120	Forward	GACAGGATGTGGCCAAGTTT	158	61	Fat	1/100
		Reverse	TTGCCCAGGGGATAGTTAAA	TGGATGGC	Exon 3/4		
<i>OXCT1</i>	NM_001076070	Forward	GCAAAGCTGCAGAAACCAC	22	61	Fat	1/10
		Reverse	GCAAATGATCCAATATCGACAA	TGGTGGAG	Exon 2/3		
<i>PCK1</i>	NM_174737	Forward	TGGATGAAATTTGACCAACAAG	147	94	Fat	1/10
		Reverse	GATTTGTCCTCACAGAGGTTCC	GCCATCAA	Exon 5/6		

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	Target tissue	Dilution of cDNA
<i>PLIN1</i>	NM_001083699	Forward	CCCTCCAGTATCCTCCTGAAA	161 GCTGAAAGG	90 Exon 3/4	Fat	1/50
<i>PNPLA2</i>	NM_001046005	Reverse	GCACACTGATGCTGTTCCCTG	27 CGGTGCTCTTCAACCATCC	64 Exon 7/8	Fat	1/50
<i>PPARG</i>	NM_181024	Reverse	ATCCACCGGATGCTCTCAG	165 GCTGCCTG	105 Exon 6/7	Fat	1/10
<i>SCD</i>	NM_173959	Forward	GCGACTTAGCAATATTATAGCTGTC	9 GAAGCCCA	67 Exon 4/5	Fat	1/100
<i>THRSP</i>	AY656814	Reverse	AGGCTTGACGACGATTTGCT	8 TGTTGATG	71 Exon 1/2	Fat	1/10

¹*RPS15A* = ribosomal protein S15a; *YWHAZ* = tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; *ACTB* = β -actin; *MRPL39* = mitochondrial ribosomal protein L39; *ACACA* = acetyl-CoA carboxylase α ; *ACSL1* = acyl-CoA synthetase long-chain family member 1; *ANGPTL4* = angiotensin-like 4; *CPT2* = carnitine palmitoyltransferase 2; *ELOVL5* = ELOVL family member 5, elongation of long-chain FA; *FABP4* = FA-binding protein 4; *FASN* = FA synthase; *GHRT* = growth hormone receptor; *GHR1A* = growth hormone receptor 1a; *GHR1B* = growth hormone receptor 1b; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *GPII* = glycerol-3-phosphate dehydrogenase 1 (soluble); *IGFALS* = insulin-like growth factor acid labile subunit; *IGFBP3* = insulin-like growth factor-binding protein 3; *INSRB* = insulin receptor b; *LIPE* = hormone sensitive lipase; *LPL* = lipoprotein lipase; *OXCT1* = 3-oxoacid CoA transferase 1; *PCK1* = phosphoenolpyruvate carboxykinase 1 (soluble); *PLIN1* = perilipin; *PNPLA2* = patatin-like phospholipase domain containing 2; *PPARG* = peroxisome proliferator-activated receptor gamma; *SCD* = stearoyl-CoA desaturase; *THRSP* = thyroid hormone responsive (SPOT14 homolog, rat).

²Primer sequences are shown 5' to 3'.

³UPL = Universal Probe Library (Roche Diagnostics Deutschland GmbH, Mannheim, Germany).

included as fixed effects, and cow, the linear trend of time within cow, spline, and the interaction of milking frequency and duration with spline were included as random effects. As treatment (milking frequency or duration or both) by time interactions ($P < 0.05$) were present, milk production, BCS, and plasma hormone and metabolite data were also analyzed at wk 1, 3, 6, and 9 using mixed models fitted with REML in GenStat, including treatment and contrasts to test milking frequency, duration, and their interaction as fixed effects, and cow as a random effect; t -tests were used for individual treatment comparisons if significant milking frequency by duration interactions were detected. Data for GH, insulin, and IGF-1 were logarithmically transformed before statistical analyses. Body condition score at 1 wk prepartum (mean $\pm$ SD; 5.1 ± 0.5 units) was used as a covariate for analysis of BCS data.

Data are presented as means with the standard error of the difference between the means (**SED**) for each treatment and time point. The $SED \times \sqrt{0.75}$ should be used to compare the mean of $1 \times$ or $3 \times$ treatments with each other and the $2 \times$ control. Differences in data were considered significant when $P < 0.05$ and declared a trend when $P < 0.10$.

Gene Expression Data. Data for each gene were logarithmically transformed before statistical analyses. Adipose gene expression data measured at 3 time points (wk 3, 6, and 9) were analyzed using a repeated-measures analysis as a mixed model in GenStat software, with cow as the random effect, and repeated measurements within cow modeled using a compound symmetry covariance structure including the fixed effects of time (wk), treatment, and their interaction. For most of the genes investigated, significant ($P < 0.05$) interactions were detected between treatment and time. Therefore, further statistical analyses were then undertaken to explore the immediate effect of milking frequency at wk 3, the interaction between milking frequency and duration at wk 6, and the carryover effects of both milking frequency and milking frequency by duration at wk 9 when all cows had been switched to $2 \times$ milking. Adipose gene expression data at each time point were analyzed using mixed models fitted with REML in GenStat, including treatments and contrasts to test milking frequency, duration, and their interaction as fixed effects, and cow as a random effect. Liver gene expression data at each of the 4 time points (wk 1, 3, 6, and 9) were also analyzed using this same model for consistency. Repeated-measures analysis for liver gene expression data was not undertaken because liver biopsies were not collected at wk 1 from cows in the $1 \times$ for 6 wk and $3 \times$ for 6 wk treatments as described in the Tissue Sampling section above.

Table 2. Effect of milking frequency (MF) on milk production, BCS, and plasma concentrations of hormones and metabolites in cows milked once daily for 3 or 6 wk immediately postpartum (1×; n = 60), twice daily for the duration of lactation (2×; n = 30), or 3 times daily for 3 or 6 wk immediately postpartum (3×; n = 60)¹

Variable	Week	Treatment						P-value					MF × duration
		1× for 6 wk	1× for 3 wk	2×	3× for 3 wk	3× for 6 wk	SED ²	1× vs. 2×	2× vs. 3×	1× vs. 3×	Duration		
Milk (kg/d)	1	16.2	17.0	20.0	21.2	20.4	0.9	<0.001	0.34	<0.001			
	3	19.7	21.0	23.9	26.2	26.0	0.9	<0.001	<0.01	<0.001			
	6	20.5	24.9	25.9	26.9	28.3	1.1	<0.001	0.08	<0.001	0.05	<0.001	
	9	25.4	26.0	26.8	28.6	28.4	1.1	0.25	0.07	<0.001	0.60	0.80	
ECM	1	19.0	19.9	23.9	24.0	23.9	1.2	<0.001	0.97	<0.001			
	3	21.3	23.3	27.0	27.6	27.5	1.1	<0.001	0.55	<0.001			
	6	21.3	26.4	28.2	28.0	28.1	1.1	<0.001	0.88	<0.001	<0.01	<0.01	
	9	26.3	27.5	29.1	29.6	29.6	1.1	<0.05	0.58	<0.001	0.48	0.46	
BCS ³	1	4.7	4.8	4.8	4.6	4.7	0.1	0.42	<0.05	0.14			
	3	4.6	4.6	4.5	4.4	4.3	0.1	0.20	0.06	<0.001			
	6	4.7	4.6	4.4	4.5	4.3	0.1	<0.05	0.60	<0.001	0.87	0.07	
	9/10	4.6	4.6	4.5	4.6	4.5	0.1	0.19	0.69	0.26	0.41	0.84	
Glucose (mmol/L)	1	3.81	3.78	3.58	3.42	3.34	0.10	<0.05	<0.05	<0.001			
	3	3.98	3.98	3.56	3.46	3.39	0.10	<0.001	0.12	<0.001			
	6	4.17	3.89	3.80	3.82	3.77	0.08	<0.001	0.92	<0.001	0.05	<0.01	
	9	4.04	4.16	4.06	4.07	3.89	0.08	0.58	0.25	<0.05	<0.05	0.53	
Growth hormone (log ₁₀ ng/mL)	1	0.25 (2.24)	0.36 (2.83)	0.26 (2.05)	0.33 (2.39)	0.35 (2.51)	0.06	0.41	0.12	0.38			
	3	0.15 (1.60)	0.22 (1.86)	0.19 (1.73)	0.30 (2.46)	0.28 (2.15)	0.06	0.96	<0.05	<0.01			
	6	0.17 (1.61)	0.25 (1.91)	0.25 (2.06)	0.21 (1.76)	0.37 (2.69)	0.05	0.30	0.42	0.05	0.31	<0.01	
	9	0.26 (1.96)	0.30 (2.16)	0.30 (2.28)	0.27 (2.07)	0.33 (2.27)	0.05	0.50	0.84	0.56	0.83	0.22	
Insulin (log ₁₀ μU/mL)	1	0.39 (2.79)	0.46 (3.19)	0.27 (2.15)	0.20 (1.77)	0.18 (1.84)	0.06	<0.01	0.14	<0.001			
	3	0.38 (2.73)	0.43 (3.51)	0.16 (1.52)	0.23 (1.81)	0.15 (1.56)	0.05	<0.001	0.55	<0.001			
	6	0.44 (3.36)	0.34 (2.38)	0.31 (2.21)	0.29 (2.12)	0.26 (1.98)	0.05	0.06	0.45	<0.01	0.30	0.07	
	9	0.37 (2.69)	0.47 (3.38)	0.35 (2.46)	0.39 (2.63)	0.32 (2.37)	0.05	0.14	0.92	0.09	<0.05	0.69	
IGF1 (log ₁₀ ng/mL)	1	0.86 (8.02)	0.84 (7.66)	0.89 (8.52)	0.88 (8.24)	0.84 (7.37)	0.05	0.34	0.46	0.80			
	3	0.94 (9.31)	0.92 (9.07)	0.81 (6.95)	0.85 (7.73)	0.83 (7.07)	0.04	<0.001	0.43	<0.01			
	6	0.98 (10.09)	0.94 (9.08)	0.91 (8.84)	0.89 (8.18)	0.88 (8.00)	0.04	0.19	0.51	<0.05	0.60	0.39	
	9	1.04 (12.50)	1.07 (12.82)	1.03 (11.82)	0.93 (9.70)	0.99 (10.46)	0.05	0.52	0.18	<0.05	0.70	0.27	
NEFA (mmol/L)	1	0.93	0.91	1.03	1.37	1.27	0.11	0.14	<0.01	<0.001			
	3	0.80	0.83	1.17	1.24	1.14	0.11	<0.001	0.79	<0.001			
	6	0.61	0.73	0.75	0.85	0.80	0.08	0.06	0.55	<0.001	0.06	0.27	
	9	0.43	0.48	0.45	0.49	0.49	0.10	0.50	0.12	0.26	0.37	0.62	

¹Growth hormone, insulin, and IGF-1 were logarithmically transformed for analysis (back-transformed values in parentheses). Milk production, ECM yield, BCS, and glucose, insulin, and NEFA concentrations have been reported previously (Grala et al., 2014).

²Standard error of the difference.

³Body condition score was on a 10-point scale. Body condition score at 1 wk prepartum (mean ± SD; 5.1 ± 0.5 units) was used as a covariate for analysis of BCS data.

In addition, *t*-tests were used for individual treatment comparisons if significant milking frequency by duration interactions were detected. Data are presented as means with the *SED* for each treatment and time point. The $SED \times \sqrt{0.75}$ should be used to compare the mean of 1× or 3× treatments with each other and the 2× control. Differences in data were considered significant when $P < 0.05$ and declared a trend when $P < 0.10$.

RESULTS

Milk Production, Body Condition, Plasma Hormone, and Metabolite Measurements

Milk production measures and BCS are described in Phyn et al. (2011); however, relevant data at wk 1, 3, 6, and 9 are presented in Table 2. In brief, daily milk and ECM yields were greater at all 4 time points in cows milked 3× postpartum relative to those milked 1×; however, although milk yield was also greater at wk 3 in cows milked 3× relative to 2×, and tended to be greater at wk 6 ($P = 0.08$) and wk 9 ($P = 0.07$), this effect was not detected for ECM yields, due to lower milk fat and protein contents (data not presented; Phyn et al., 2011). Furthermore, cows milked 1× postpartum had lower yields of milk and ECM at wk 1, 3, and 6 compared with cows milked 2×. At wk 6, milking frequency by duration interactions were detected, as cows that remained on 1× milking for 6 wk had lower milk and ECM yields relative to cows milked 1× for only 3 wk. Energy-corrected milk yields remained lower at wk 9 in cows previously milked 1× relative to 2×, but this negative carryover response was not affected by the duration of reduced milking frequency following parturition.

Body condition score at wk 1 postpartum was lower in cows milked 3× relative to those milked 2×. Cows milked 3× also had a lower BCS at wk 3 postpartum relative to cows milked 1×, and tended ($P = 0.06$) to have a lower BCS relative to cows milked 2×. By 6 wk, all cows milked 1× had a greater BCS than cows milked 2× or 3×; however, a trend ($P = 0.07$) for a milking frequency by duration interaction indicated that differences were greatest for cows milked 1× for 6 wk postpartum.

Plasma concentrations of glucose, insulin and NEFA are described in Grala et al. (2014) and are presented with IGF-1 and GH in Table 2 for clarity. Plasma concentrations of IGF-1 at wk 3 postpartum were greater in cows milked 1× relative to 2× or 3×, but at wk 6 and 9, cows milked 1× postpartum only had greater concentrations relative to those milked 3×. At wk 3 postpartum, concentrations of GH were greater in cows milked 3× relative to those milked 2×. At wk 6 post-

partum, a milking frequency by duration interaction indicated that cows milked 3× for 6 wk continued to have greater GH concentrations relative to all other treatment groups. Plasma NEFA concentrations at wk 1 were greater in cows milked 3× compared with those milked 2×. Cows milked 1× postpartum had lower NEFA at wk 3 and tended ($P = 0.06$) to have lower NEFA at wk 6 relative to cows milked 2×; however, relative to cows milked 3×, they had lower NEFA at wk 1, 3, and 6. Glucose concentrations were reduced in cows milked 3× at wk 1 relative to cows milked 2×; however, glucose and insulin concentrations were greater at wk 1 and 3 in cows milked 1×. At wk 6, milking frequency by duration interactions indicated that cows milked 1× for 6 wk continued to have greater glucose and insulin than cows milked 2× or 3×. At wk 9, glucose concentrations were still greater and insulin tended ($P = 0.09$) to be greater in cows previously milked 1× relative to cows previously milked 3×.

Somatotropic Axis Gene Expression

Liver. Expression values are presented in Table 3. At wk 3 postpartum, expression of total GHR (*GHRT*) and *GHR1A* in the liver was lower for cows milked 3× compared with 2× or 1×. Expression of *IGF1* and IGF acid labile subunit (*IGFALS*) was lower and IGF-binding protein 3 (*IGFBP3*) tended ($P = 0.06$) to be lower, whereas insulin receptor (*INSR*) B transcription was greater in the liver of cows milked 3× compared with those milked 1×.

Adipose. Expression values are presented in Table 4. At wk 6, *GHRT* expression in adipose tissue was lower in cows milked 3× postpartum compared with cows milked 2× or 1×. Expression of *GHR1B* also tended ($P = 0.06$) to be lower in cows milked 3× compared with 2×, and was significantly lower compared with 1×. A milking frequency by duration interaction was detected for *IGF1* transcription at wk 6 postpartum. Expression of *IGF1* was greater in cows milked 1× for 6 wk, compared with all other treatments, which did not differ from each other. At wk 9, milking frequency by duration interactions for *GHRT* and *GHR1B* expression occurred, such that cows previously milked 3× for 6 wk, but not 3 wk, had lower expression compared with cows milked 2×.

Adipose Lipolytic Gene Expression

Expression values are presented in Table 5. At wk 3, no treatment differences existed in the expression of the 3 main lipolytic genes measured [hormone-sensitive lipase (*LIPE*), perilipin 1 (*PLIN1*), and patatin-like phospholipase domain-containing protein 2 (*PNPLA2*)].

Table 3. Effect of milking frequency (MF) on hepatic mRNA expression of somatotrophic axis genes in cows milked once daily for 3 or 6 wk immediately postpartum (1×; n = 24), twice daily for the duration of lactation (2×; n = 12), or 3 times daily for 3 or 6 wk immediately postpartum (3×; n = 24)¹

Gene ²	Week	Treatment						P-value				
		1× for 6 wk	1× for 3 wk	2×	3× for 3 wk	3× for 6 wk	SED ³	1× vs. 2×	2× vs. 3×	1× vs. 3×	Duration	MF × duration
<i>GHRT</i>	1		0.74	0.70	0.69		0.10	0.68	0.83	0.54		
	3	0.69	0.70	0.67	0.57	0.57	0.05	0.72	0.05	<0.01		
	6	0.70	0.63	0.71	0.57	0.70	0.06	0.47	0.35	0.78	0.05	0.61
	9	0.69	0.75	0.66	0.65	0.67	0.07	0.40	0.98	0.33	0.70	0.42
<i>GHR1A</i>	1		0.88	0.95	0.95		0.23	0.73	0.90	0.83		
	3	1.11	1.10	1.09	0.77	0.93	0.09	0.91	<0.05	<0.01		
	6	1.08	1.04	1.14	0.99	1.12	0.09	0.36	0.42	0.92	0.31	0.53
	9	1.08	1.25	1.08	1.10	1.15	0.11	0.45	0.64	0.74	0.38	0.25
<i>IGF1</i>	1		1.65	1.70	1.79		0.27	0.82	0.73	0.58		
	3	1.89	1.84	1.67	1.43	1.50	0.13	0.12	0.12	<0.001		
	6	1.90	1.93	1.87	1.89	1.83	0.14	0.71	0.91	0.56	0.71	0.90
	9	1.85	2.16	1.85	1.97	1.98	0.16	0.30	0.41	0.83	0.18	0.22
<i>IGFBP3</i>	1		0.27	0.24	0.21		0.06	0.45	0.70	0.26		
	3	0.28	0.25	0.25	0.20	0.22	0.04	0.67	0.24	0.06		
	6	0.35	0.23	0.32	0.23	0.28	0.03	0.29	0.10	0.45	<0.01	0.22
	9	0.33	0.33	0.31	0.32	0.34	0.04	0.46	0.49	0.98	0.76	0.79
<i>IGFALS</i>	1		0.80	0.83	0.81		0.12	0.73	0.90	0.64		
	3	0.88	0.89	0.83	0.61	0.77	0.08	0.49	0.11	<0.01		
	6	0.87	0.86	0.94	0.77	0.86	0.07	0.29	0.14	0.56	0.46	0.55
	9	0.87	0.96	0.82	0.93	0.93	0.07	0.20	0.14	0.76	0.40	0.47
<i>INSRB</i>	1		0.26	0.29	0.27		0.05	0.48	0.75	0.31		
	3	0.26	0.25	0.28	0.31	0.32	0.04	0.39	0.36	<0.05		
	6	0.24	0.23	0.29	0.23	0.28	0.04	0.12	0.43	0.37	0.42	0.51
	9	0.26	0.25	0.27	0.24	0.31	0.04	0.70	0.67	0.33	0.24	0.34

¹Cows milked 1× or 3× were switched to 2× milking after 3 or 6 wk on the alternative milking frequency. Data are expressed as logarithmically transformed arbitrary units, normalized to the expression of ribosomal protein S15a and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide.

²*GHRT* = growth hormone receptor total; *GHR1A* = growth hormone receptor 1a; *IGF1* = insulin-like growth factor 1; *IGFBP3* = insulin-like growth factor-binding protein 3; *IGFALS* = insulin-like growth factor acid labile subunit; *INSRB* = insulin receptor b.

³Standard error of the difference.

Table 4. Effect of milking frequency (MF) on adipose mRNA expression of somatotrophic axis genes in cows milked once daily for 3 or 6 wk immediately postpartum (1×; n = 24), twice daily for the duration of lactation (2×; n = 12), or 3 times daily for 3 or 6 wk immediately postpartum (3×; n = 24)¹

Gene ²	Week	Treatment							P-value			MF × duration
		1× for 6 wk	1× for 3 wk	2×	3× for 3 wk	3× for 6 wk	SED ³	1× vs. 2×	2× vs. 3×	1× vs. 3×	Duration	
<i>GHRT</i>	3	0.79	0.74	0.80	0.77	0.82	0.08	0.60	0.94	0.59		
	6	0.78	0.76	0.69	0.60	0.53	0.08	0.23	<0.05	<0.001	0.67	0.36
	9	0.78	0.63	0.72	0.72	0.51	0.09	0.93	0.13	0.09	0.59	<0.001
<i>GHR1B</i>	3	0.90	0.78	0.83	0.84	0.84	0.08	0.85	0.08	0.91		
	6	0.91	0.73	0.68	0.52	0.51	0.10	0.11	0.06	<0.001	0.22	0.21
	9	0.81	0.62	0.75	0.71	0.50	0.12	0.79	0.11	0.11	0.81	<0.05
<i>IGF1</i>	3	0.43	0.36	0.36	0.37	0.31	0.07	0.50	0.73	0.23		
	6	0.69	0.39	0.37	0.47	0.50	0.08	<0.05	0.09	0.37	<0.001	<0.05
	9	0.59	0.63	0.61	0.65	0.55	0.08	0.99	0.79	0.74	0.22	0.53

¹Cows milked 1× or 3× were switched to 2× milking after 3 or 6 wk on the alternative milking frequency. Data are expressed as logarithmically transformed arbitrary units, normalized to the expression of β-actin and mitochondrial ribosomal protein L39.

²*GHRT* = growth hormone receptor total; *GHR1B* = growth hormone receptor 1b; *IGF1* = insulin-like growth factor 1.

³Standard error of the difference.

However, expression of angiopoietin-like 4 (*ANGPTL4*) was lower in cows milked 1× compared with cows milked 2× and tended ($P = 0.09$) to be lower compared with cows milked 3×.

At wk 6, expression of *LIPE* and *PLIN1* was greater in all cows milked 1× compared with all cows milked 3×. In addition, all cows milked 1× tended ($P = 0.07$) to have greater *LIPE* expression than cows milked 2×. A milking frequency by duration interaction was detected for *PNPLA2* expression at wk 6. Cows milked 1× for 6 wk had greater *PNPLA2* expression compared with cows milked 2× or 3×; however, cows milked 1× for 3 wk only had greater *PNPLA2* expression compared with cows milked 3× for 6 wk. Also at wk 6 postpartum, FA-binding protein 4 (*FABP4*) was downregulated in cows milked 3× compared with cows milked 2× or 1×.

At wk 9, all cows previously milked 3× had lower expression of *LIPE*, *PLIN1*, *PNPLA2*, and *ANGPTL4* compared with cows milked 2×. In addition, cows previously milked 3× had lower expression of *LIPE* and *PNPLA2*, and tended ($P = 0.07$) to have lower *PLIN1* expression compared with cows milked 1×. A trend ($P = 0.06$) existed for a milking frequency by duration interaction for *FABP4* expression, such that cows milked 3× for 6 wk had lower expression compared with cows milked 1× for 6 wk or 2×. Additionally, all cows previously milked 1× tended ($P = 0.09$) to have lower *ANGPTL4* expression compared with cows milked 2×.

Adipose β-Oxidation Gene Expression

Expression values are presented in Table 5. At wk 6 postpartum, milking frequency by duration interactions were detected for acyl-CoA synthetase long-chain family member 1 (*ACSL1*) and carnitine palmitoyltransferase 2 (*CPT2*). Expression of *ACSL1* was greater in cows milked 1× for 6 wk, and lower in cows milked 3× for 6 wk, compared with the other treatments, which did not differ from each other. Expression of *CPT2* was greater in cows milked 1× for 6 wk than all other treatments, which did not differ from each other.

At wk 9, expression of *ACSL1* was lower in all cows previously milked 3× compared with 1×. A milking frequency by duration interaction existed for *CPT2* and expression at wk 9 was greater in cows milked 1× for 6 wk, and lower in cows milked 3× for 6 wk, compared with other treatments, which did not differ from each other.

Adipose Lipogenic and Reesterification Gene Expression

Expression values are presented in Table 6. At wk 3, the transcription of genes involved with lipogenesis and

Table 5. Effect of milking frequency (MF) on adipose mRNA expression of lipolysis and β -oxidation genes in cows milked once daily for 3 or 6 wk immediately postpartum (1 $\times$; n = 24), twice daily for the duration of lactation (2 $\times$; n = 12), or 3 times daily for 3 or 6 wk immediately postpartum (3 $\times$; n = 24)¹

Gene ²	Week	Treatment						P-value				
		1 $\times$ for 6 wk	1 $\times$ for 3 wk	2 $\times$	3 $\times$ for 3 wk	3 $\times$ 6 wk	SED ³	1 $\times$ vs. 2 $\times$	2 $\times$ vs. 3 $\times$	1 $\times$ vs. 3 $\times$	Duration	MF $\times$ duration
<i>LIPE</i>	3	2.19	2.10	2.20	2.10	2.13	0.13	0.61	0.41	0.70		
	6	2.16	2.11	1.81	1.65	1.63	0.21	0.07	0.34	<0.001	0.93	0.83
	9	1.89	1.78	1.88	1.65	1.45	0.18	0.78	<0.05	<0.05	0.68	0.23
<i>PLIN1</i>	3	1.60	1.44	1.65	1.54	1.61	0.12	0.20	0.45	0.54		
	6	1.48	1.44	1.31	1.16	1.16	0.18	0.33	0.31	<0.05	0.87	0.87
	9	1.16	1.16	1.33	1.09	0.85	0.16	0.21	<0.01	0.07	0.29	0.28
<i>PNPLA2</i>	3	1.28	1.19	1.19	1.11	1.18	0.11	0.54	0.69	0.22		
	6	1.37	1.13	1.00	0.96	0.78	0.14	<0.05	0.23	<0.001	0.73	<0.05
	9	1.14	1.08	1.13	0.93	0.72	0.15	0.91	<0.05	<0.01	0.46	0.20
<i>ANGPTL4</i>	3	1.17	1.02	1.31	1.22	1.22	0.10	<0.05	0.28	0.09		
	6	1.16	1.23	1.19	1.13	1.00	0.14	0.97	0.26	0.15	0.31	0.77
	9	0.86	0.89	1.09	0.92	0.66	0.15	0.09	<0.05	0.35	0.18	0.29
<i>FABP4</i>	3	1.30	1.21	1.46	1.32	1.42	0.14	0.09	0.46	0.25		
	6	1.33	1.24	1.16	0.93	0.85	0.15	0.34	<0.05	<0.001	0.93	0.44
	9	1.10	1.00	1.10	1.01	0.79	0.12	0.71	<0.05	0.05	0.47	0.06
<i>ACSL1</i>	3	0.65	0.71	0.61	0.65	0.69	0.10	0.45	0.49	0.94		
	6	1.02	0.68	0.62	0.64	0.39	0.12	<0.05	0.22	<0.001	0.53	<0.001
	9	0.90	0.86	0.74	0.76	0.49	0.14	0.22	0.27	<0.01	0.24	0.12
<i>CPT2</i>	3	0.29	0.24	0.23	0.25	0.18	0.05	0.39	0.83	0.20		
	6	0.49	0.26	0.24	0.26	0.24	0.06	<0.01	0.89	<0.01	<0.01	<0.01
	9	0.43	0.33	0.31	0.34	0.23	0.04	0.05	0.45	<0.01	0.86	<0.01

¹Cows milked 1 $\times$ or 3 $\times$ were switched to 2 $\times$ milking after 3 or 6 wk on the alternative milking frequency. Data are expressed as logarithmically transformed arbitrary units, normalized to the expression of β -actin and mitochondrial ribosomal protein L39.

²*LIPE* = hormone-sensitive lipase; *PLIN1* = perilipin; *PNPLA2* = patatin-like phospholipase domain containing 2; *ANGPTL4* = angiotensin-like 4; *FABP4* = FA-binding protein 4; *ACSL1* = acyl-CoA synthetase long-chain family member 1; *CPT2* = carnitine palmitoyltransferase 2.

³Standard error of the difference.

Table 6. Effect of milking frequency (MF) on adipose mRNA expression of lipogenic genes in cows milked once daily for 3 or 6 wk immediately postpartum (1×; n = 24), twice daily for the duration of lactation (2×; n = 12), or 3 times daily for 3 or 6 wk immediately postpartum (3×; n = 24)¹

Gene ²	Week	Treatment						P-value				
		1× for 6 wk	1× for 3 wk	2×	3× for 3 wk	3× for 6 wk	SED ³	1× vs. 2×	2× vs. 3×	1× vs. 3×	Duration	MF × duration
<i>ACACA</i>	3	0.90	0.93	0.57	0.54	0.56	0.13	<0.01	0.84	<0.001		
	6	1.37	0.84	0.57	0.67	0.46	0.13	<0.001	0.87	<0.001	0.07	<0.0001
	9	1.32	1.23	1.02	0.96	0.70	0.16	0.06	0.14	<0.001	0.44	0.13
<i>ELOVL5</i>	3	0.63	0.73	0.52	0.47	0.48	0.09	<0.05	0.59	<0.01		
	6	1.02	0.66	0.49	0.50	0.52	0.09	<0.001	0.73	<0.001	<0.01	<0.05
	9	0.94	0.88	0.68	0.79	0.60	0.11	<0.05	0.94	<0.01	0.40	0.09
<i>FASN</i>	3	1.46	1.52	0.64	0.78	0.63	0.19	<0.001	0.73	<0.001		
	6	2.23	1.50	1.07	1.15	1.02	0.19	<0.001	0.95	<0.001	<0.05	<0.01
	9	2.10	2.02	1.86	1.83	1.48	0.19	0.21	0.15	<0.01	0.29	0.10
<i>GPAM</i>	3	1.25	1.13	0.78	0.84	1.04	0.15	<0.01	0.20	<0.05		
	6	1.65	1.01	0.81	0.73	0.74	0.15	<0.001	0.57	<0.001	<0.01	<0.01
	9	1.45	1.26	1.21	1.10	0.82	0.19	0.33	0.10	<0.01	0.72	0.08
<i>GPD1</i>	3	1.30	1.22	0.90	0.94	0.91	0.15	<0.01	0.86	<0.01		
	6	1.67	1.18	1.09	1.03	0.73	0.14	<0.01	0.07	<0.001	0.29	<0.0001
	9	1.55	1.37	1.44	1.36	1.06	0.15	0.82	0.06	<0.05	0.57	<0.05
<i>LPL</i>	3	0.81	0.86	0.45	0.49	0.35	0.12	<0.001	0.75	<0.001		
	6	1.17	0.92	0.55	0.50	0.51	0.11	<0.001	0.67	<0.001	0.10	0.14
	9	1.10	1.00	0.92	0.84	0.68	0.14	0.26	0.15	<0.01	0.73	0.19
<i>OXCT1</i>	3	0.55	0.55	0.44	0.43	0.35	0.06	<0.05	0.31	<0.001		
	6	0.65	0.45	0.37	0.42	0.33	0.07	<0.01	0.99	<0.001	0.24	<0.01
	9	0.77	0.58	0.60	0.59	0.38	0.08	0.26	0.07	<0.001	0.80	<0.001
<i>PCK1</i>	3	1.81	1.63	1.65	1.77	1.55	0.24	0.69	0.97	0.67		
	6	2.23	1.35	1.61	1.60	1.21	0.26	0.43	0.31	<0.05	0.16	<0.01
	9	1.61	1.26	1.50	1.25	1.05	0.26	0.82	0.10	0.09	0.71	0.14
<i>PPARG</i>	3	1.24	1.17	1.19	1.16	1.22	0.10	0.84	0.95	0.873		
	6	1.26	1.11	0.96	0.81	0.89	0.13	<0.05	0.36	<0.001	0.22	0.70
	9	1.12	1.01	1.02	0.93	0.85	0.13	0.65	0.25	0.06	0.88	0.30
<i>SCD</i>	3	1.11	1.21	0.80	0.61	0.70	0.19	<0.05	0.42	<0.001		
	6	1.74	1.15	0.86	0.87	0.66	0.18	<0.001	0.48	<0.001	0.13	<0.01
	9	1.66	1.65	1.41	1.53	1.04	0.18	0.11	0.26	<0.01	0.07	0.05
<i>THRSP</i>	3	1.45	1.53	1.06	1.10	0.99	0.19	<0.05	0.91	<0.01		
	6	2.03	1.54	1.04	1.10	0.85	0.20	<0.001	0.65	<0.001	0.39	<0.05
	9	1.91	1.85	1.65	1.66	1.14	0.21	0.21	0.11	<0.01	0.12	0.06

¹Cows milked 1× or 3× were switched to 2× milking after 3 or 6 wk on the alternative milking frequency. Data are expressed as logarithmically transformed arbitrary units, normalized to the expression of β -actin and mitochondrial ribosomal protein L39.

²*ACACA* = acetyl-CoA carboxylase α ; *ELOVL5* = ELOVL family member 5, elongation of long-chain FA; *FASN* = FA synthase; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *GPD1* = glycerol-3-phosphate dehydrogenase 1 (soluble); *LPL* = lipoprotein lipase; *OXCT1* = 3-oxoacid CoA transferase 1; *PCK1* = phosphoenolpyruvate carboxykinase 1 (soluble); *PPARG* = peroxisome proliferator-activated receptor gamma; *SCD* = stearoyl-CoA desaturase; *THRSP* = thyroid hormone responsive (SPOT14 homolog, rat).

³Standard error of the difference.

reesterification [lipoprotein lipase (*LPL*), acetyl-CoA carboxylase α (*ACACA*), FA synthase (*FASN*), stearoyl-CoA desaturase (*SCD*), elongation of long-chain FA member 5 (*ELOVL5*), glycerol-3-phosphate acyltransferase (*GPAM*), glycerol-3-phosphate dehydrogenase 1 (*GPD1*), 3-oxoacid CoA transferase 1 (*OXCT1*), and thyroid hormone responsive (*THRSP*)] was greater in cows milked 1 $\times$ compared with 2 $\times$ or 3 $\times$.

At wk 6, there were many milking frequency by duration interactions. Cows milked 1 $\times$ for 6 wk had greater expression of *ACACA*; *ELOVL5*; *FASN*; *GPAM*; *GPD1*; *OXCT1*; phosphoenolpyruvate carboxykinase 1, soluble (*PCK1*), *SCD*; and *THRSP* compared with all other treatments. Additionally, at wk 6, cows milked 1 $\times$ for 3 wk had greater expression of *ACACA*, *FASN*, and *THRSP* than cows milked 2 $\times$ and greater *ACACA*, *FASN*, *SCD*, and *THRSP* expression than cows milked 3 $\times$ for 6 wk. Furthermore, expression of *GPD1* was lower in cows milked 3 $\times$ for 6 wk compared with all other treatments. At wk 6, there were no interactions between milking frequency and duration for *LPL* or *PPARG*, and expression of *LPL* and *PPARG* was greater in all cows milked 1 $\times$ relative to 2 $\times$.

At wk 9, greater *ACACA*, *FASN*, and *LPL* expression was detected, and trends ($P = 0.09$ and $P = 0.06$, respectively) for greater *PCK1* and *PPARG* expression were detected in all cows previously milked 1 $\times$ compared with 3 $\times$. Cows previously milked 1 $\times$ also tended ($P = 0.06$) to have greater *ACACA* expression relative to those milked 2 $\times$ from calving. Milking frequency by duration interactions existed for *GPD1*, *OXCT1*, and *SCD*, and trends ($P = 0.09$, $P = 0.08$, and $P = 0.06$, respectively) for interactions existed for *ELOVL5*, *GPAM*, and *THRSP*. Cows previously milked 3 $\times$ for 6 wk had lower expression of *GPD1*, *SCD*, *GPAM*, and *THRSP* compared with all other treatments. Expression of *OXCT1* was greater in cows previously milked 1 $\times$ for 6 wk, and lower in cows previously milked 3 $\times$ for 6 wk, compared with all other treatments. Cows previously milked 1 $\times$ for 6 wk had greater expression of *ELOVL5* than cows previously milked 2 $\times$ or 3 $\times$ for 6 wk; however, cows milked 1 $\times$ for 3 wk postpartum only had greater *ELOVL5* expression compared with cows milked 3 $\times$ for 6 wk.

DISCUSSION

Differences in the plasma measurements and transcription of genes involved in the somatotrophic axis and lipid metabolism indicate that increased milking frequency postpartum increases energy demands to sustain galactopoiesis, whereas these adaptations are required to a lesser degree in cows milked 1 $\times$. The increase in mammary energy output in cows milked 3 $\times$

requires carefully coordinated homeorhetic adjustments across different body tissues.

Greater Somatotrophic Axis Uncoupling Occurred in Cows Milked 3 $\times$

Reduced hepatic transcription of *GHR1A* at wk 3 postpartum in cows milked 3 $\times$ is indicative of greater somatotrophic axis uncoupling compared with cows milked less frequently. Reduced *GHR1A* would decrease the sensitivity of the liver to GH signaling, therefore modulating the transcription of *GHR1A*, *IGF1*, *IGFALS*, and *IGFBP3* (Jiang et al., 2007; Vijayakumar et al., 2010). This expression modulation explains the reduced transcription of these genes in the liver of cows milked 3 $\times$. The increased plasma GH and decreased IGF-1 concentrations in cows milked 3 $\times$ support the gene transcription measures and indicate that the somatotrophic axis of 3 $\times$ cows was more uncoupled, particularly compared with 1 $\times$ cows. Although plasma GH was only measured weekly and GH is secreted in a pulsatile fashion, the GH changes in the current study support the gene expression results and plasma IGF-1 changes, consistent with previous studies using weekly plasma GH measurements in this manner (Kobayashi et al., 1999; Grala et al., 2011).

It has been reported that insulin is involved in stimulating *GHR1A* transcription, and, hence, recoupling of the somatotrophic axis (Butler et al., 2003). In the present study, insulin concentrations and expression of *GHR1A*, *IGF1*, and *IGFALS* in the liver were similar at wk 6 postpartum between cows milked 3 $\times$ and those milked 2 $\times$. By wk 9, no treatment effects existed, indicating that the somatotrophic axis was recoupled and the effect of postpartum milking frequency was transient. The somatotrophic axis is also altered by nutritional status (McGuire et al., 1995), and due to the lack of individual feed intake data in the current study, differences in nutrition may have contributed to the uncoupling despite the generous pasture allowance and supplementary feed provided.

Cows milked 3 $\times$ had greater BCS loss during the first week postpartum than cows milked 2 $\times$ (Phyn et al., 2011), indicating that the adipose tissue of these cows was initially responsive to the effects of GH and more body fat was mobilized to support the mammary gland's greater energy requirements for milk production. This was confirmed by the increase in NEFA concentrations at this time. These effects, however, were short lived, as NEFA concentrations did not differ between cows milked 3 $\times$ and 2 $\times$ thereafter and BCS converged from wk 4 onwards (Phyn et al. 2011), indicating that in cows milked 3 $\times$, mobilization of subcutaneous fat stores was not greater after wk-1 postpartum. This re-

sult is unexpected considering that cows milked 3× had elevated plasma GH concentrations at wk 3 postpartum, and those animals that remained on 3× milking had greater GH concentrations at wk 6. However, by wk 6 postpartum, adipose tissue transcription of *GHRT* was reduced and the main variant in adipose, *GHR1B*, tended to be reduced in cows milked 3× compared with 2×, potentially indicating some resistance to GH signaling in response to 3× daily milking.

Reduced *GHRT* expression after 6 wk of 3× milking may result from negative feedback from reduced adipose stores, as downregulation of *GHR* expression in adipose tissue would limit further efflux of FFA, thereby preserving lipid reserves and preventing excessive body fat mobilization during early lactation. Indeed, NEFA concentrations at wk 3 and 6 were similar between cows milked 2× and 3×, indicating that net export of FA from adipose reserves was also similar and, therefore, in cows milked 3×, the export of FA was not greater after wk-1 postpartum. In adipose-specific *GHR* KO mice models, adiposity was increased (List et al., 2013), supporting the hypothesis that reduced *GHR* mRNA expression would minimize body fat mobilization. The *GHR* abundance in adipose tissue after 2 wk of negative energy balance has been studied in cows with no effect on *GHR* mRNA, but reductions in *GHR* protein expression have been reported (Rhoads et al., 2007). This indicates that *GHR* expression is altered by changes to energy balance and the differences in *GHR* mRNA expression between the study of Rhoads et al. (2007) and the current study may be due to the length or severity of the negative energy balance.

Furthermore, the decreased adipose transcription of *GHR* at wk 6 and lack of continued BCS loss in cows milked 3× relative to 2× in the current study may be a result of the pasture-based farming system and the genetics of the New Zealand strain of Holstein-Friesian cow. Milking these grazing cows 3× for 6 wk postpartum did not increase milk production beyond what was achieved in cows milked 3× for only 3 wk (Phyn et al., 2011), indicating that a production ceiling was reached in animals genetically selected for and managed in a pasture-based system where excessive body fat mobilization is detrimental to maintain an annual, seasonally concentrated breeding cycle.

Lipolysis and Lipogenesis in Cows Milked 3×

Increased adipose tissue lipolysis and FA export in cows milked 3× relative to 2× was not evident from gene transcription measured at wk 3 and 6, despite these cows having an increased milk yield and lower BCS during the first 3 wk of lactation (Phyn et al., 2011). This finding may simply reflect that the greatest

differences in BCS loss and plasma NEFA concentrations between cows milked 3× and 2× occurred at wk 1 postpartum when lipolytic gene expression was not measured. Nevertheless, authors measuring actual rates of lipolysis in bovine adipose tissue (McNamara and Hillers, 1986; Sumner and McNamara, 2007; Rocco and McNamara, 2013) have not reported high concordance with mRNA expression, which they suggest is due to posttranslational control of lipolysis. Additionally, gene expression studies over the transition period indicate only minor lipolytic gene expression changes during early lactation in combination with increased NEFA concentrations (Khan et al., 2013), consistent with the results presented here, indicating that mRNA abundance may not be a good indicator of lipolysis. It is also possible that the lack of an increase in lipolytic mRNA expression may be due to the depot of adipose tissue biopsied, as the subcutaneous depot may be less responsive to GH signaling than omental depots (Ermann et al., 2011).

Instead, gene expression data in the current study indicates that cows milked 3× for 6 wk may have increased nutrient availability for milk production by downregulating genes involved in de novo FA synthesis and reesterification. This downregulation was maintained at wk 9, after cows had been switched to 2× milking for 3 wk, indicating delayed adaptation to the new regimen. Lipogenic gene transcription decreases during early lactation (Janovick et al., 2009; Khan et al., 2013), indicating that milk production limits adipose lipogenesis. Additionally, all cows previously milked 3× had similar plasma concentrations of NEFA at wk 3, 6, and 9 to cows milked 2×, but had reduced lipolytic gene transcription at wk 9. This result may indicate inhibition of lipolysis in cows with less tissue reserves to mobilize, or a reduction in lipolysis because of the reduced energy required for milk production after cows are switched to 2× milking. Further work is required to explore the inhibition of lipolysis at this time. In general, however, cows milked 3× were able to partition more energy for milk production through reducing lipogenic gene transcription in adipose tissue.

Lipogenesis Is Greater in Cows Milked 1× at 3 wk Postpartum

All 10 lipogenic and reesterification genes measured in adipose tissue were greater in cows milked 1×, compared with cows milked 2× or 3×, both during and after the period of 1× milking. Increased lipogenesis and reesterification relative to similar rates of lipolysis would encourage the formation of adipose stores in cows milked 1×. Lipolytic gene expression was not altered at 3 wk but plasma NEFA concentrations were

reduced in cows milked 1×, indicating reduced lipolysis, or increased reesterification of FA compared with cows milked 2× and 3×. In the current study, transcription of *PPARG* was also greater at 6 wk in all cows milked 1× postpartum, and adipose *PCK1* transcription was greater in cows milked 1× for 6 wk compared with cows milked 1× for 3 wk or 2×, indicating an increase in glycerol-3-phosphate production for FA esterification into TAG. This process protects the animal against excess FA and subsequent insulin resistance (Beale et al., 2007). Increased transcription of *PPARG* at this time may have enhanced the transcription of *PCK1* through the *PPARG*-binding site in the promoter region of *PCK1* (Millward et al., 2010).

Consistent with an effect of 1× milking on increasing adipose storage of TAG, NEFA concentrations in plasma were lower and insulin concentrations greater in cows milked 1× compared with 2× or 3×. An increased capability to store TAG may be a priority pathway in cows milked 1× in response to their lower energy requirements for milk production by the mammary gland.

Lipolysis and FA Oxidation Are Greater in Cows Milked 1×

Interestingly, at wk 6, all cows currently or previously on 1× milking had greater basal and stimulated lipolytic gene transcription relative to cows milked 3×. This effect indicates that cows milked 1×, once switched to 2×, may increase lipolytic gene expression to cope with increased energy demand from the greater milk production (McNamara, 1991), or to regulate adiposity (Kennedy, 1953). Alternatively, the enhanced lipolysis and reesterification may be important in the fine-tune regulation of metabolic demands (Kalderon et al., 2000).

Lipolysis of TAG results in reesterification of part of the FA released, back into TAG (Hammond and Johnston, 1987; Wang et al., 2003), resulting in a futile TAG/FA cycle. This cycle requires ATP (Baldwin, 1970), which can be supplied by glucose oxidation (Flatt, 1970), de novo lipogenesis from glucose, or FA oxidation (Flachs et al., 2013). However, in lactating ruminants, glucose is preferentially used for milk production (Aschenbach et al., 2010); therefore, energy is not likely supplied from glucose. Transcription of *ACSL1* and *CPT2* were both greater at wk 6 in cows still being milked 1×, indicating increased FA oxidation compared with cows that were being milked 2× or 3× at that time point. Although white adipose tissue is not traditionally thought to have high oxidative capacity, recent research has identified that the activation of lipolysis associates with stimulation of FA oxidation (Cho et al., 2009). It is possible that the metabolic

flexibility of adipocytes is increased in cows milked 1× for 6 wk due to increased futile cycling possible through increased oxidation. This, however, is not a mechanism desired in a dairy cow, as energy is being utilized in a futile manner, rather than for milk production, which may reduce overall animal efficiency.

CONCLUSIONS

Temporary alterations to milking frequency immediately postpartum have immediate and short-term carryover effects on the metabolism of dairy cows. Cows milked 1× had greater rates of lipogenesis, reesterification, lipolysis, and FA oxidation during 1× milking, and greater lipogenesis and FA oxidation after treatments. Cows milked 3× had greater somatotrophic axis uncoupling in the liver during treatment and cows milked 3× for 6 wk had reduced *GHR* mRNA expression in adipose tissue at 6 wk postpartum. Changes to milking frequency in early lactation altered metabolism during and after the change as the transcription of key genes was modified to support nutrient supply for milk production as well as maintaining adipose stores.

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