Once-daily milking during late lactation in pasture-fed dairy cows has minor effects on feed intake, body condition score gain, and hepatic gene expression

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ABSTRACT

Milking cows once daily (1×) is a management practice occasionally used during mid/late lactation in pasture-based systems. It has been postulated that 1× milking will reduce dry matter intake (DMI) and increase body condition score (BCS) gain; however, this has not been quantified. Lactating, pregnant Holstein-Friesian dairy cows ($n = 52$) were allocated to either 1× or twice-daily (2×) milking in mid-January (summer, 175 d in milk). To obtain accurate DMI measurements, cows underwent 4 periods in a Calan gate indoor feeding facility, interspersed with grazing outdoors. Milk production, body weight (BW), and BCS were recorded 2 wk before treatment start (−2 wk) and weekly thereafter. Blood variables were recorded at −2 wk and weekly when indoors. Liver was biopsied at −2, 2, and 10 wk, and hepatic gene expression measured using quantitative PCR. Milking cows 1× tended to lower DMI (17.8 vs. 18.2 kg of dry matter), but increased BCS gain (0.36 vs. 0.13 BCS units) and BW (546 vs. 533 kg) at wk 12 relative to 2×. The greater BCS and BW of cows milked 1× compared with 2× were reflected in lower plasma concentrations of nonesterified fatty acids and lower transcription of genes involved in the oxidation of fatty acids, indicating reduced release and processing of fatty acids. Cows milked 1× produced 20% less milk, and although milk fat and protein concentrations were increased relative to cows milked 2×, yields of fat and protein were 14 and 17% less, respectively. The reduction in milk production with 1× milking (14.1 vs. 16.8 kg/cow per d energy-corrected milk) was accompanied by increases in blood concentrations of glucose and insulin, with a concurrent decrease in the transcription of the insulin receptor and gluconeogenic genes. These results indicate a coordinated response to reduce glu-

cose production due to decreased mammary demand. Expression of 2 genes linked to inflammation and adipokine signaling was reduced in cows milked 1× and may indicate a lower inflammatory state in the liver of cows milked 1× in late lactation. No effect was found of milking frequency during late lactation on milk production in the subsequent lactation. In summary, although 1× milking tended to reduce DMI and increase BCS in late lactation, these effects were lower than what is commonly supposed in pasture-based dairy systems. The modest BCS gains need to be considered with the reduced milk production when adopting 1× milking as a management strategy.

Key words: milking frequency, dry matter intake, once-daily milking, body condition score

INTRODUCTION

In pasture-based dairy systems, cows are usually milked twice daily (2×). However, milking cows once daily (1×) in late lactation is a strategy occasionally used by farmers to reduce the amount and cost of labor associated with milking (Clark et al., 2006; Bewsell et al., 2008; Stelwagen et al., 2013), to reduce feed demand, to increase BCS, or a combination of these. In particular, because the success of a pasture-based dairy system is reliant on low input costs, 1× milking in late lactation has become increasingly popular in these production systems because it is a low-cost strategy to help achieve calving BCS targets that optimize milk production and reproduction during the subsequent lactation (O'Driscoll et al., 2010; Douglas et al., 2011).

The effect of 1× milking in late lactation on BW or BCS, DMI, and nutrient partitioning is, however, not well characterized, despite research consistently indicating a decrease in milk production relative to 2× milking (Lynch et al., 1991; O'Brien et al., 2002; Ferris et al., 2008). This lower milk production in cows milked 1× should improve energy status and BCS gain if feed intake is not reduced to the same degree. In grazing trials, where individual DMI was not measured, the BW

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of cows milked 1× was 13 to 15 kg greater than the BW of cows milked 2× (Lynch et al., 1991; O'Brien et al., 2002); however, BCS was either not affected (O'Brien et al., 2002) or not measured (Lynch et al., 1991). In an Irish study, Ferris et al. (2008) used a Calan gate feeding system and reported that housed, grass-silage- and concentrate-fed cows milked 1× did not differ from their 2× counterparts in DMI, BW, or BCS. Despite this research, DMI and BCS changes in pasture-fed cows milked 1× during late lactation have not been accurately quantified.

We have previously reported improvements in the metabolic status of grazing cows when milked 1× either immediately postcalving or during a period of feed restriction in early lactation (Kay et al., 2013; Phyn et al., 2014). Plasma concentrations of glucose and insulin were greater, and nonesterified fatty acids (NEFA) were reduced, in cows milked 1× compared with those milked 2× (Kay et al., 2013; Phyn et al., 2014), indicating a more positive energy balance. Additionally, hepatic expression of genes involved in gluconeogenesis (glucose-6-phosphatase catalytic subunit, *G6PC*; phosphoenolpyruvate carboxykinase 1, *PCK1*), fatty acid β -oxidation (carnitine palmitoyltransferase 1A, *CPT1A*; carnitine palmitoyltransferase 2, *CPT2*), and ketogenesis (3-hydroxy-3-methylglutaryl-CoA synthase 2, *HMGCS2*), were lower in cows milked 1×, supporting the positive metabolic effects reported (Grala et al., 2014a). However, to our knowledge, no published studies are available investigating the underlying metabolic and physiological response to 1× milking during late lactation, which may not be as great due to the smaller decrease in milk production at this stage of lactation.

We have also previously reported that short periods of 1× milking during early lactation had short-term positive carry-over effects on metabolic status once cows were switched to 2× milking as plasma glucose and insulin concentrations remained greater and NEFA concentrations remained lower (Phyn et al., 2014). However, only one published study has measured carry-over effects of 1× milking in late lactation (Ferris et al., 2008). In this experiment, 1× milking in late lactation did not affect milk production in the subsequent lactation when cows were milked 2×. Therefore, although it is unlikely that 1× milking in late lactation will have positive effects on blood hormone and metabolite indicators of metabolic status in the subsequent lactation, this hypothesis has not been examined in pasture-based systems.

The effects of 1× milking in late lactation on milk production, BCS, and DMI need to be quantified to enable farmers to plan feed budgets, cash flows, and manage cows to reach calving BCS targets (Roche et al., 2009). Furthermore, little is known about the

metabolic effects of 1× milking during late lactation or the effects on performance in the subsequent lactation. Therefore, the objectives of our experiment were (1) to measure the DMI, BW, and BCS, milk production parameters, plasma hormones and metabolites, and hepatic gene expression of pasture-fed cows milked 1× relative to 2× for 12 wk in late lactation, and (2) to determine the effects of 1× milking on milk production, and plasma hormone and metabolite concentrations in the subsequent lactation when milked 2×.

MATERIALS AND METHODS

This study was conducted at the DairyNZ Lye Farm, Hamilton, New Zealand (37°46'S, 175°18'E) from January 2013 to September 2013. All procedures had prior approval (RAEC 12800 and RAEC 12952) of the Ruakura Animal Ethics Committee, Hamilton, New Zealand.

Experimental Design and Treatments

In mid-January 2013 (summer), 52 multiparous, pregnant Holstein-Friesian cows (175 ± 18 DIM; mean $\pm$ SD) were randomly assigned to either 1× or 2× milking ($n = 26$ per treatment) until dry-off (mean = 259 DIM). All cows had been previously milked 2× from calving in July–August 2012 and were artificially bred in September–October 2012. Pregnancy was confirmed by ultrasonography in early January 2013 (108 d after the planned start of mating; September 23, 2012). Daily milking times were 0700 h for 1× milking (24 h interval), and 0700 and 1500 h for 2× milking (16/8 h interval). Cows were dried off individually with the timing based on milk production and BCS as outlined by Macdonald and Penno (1998) and Macdonald et al. (2005). The final dry-off date was 56 d (May 10) before the planned start of calving in July 2013. During the nonlactating period, all cows were managed to reach a BCS target of 5.0 at calving (10-point scale, where 1 is emaciated and 10 is obese; Roche et al., 2004). Following calving in July 2013, all cows were milked 2×.

Grazing Management and Supplementary Feeding

The cows grazed perennial ryegrass-white clover pasture from calving in July–August 2012. From the January 14, 2013, cows underwent 4 periods of 18 d in a Calan gate (American Calan, Northwood, NH) indoor feeding facility (Hamilton, New Zealand), interspersed with 3 periods of 10 d grazing outdoors.

Indoors. In the Calan gate facility, cows were individually offered freshly cut pasture and pasture silage (2:1 ratio) ad libitum twice daily, allowing for at least

a 10% refusal. The amount (kg of DM/cow) of feed offered and refusals were recorded for each cow twice daily and DM content (%) was measured from a subsample of each feed to calculate individual daily DMI.

Outdoors. While outdoors, 1× and 2× cows were grazed in the same paddock separated by a temporary electric fence and were offered a generous allowance of perennial ryegrass/white clover pasture (~35 kg of DM/cow per d to ground level). Group DMI was estimated from pre- and postgrazing herbage mass estimated every 3 d for each grazing period using a rising plate meter with an electronic counter (Farmworks, Palmerston North, New Zealand) calibrated as described by Roche et al. (2005). Pregrazing pasture mass was 3,630 kg of DM/ha. Pasture silage was offered to maintain pasture postgrazing residuals of 1,800 kg of DM/ha when pasture availability was limited: 4.6 kg of DM/cow per d from February 5 to 8, 8.3 kg of DM/cow per d March 1 to 8, and 10.8 kg of DM/cow per d April 2 to 5.

Nonlactating Period and Subsequent Lactation. Cows were allocated to either a high (BCS 4.5 and greater) or low (BCS 4.0 and below) group at dry off. All cows grazed in the same paddock separated by an electric fence and were offered maize silage at 25% of the diet. Cows in the high group were fed to target a DMI of 12 kg of DM/cow per d; whereas cows in the low group targeted an intake of 10 kg of DM/cow per d. The low group had eighteen 1× cows and eight 2×

cows, and the high group contained nine 1× cows and sixteen 2× cows.

All cows grazed perennial ryegrass-white clover pasture and were offered ~29 kg of DM/cow per d (measured to ground level) from calving in July–August 2013, until the end of the experimental period (10 wk). Pre-grazing pasture mass was 3,450 kg of DM/ha targeting postgrazing residuals of 1,800 kg of DM/ha.

Feed Sampling and Measurements

Representative pasture samples were collected on 5 consecutive days each week. When the cows were outdoors, pasture was hand-clipped to the predicted grazing height from paddocks immediately before grazing. Samples of pasture silage were collected each day that it was offered to cows and bulked weekly. Duplicate sub-samples were oven-dried at either 95°C for 72 h to determine DM content or at 65°C for 48 h for subsequent analysis of nutrient composition. Samples were then ground to pass through a 0.5-mm sieve (Christy Laboratory Mill, Suffolk, UK), and analyzed for chemical composition by near-infrared reflective spectroscopy (FeedTech, Palmerston North, New Zealand). Composition of pasture and pasture silage is presented in Table 1.

In addition, botanical composition of pasture offered was evaluated; on average ($\pm$ SD), freshly cut pastures

Table 1. Chemical composition % of DM (unless otherwise noted) of pasture and pasture silage as offered to 52 dairy cows milked once daily or twice daily for 12 wk during late lactation¹

Variable	Pasture		Pasture silage	
	Indoor	Outdoor	Indoor	Outdoor
January–February				
DM, %	27.3 $\pm$ 3.1	NA	29.9 $\pm$ 2.7	19.6 $\pm$ 3.6
ME, ² MJ/kg of DM	12.0 $\pm$ 0.2	12.4	12.2 $\pm$ 0.7	11.7
ADF	27.0 $\pm$ 1.6	25.3	31.8	21.8
Ash	8.5 $\pm$ 0.5	9.8	10.8	10.8
CP	14.8 $\pm$ 1.3	18.9	22.4	20.0
Lipid	2.1 $\pm$ 0.4	2.3	2.3	4.3
NDF	53.0 $\pm$ 2.6	53.2	54.3	53.0
OMD, % of OM	70.6 $\pm$ 2.6	74.4	74.9	77.6
SSS	7.9 $\pm$ 2.1	5.9	1.2	<1
March–April				
DM, %	17.3 $\pm$ 2.4	NA	35.2 $\pm$ 2.9	38.7 $\pm$ 2.6
ME, ² MJ/kg of DM	12.8 $\pm$ 0.2	12.8 $\pm$ 0.7	12.7 $\pm$ 0.1	12.2 $\pm$ 0.3
ADF	25.0 $\pm$ 1.3	23.9 $\pm$ 2.2	33.5 $\pm$ 1.7	32.4 $\pm$ 1.1
Ash	10.2 $\pm$ 0.6	10.5 $\pm$ 0.6	12.1 $\pm$ 0.6	10.6 $\pm$ 0.6
CP	22.1 $\pm$ 2.8	22.6 $\pm$ 5.5	22.4 $\pm$ 2.1	21.2 $\pm$ 1.9
Lipid	2.5 $\pm$ 0.1	3.2 $\pm$ 0.6	2.6 $\pm$ 0.3	2.9 $\pm$ 0.1
NDF	48.6 $\pm$ 4.2	45.8 $\pm$ 7.8	46.3 $\pm$ 0.3	52.1 $\pm$ 5.6
OMD, % of OM	76.3 $\pm$ 4.7	77.2 $\pm$ 8.3	72.5 $\pm$ 0.6	74.6 $\pm$ 4.6
SSS	5.2 $\pm$ 2.2	8.8 $\pm$ 1.2	1.8 $\pm$ 1.8	3.9 $\pm$ 4.2

¹Cows were fed in a Calan gate indoor feeding facility for four 18-d periods (indoor), interspersed with 3 periods grazing outdoors (outdoor). Composition was measured by near-infrared reflective spectroscopy. Means $\pm$ SD are presented. OMD = organic matter digestibility; SSS = soluble sugars and starch. NA = not applicable.

²Metabolizable energy calculated from DM digestibility $\times$ 0.172 – 1.707 (CSIRO, 2007).

offered indoors consisted of $62.9 \pm 9.4\%$ of DM perennial ryegrass, $6.0 \pm 4.3\%$ of DM other grasses, $8.7 \pm 5.5\%$ of DM white clover, $6.6 \pm 5.5\%$ of DM weeds, and $15.8 \pm 4.8\%$ of DM dead material. Pastures grazed outdoors consisted of $77.6 \pm 14.5\%$ of DM perennial ryegrass, $1.0 \pm 0.8\%$ of DM other grasses, $2.1 \pm 0.9\%$ of DM white clover, $7.3 \pm 7.3\%$ of DM weeds, and $12.0 \pm 6.1\%$ of DM dead material.

Cow Measurements

Milk. Individual milk yields were recorded at each milking (Westfalia Surge Metatron Milk Meter; GEA Farm Technologies, Oelde, Germany), and milk samples (1.25% of total volume) were collected once each week using in-line milk meters at an a.m. milking ($1\times$) or consecutive p.m. and a.m. milkings ($2\times$). Milk samples from $2\times$ were combined to give one daily sample per cow. Milk fat, total protein, and lactose concentrations in milk were determined by an infrared milk analyzer [Fourier Transform Infrared Spectroscopy (FT120); Foss Electric, Hillerød, Denmark]. A subset of samples was also analyzed by reference techniques (milk fat by Röse-Gottlieb method, IDF, 1987; protein by Kjeldahl techniques, Barbano et al., 1991) to correct the FT120 results for matrix effects associated with milking frequency, nutrition and stage of lactation. Energy-corrected milk was calculated as kg of ECM/cow per d = $\text{kg milk/d} \times [(383 \times \text{fat}\%) + (242 \times \text{protein}\%) + 783.2)]/3,140$ (Sjaunja et al., 1990). Milk SCC was determined fortnightly using a Fossomatic automated cell counter (DK-3400, Foss Electric). Pre-trial SCC ranged from 27,000 to 532,000 cells/mL with a mean of 78,700 cells/mL.

BW and BCS. Body weight and BCS were determined 2 wk before treatment start then weekly during the treatment period. During the nonlactating period, BCS was determined fortnightly and postcalving, BCS was measured weekly for 10 wk. Calves were weighed within 24 h of birth.

Energy Balance Calculations and Estimates

Energy balance was calculated for the 4 indoor periods for each cow using equations described previously (NRC, 2001; Nicol and Brookes, 2007). Briefly, the metabolizable energy of intake (ME_I ; MJ/d) was determined by multiplying DMI by the calculated mean ME density of the diet. The ME (MJ/kg of DM) value for pasture and pasture silage was calculated from in vitro true digestibility (after 24 h of incubation, Ankom Technology Method 3, Dairy One Forage Laboratory Services, Ithaca, NY) as $\text{DM digestibility} \times 0.172 - 1.707$ (CSIRO, 2007). The ME value of each individual

feed was then used to calculate the mean ME content of the diet. The $\text{ME}_{\text{maintenance}}$ (MJ/kg) was calculated as $\text{BW}^{0.75} \times 0.56$ with a correction made for the ME of the feed (add/subtract 5% per MJ of ME for diets below/above 11 MJ of ME/kg of DM; Nicol and Brookes, 2007). The metabolizable energy requirement for pregnancy (ME_P ; MJ/d) was calculated as $4.186 \times [(0.00318 \times \text{day of gestation} - 0.0352) \times (\text{calf birth weight}/45)]/0.14$ (NRC, 2001). The ME requirement for lactation (ME_{milk} ; MJ/kg) was calculated as $\text{milk yield} \times [4.186 \times (0.0929 \times \text{fat}\% + 0.0547 \times \text{protein}\% + 0.0395 \times \text{lactose}\%)]/0.64$ (NRC, 2001). A correction was made for the ME of the feed (add/subtract 8% per MJ of ME for diets below/above 11 MJ of ME/kg of DM; Nicol and Brookes, 2007). The equation used to calculate energy balance (**EBAL**; MJ of ME/d) was $\text{EBAL} = \text{ME}_I - (\text{ME}_{\text{MILK}} + \text{ME}_P + \text{ME}_{\text{maintenance}})$.

It was assumed that 19 MJ of NE is required per kilogram of BW gain in dairy cattle grazing pasture (Holmes et al., 2002) and that the efficiency of autumn pasture for BW gain is 0.34 (Mandok et al., 2014); therefore, it was calculated that 55.9 MJ of ME was required per kilogram of BW gain. The estimated additional BW gain (kg) in cows milked $1\times$ was calculated as $(\text{additional MJ of ME available for BW gain} \times \text{length of trial in d})/55.9$ MJ of ME. The BCS was calculated based on a 550-kg cow where a BCS unit is equivalent to 36 kg of BW (DairyNZ Limited, 2012).

Blood Sampling and Analysis

Blood samples were taken from each cow 2 wk before the start of treatment and then on the same day of wk 1, 4, 5, 8, and 12, so that sampling always occurred after the cows had been in the Calan gates system for at least 7 d. Blood was also sampled weekly for the first 10 wk following calving in July 2013. For all blood sampling, two 10-mL evacuated blood tubes (containing K_2EDTA for hormone analysis or sodium fluoride for metabolite analysis; Becton Dickinson, Franklin Lakes, NJ) were collected from each cow by coccygeal venipuncture immediately before the morning milking (approximately 0700 h). Samples were placed on ice before centrifugation 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, glucose) and hormones (insulin, leptin).

Analyses of metabolites were performed on a Modular P800 analyzer (Roche, Basel, Switzerland) at 37°C by Gribbles Veterinary Pathology Ltd. (Hamilton, New Zealand). The NEFA were measured (mmol/L) by the acyl Co-A synthetase, acyl-CoA oxidase (ACS-ACOD) colorimetric method using the NEFA C Kit from Wako (Osaka, Japan), and glucose was determined (mmol/L)

photometrically by the GOD-PAP (glucose oxidase/4-aminophenazone/phenol) method. The inter- and intra-assay CV were <2% for all assays.

Insulin ($\mu\text{U/mL}$) was measured in duplicate using the Porcine Insulin RIA Kit (PI-12K, Merck Millipore, Auckland, New Zealand). Bovine cross-reactivity was 90% (Bellmann et al., 2004; Hammon et al., 2009) with inter- and intra-assay CV <10%. The assay detection limits were 2 $\mu\text{U/mL}$. Leptin concentrations (ng/mL) were determined using the Bovine LEP ELISA kit (SEA084Bo, USCNK, Wuhan, China) with interassay CV <12% and intraassay CV <10%. The minimal detectable concentration of leptin was 0.06 ng/mL.

Liver Tissue Sampling

Liver tissue was biopsied from a randomly selected subset of animals ($n = 12$) in each treatment group at 2 wk before the start of treatment and at wk 2 and 10 of the treatment period while indoors. Tissue was sampled as per Lucy et al. (2009). In brief, region on the right rib cage at the 11th intercostal space was clipped and cleansed. Local anesthetic was administered and the skin penetrated with a scalpel. Samples of approximately 200 mg were collected using a liver biopsy needle and immediately placed in screw-capped microcentrifuge tubes and frozen in liquid nitrogen. Samples were stored at -80°C until RNA was extracted.

Extraction of RNA and cDNA Synthesis

Total cellular RNA was isolated from liver samples (~30 mg) using a TissueLyser II and RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, then treated with recombinant DNase (Ambion DNA-free kit; Ambion, Austin, TX). The RNA quantity and purity were determined by spectrophotometry using a NanoDrop ND-1000 (Nano-Drop Technologies, Wilmington, DE). The absorbance ratio at 260:280 nm of all samples was >1.9. The RNA integrity was determined using the Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA), and an RNA integrity score minimum cut-off of 6.9 (7.5 ± 0.4 ; mean $\pm$ SD) was used. The RNA samples were stored at -80°C .

Using the SuperScript III First-Strand Synthesis SuperMix kit (Life Technologies, Auckland, New Zealand), cDNA was synthesized from 2 μg of each RNA sample (final volume = 40 μL). Total RNA was reverse transcribed using a final concentration of 27 μM random pentadecamer primers according to the manufacturer's instructions. Reverse transcriptase negative controls were generated by excluding the enzyme. The cDNA samples were stored at -20°C .

Quantitative PCR

Using reverse-transcription quantitative PCR (RT-qPCR) analysis, hepatic expression of 31 target genes was measured (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2015-10412>). Genes were targeted for their involvement in the somatotrophic axis, nutrient utilization, lipid synthesis, and transport as these pathways have been previously reported to be changed by milking frequency during early lactation (Grala et al., 2014a,b). In addition, genes involved in adipokine signaling were selected as recent research indicates expression is altered with changes in energy balance (Koltes and Spurlock, 2012).

Assays were designed to publicly available bovine gene sequences from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/gene>). Assays were designed using Roche Universal Probe Library design software to span an intron-exon boundary to prevent amplification of genomic DNA and the specificity of assays tested using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; Altschul et al., 1990). The primer and probe sequences and location of the assays are presented in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2015-10412>). Primers were manufactured by Invitrogen at 50 nmol or Integrated DNA Technologies (Coralville, IA) at 25 nmol concentration and purified by desalting.

The RT-qPCR was performed using Roche master mix (LightCycler 480 Probes Master) and Roche Universal Probe Library assays analyzed on the Roche LightCycler 480 (Roche Diagnostics, Mannheim, Germany). Each 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 each of 5 μM primer, 0.05 or 0.1 μL probe, and 0.15 or 0.1 μL of H_2O). Reactions were set-up using an epMotion 5075 robot (Eppendorf, 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, 60°C for 30 s) $\times$ 45 cycles, and 40°C for 40 s. Each RT-qPCR run included 8 negative controls ($n = 2$ no cDNA, $n = 6$ no reverse transcriptase). Triplicate measurements were performed for all samples. Assays were tested for specificity by running an agarose gel of the PCR products to ensure amplicon specificity and length.

A relative standard curve was generated for each assay using a 6-point serial cDNA dilution, ranging from $3.33\text{e}-1$ to $1.37\text{e}-3$ or $2.00\text{e}-1$ to $3.2\text{e}-4$, and the Roche LightCycler 480 software second-derivative maximum analysis. Standard curves where the CV for each triplicate measurement was <2% were subsequently used to quantify the experimental samples. The PCR

efficiency of each assay was between 1.8 and 2.2 (100% efficiency = 2.0). Endogenous control genes were tested across all samples and their suitability determined using NormFinder and GeNorm (Vandesompele et al., 2002; Andersen et al., 2004); cytochrome c oxidase subunit IV isoform 1 (*COX4I1*) and eukaryote initiation factor 3K (*EIF3K*) were the 2 most stably expressed genes; stability values for *COX4I* were 0.278 (GeNorm) and 0.022 (Normfinder), and stability values for *EIF3K* were 0.308 (GeNorm) and 0.042 (Normfinder). The Roche LightCycler 480 Software was used to perform advanced relative quantification analysis of each target gene expression using the normalization factor of each of the 2 endogenous control genes. The geometric mean of the resulting target: endogenous control ratio for each sample was then calculated. Expression ratios were subsequently normalized to the sample with the lowest ratio to simplify statistical analysis and interpretation.

Statistical Analyses

Milk production, SCC, BW, BCS, and hormone and metabolite measures from 2 wk before treatment start were used as covariates in their respective statistical analyses. Repeated measurements through time were modeled for milk production and composition, BW, and BCS, and plasma hormone and metabolite data using REML, including covariate, biopsy (yes vs. no), location (indoors vs. outdoors), and milking frequency treatment (1× vs. 2×) as fixed effects, and cow as a random effect in GenStat (version 16; VSN International, Hemel Hempstead, UK). As treatment by time interactions ($P < 0.001$) were present, data were also analyzed by each week. Data for SCC, insulin, NEFA, and leptin were $\log_{10}$ -transformed before statistical analyses.

The DMI data were analyzed by calculating the mean daily intake for each cow over the 4 indoor periods and then performing a repeated measurements analysis using mixed models in GenStat. Pretreatment intake data were used as a covariate, and indoor measurement period, milking frequency treatment, and the interaction of measurement period with treatment were fixed effects and cow was a random effect.

Data for each gene were $\log_{10}$ -transformed before analysis due to heterogeneity of variance and data from 2 wk before treatment start were used as covariates. Data were then analyzed using ANOVA in GenStat, including cow as a blocking factor with milking frequency treatment, time (week of treatment period), and the interaction of treatment with time as fixed effects. Significant interactions were not observed between wk 2

and 10 samples, so data were subsequently analyzed for treatment effects using averaged wk 2 and 10 data.

Treatment means and the SE of the difference between means (**SED**) are presented. Differences in data are considered significant when $P < 0.05$ and declared a trend when $P \leq 0.10$.

RESULTS

Milk Production and Composition

The cows milked 1× for 12 wk from mid-January produced 20% less milk than those milked 2× during that period (Table 2; Figure 1). The milk fat and protein concentrations were greater in cows milked 1×; however, they still produced 14% less milk fat and 17% less protein relative to cows milked 2×. This effect resulted in a decrease in cumulative ECM yield of 31.4 kg/cow (16%) in cows milked 1× (2.6 kg/d). We detected no difference in SCC until after 9 wk of 1× milking, after which point, SCC were greater in cows milked 1× (79,433 vs. 50,110 cells/mL; Figure 1). Milking 1× had no effect on total DIM (276 ± 22 d). Ten cows were dried off before the end of the experiment (during wk 11; $n = 4$ vs. 6 for 1× and 2× milked cows, respectively). All the cows milked 2× were dried off based on BCS (4.0 or lower), whereas 2 out of 4 cows milked 1× were dried off for low milk yield (<5 L/d for 5 d). No effect was observed of previous milking frequency on any milk production parameters measured in the subsequent lactation (Table 3).

DMI, BCS, and BW

During the indoor periods, the cows milked 1× tended ($P = 0.05$) to eat less (-0.4 kg of DM/cow per d) than those milked 2× (Table 2). We found no effects of time or interactions between milking frequency and time on DMI.

At the end of the 12-wk experiment, cows milked 1× had a greater BCS ($+0.23$ BCS units; Figure 2; $P < 0.05$) and greater BW (546 vs. 533 kg $\pm$ 5.5; mean $\pm$ SD; $P < 0.05$) compared with those milked 2×. Significant differences in BCS were present from wk 7 onward.

At calving, no differences were found between groups for BCS (5.4 in cows previously milked 1×, 5.3 in cows previously milked 2×; SED = 0.14; $P = 0.40$) or BW (622 kg in cows previously milked 1×, 609 in cows previously milked 2×; SED = 14; $P = 0.37$). We found no differences in BW or BCS immediately precalving nor for the 10 wk immediately postcalving in the next lactation ($P = 0.57$ and 0.87, respectively). Nor were differences found in the birth weight of calves born to

Table 2. Average milk production, SCC, ECM, and daily DMI from pasture-fed dairy cows (n = 52) milked once daily (1×; n = 26) or twice daily (2×; n = 26) for 12 wk during late lactation¹

Item	1×	2×	SED	P-value
ECM, kg/cow per d	14.1	16.8	0.4	<0.001
Milk yield, kg/d	11.9	14.9	0.4	<0.001
Protein, %	4.05	3.73	0.04	<0.001
Fat, %	5.23	4.89	0.10	<0.001
Lactose, %	4.63	4.69	0.03	0.08
Protein yield, kg/d	0.48	0.55	0.01	<0.001
Fat yield, kg/d	0.62	0.72	0.02	<0.001
Lactose yield, kg/d	0.55	0.70	0.02	<0.001
SCC, ² log ₁₀ × 1,000 cells/mL	1.78	1.66	0.09	0.14
DMI, kg of DM per cow/d	(60.2)	(45.7)	0.2	0.052
	17.8	18.2		

¹Means and the SE × 1,000 of the difference (SED) are presented.²Back-transformed means in parentheses. **Figure 1.** Energy-corrected milk yield, SCC, fat percentage, and protein percentage from pasture-fed dairy cows (n = 52) milked once daily (1×; n = 26; ■) or twice daily (2×; n = 26; ○) for 12 wk during late lactation. At wk 12, 22 cows remained that were milked 1× and 20 cows that were milked 2×, as 10 cows were dried off early. Grey shaded panels indicate times when cows were outdoors. Means and the SE of the difference are presented.

Table 3. Average milk production parameters in the 10 wk immediately postcalving from pasture-fed dairy cows ($n = 52$) milked twice daily ($2\times$) during early lactation but milked once daily ($1\times$; $n = 26$) or $2\times$ ($n = 26$) for 12 wk during the previous late lactation period¹

Item	1×	2×	SED	P-value
ECM, kg/cow per d	31.9	31.1	0.9	0.37
Milk yield, kg/d	28.7	28.2	0.8	0.54
Protein, %	3.63	3.61	0.05	0.70
Fat, %	4.80	4.72	0.14	0.57
Lactose, %	4.80	4.83	0.02	0.20
Protein yield, kg/d	1.04	1.02	0.02	0.48
Fat yield, kg/d	1.37	1.33	0.05	0.37
Lactose yield, kg/d	1.38	1.36	0.04	0.68
SCC, $\log_{10} \times 1,000$ cells/mL	1.59	1.66	0.11	0.54

¹Means and the SE of the difference (SED) are presented.

cows previously milked $2\times$ ($40.2 \text{ kg} \pm 4.7$; mean $\pm$ SD) compared with calves born to cows previously milked $1\times$ ($39.8 \text{ kg} \pm 4.4$; mean $\pm$ SD).

Energy Balance

All cows were in positive EBAL during late lactation. The cows milked $1\times$ had a greater EBAL than the cows milked $2\times$, but only during the first indoor period (79.5 vs. $71.6 \text{ MJ of ME/d} \pm 3.1$; mean $\pm$ SD; $P < 0.05$). The additional daily energy available for BW gain (5.99 MJ of ME/d) in cows milked $1\times$ was used to calculate the cumulative energy available by the end of the 12-wk treatment period. This energy was predicted to supply 9.0 kg of additional BW gain, which is consistent with 0.23 BCS units .

Hormones and Metabolites

We determined milking frequency by time interactions for plasma glucose, NEFA, and insulin concentrations (Figure 3). The glucose concentrations were greater at wk 1, 8, and 12, and the insulin concentrations were greater at wk 1, 4, and 8, in cows milked $1\times$ compared with those milked $2\times$. Concentrations of NEFA were lower at wk 4 in cows milked $1\times$ compared with $2\times$; however, no differences were found at the other time points. Leptin concentrations were not altered by $1\times$ milking.

In the following lactation, no interactions were found between treatment and week postcalving. Concentrations of glucose were lower in cows previously milked $1\times$; however, plasma concentrations of insulin, NEFA, and leptin were not affected (Table 4).

Hepatic Gene Expression

We found no effects of sampling time (wk 2 vs. 10) or interactions between milking frequency and sampling

time on hepatic gene expression. Milking frequency did, however, affect hepatic gene expression. Hepatic expression of *G6PC*, angiotensin-like 4 (*ANGPTL4*), and adiponectin receptor (*ADIPOR*) 2 was lower and expression of *PCK1* tended ($P = 0.07$) to be lower in cows milked $1\times$ relative to $2\times$ (Table 5). The expression of retinol binding protein 4 (*RBP4*) also tended ($P = 0.06$) to be lower in cows milked $1\times$, whereas *ADIPOR1* expression tended ($P = 0.08$) to be greater relative to cows milked $2\times$. In addition, insulin receptor-B isoform (*INSRB*), *CPT2*, and peroxisome proliferator-activated receptor gamma coactivator 1- α (*PPARGC1A*) tended ($P = 0.06, 0.07$, and 0.09 , respectively) to be lower in cows milked $1\times$ compared with $2\times$.

Figure 2. Body condition score of pasture-fed dairy cows ($n = 52$) milked once daily ($1\times$; $n = 26$; ■) or twice daily ($2\times$; $n = 26$; ○) for 12 wk during late lactation. At wk 12, 22 cows remained that were milked $1\times$ and 20 cows milked $2\times$, as 10 cows were dried off early. Grey shaded panels indicate times when cows were outdoors. Means and the SE of the difference are presented.

Table 4. Average concentrations of plasma hormones and metabolites in the 10 wk immediately postcalving from pasture-fed dairy cows ($n = 52$) milked twice daily ($2\times$) during early lactation but milked once daily ($1\times$; $n = 26$) or $2\times$ ($n = 26$) for 12 wk during the previous late lactation period¹

Item	$1\times$	$2\times$	SED	<i>P</i> -value
Glucose, mmol/L	3.03	3.16	0.06	<0.05
NEFA, $\log_{10}$ mmol/L	-0.12 (0.76)	-0.13 (0.74)	0.03	0.65
Insulin, $\log_{10}$ μ U/mL	0.35 (2.2)	0.39 (2.5)	0.04	0.33
Leptin, $\log_{10}$ mmol/L	-0.42 (0.38)	-0.42 (0.38)	0.05	0.93

¹Plasma nonesterified fatty acids (NEFA), insulin, and leptin concentrations are presented as $\log_{10}$ -transformed, with back-transformed values in parentheses. Means and the SE of the difference (SED) are presented.

DISCUSSION

Our results show that reducing milking frequency from $2\times$ to $1\times$ during late lactation in pasture-based cows decreased milk production by 20% but only tended

to lower DMI by 2%, which resulted in positive effects on EBAL. This increase in EBAL in cows milked $1\times$ resulted in gains of both BW and BCS, consistent with predicted gains based on energetic equations (NRC, 2001; Nicol and Brookes, 2007). Our plasma hormone

Figure 3. Circulating concentrations of glucose, nonesterified fatty acids (NEFA), insulin, and leptin from pasture-fed dairy cows ($n = 52$) milked once daily ($1\times$; $n = 26$; $\circ$) or twice daily ($2\times$; $n = 26$; $\blacksquare$) for 12 wk during late lactation. At wk 12, 22 cows remained that were milked $1\times$ and 20 cows milked $2\times$, as 10 cows were dried off early. Grey shaded panels indicate times when cows were outdoors. Means and the SE of the difference are presented. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 5. Hepatic gene expression from pasture-fed dairy cows (n = 24) milked once daily (1×; n = 12) or twice daily (2×; n = 12) for 12 wk during late lactation¹

Gene ²	1 ×	2 ×	SED	P-value
Gluconeogenesis				
<i>G6PC</i>	0.19	0.27	0.03	<0.01
<i>INSRB</i>	0.20	0.27	0.03	0.06
<i>PC</i>	0.38	0.39	0.05	0.87
<i>PCK1</i>	0.27	0.33	0.03	0.07
<i>PCK2</i>	0.20	0.22	0.03	0.65
<i>PDK4</i>	0.24	0.29	0.04	0.19
Adipokine signaling				
<i>ADIPOR1</i>	0.25	0.21	0.03	0.08
<i>ADIPOR2</i>	0.24	0.32	0.03	<0.05
<i>ANGPTL4</i>	0.24	0.42	0.05	<0.01
<i>LEPR</i>	0.33	0.36	0.03	0.25
<i>RBP4</i>	0.21	0.26	0.03	0.06
Fatty acid oxidation and ketogenesis				
<i>ACSL1</i>	0.21	0.24	0.03	0.33
<i>CPT1A</i>	0.25	0.26	0.04	0.80
<i>CPT2</i>	0.13	0.17	0.02	0.07
<i>HMGCS2</i>	0.63	0.67	0.06	0.53
Somatotropic axis				
<i>GHR1A</i>	0.83	0.88	0.05	0.37
<i>IGF1</i>	0.38	0.33	0.04	0.30
<i>IGFBP3</i>	0.18	0.19	0.02	0.60
<i>IGFALS</i>	0.57	0.52	0.05	0.28
Transcription factors				
<i>FOXO1</i>	0.34	0.39	0.05	0.26
<i>PPARA</i>	0.32	0.35	0.04	0.37
<i>PPARGC1A</i>	0.43	0.53	0.06	0.09
<i>SREBF1</i>	0.24	0.27	0.03	0.33
Lipid synthesis and transport				
<i>ACACA</i>	0.36	0.42	0.04	0.18
<i>APOA1</i>	0.31	0.36	0.04	0.22
<i>APOB</i>	0.21	0.20	0.03	0.80
<i>DGAT1</i>	0.21	0.27	0.04	0.18
<i>GPAM</i>	0.24	0.24	0.03	0.88
<i>LIPE</i>	0.03	0.03	0.03	0.96
<i>MID1IP1</i>	0.29	0.29	0.04	0.83
<i>THRSP</i>	0.61	0.62	0.07	0.97

¹Means and the SE of the difference (SED) are presented. Data were normalized to *COX4I1* and *EIF3K* gene expression and are expressed as log₁₀-transformed arbitrary units.

²*ACACA* = acetyl-CoA carboxylase α; *ACSL1* = acyl-CoA synthetase long-chain family member 1; *ADIPOR1* = adiponectin receptor 1; *ADIPOR2* = adiponectin receptor 2; *ANGPTL4* = angiopoietin-like 4; *APOA1* = apolipoprotein A-I; *APOB* = apolipoprotein B; *CPT1A* = carnitine palmitoyltransferase 1A; *CPT2* = carnitine palmitoyltransferase 2; *DGAT1* = diacyl-glycerol transferase 1; *G6PC* = glucose-6-phosphatase, catalytic sub-unit; *GHR1A* = growth hormone receptor 1A; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *HMGCS2* = 3-hydroxy-3-methylglutaryl-CoA synthase 2, mitochondrial; *IGF1* = insulin-like growth factor 1; *IGFALS* = insulin growth factor binding protein, acid labile subunit; *IGFBP3* = insulin growth factor binding protein 3; *INSRB* = insulin receptor-B isoform; *LEPR* = leptin receptor; *LIPE* = hormone sensitive lipase; *PC* = pyruvate carboxylase; *MID1IP1* = MID1 (Midline 1) interacting protein 1; *PCK1* = phosphoenolpyruvate carboxykinase 1 (soluble); *PCK2* = phosphoenolpyruvate carboxykinase 2, mitochondrial; *PDK4* = pyruvate dehydrogenase kinase, isozyme 4; *RBP4* = retinol binding protein 4; *THRSP* = thyroid hormone responsive; *FOXO1* = forkhead box O1; *PPARA* = peroxisome proliferator activated receptor α; *PPARGC1A* = peroxisome proliferator activated receptor gamma, coactivator 1 α; *SREBF1* = sterol regulatory element binding transcription factor 1.

and metabolites, and hepatic gene expression results were consistent with the changes in milk production and EBAL, and may indicate an anti-inflammatory effect of 1× milking. Fewer changes were found in hepatic gene expression than previously reported by 1× milking during early lactation, which is unsurprising given the smaller magnitude of milk production loss at this later stage of lactation.

Milking Cows 1× in Late Lactation Had Small Effects on DMI and BCS Gain

This is the first report to quantify individual DMI and BCS gain in pasture-fed dairy cows milked 1× compared with 2× during late lactation. The tendency for only a small reduction in DMI (0.4 kg of DM/cow per d, ~2%) when cows were milked 1× is, perhaps,

not unexpected, because estimates of pasture disappearance from pre- and postgrazing measurements in a recent short-term 1× milking experiment in early lactation support a similar intake in cows milked 1× and 2×, despite their different milk production (Kay et al., 2013). Furthermore, although it is difficult to measure intakes accurately while cows are grazing, Holmes et al. (1992) used an intraruminal slow-release chromium tracer method to obtain estimates of individual cow DMI during late lactation in grazing cows milked 1× for the whole lactation and reported a tendency for lower DMI (1 kg of DM/d) compared with cows milked 2×. Similarly, Ferris et al. (2008) reported a tendency for cows milked 1× to consume 0.6 kg of DM/cow per d less in housed cows fed concentrates and silage. Additionally, a review of French experiments in which cows were housed indoors and fed TMR, 1× milking during early lactation did not significantly decrease feed intake for the first 6 to 8 wk, but tended to decrease if milked 1× for longer (Rémond and Pomiès, 2005). Therefore, short periods of 1× milking do not result in large reductions to DMI.

The minimal decrease in DMI, despite a 16% reduction in milk energy output in cows milked 1×, resulted in a more positive EBAL and indicates that additional energy would have been available for BW and BCS gain. Indeed, the cows milked 1× gained 13 kg of BW and 0.23 BCS units more than the cows milked 2× during the 12-wk late-lactation experimental period. These differences were similar to our predicted values (9 kg of BW and 0.23 BCS units), which were based on EBAL equations and assumed the efficiency of BW gain from pasture is low at this stage of the grazing season (Mandok et al., 2014). Lynch et al. (1991) and O'Brien et al. (2002) reported increases in BW (3.6 and 2.2%, respectively) when cows were milked 1× in late lactation, which is similar to the BW gain in the current study (2.4%). In contrast, Ferris et al. (2008) reported no significant differences in BW.

The greater BCS gain in cows milked 1× in the current study is in contrast to other studies, where no differences were found in BCS between cows milked 1× and 2× in late lactation (O'Brien et al., 2002; Ferris et al., 2008). Ferris et al. (2008) reported that the EBAL of cows milked 1× was only 4.0 MJ/d greater than for cows milked 2×, which was insufficient for significant BCS gain over the 6 to 8 wk of their experiment. However, the current study had EBAL differences of 6.0 MJ/cow per d for 12 wk. The difference in BCS did not become significant until wk 9; therefore, to achieve additional BCS gains, longer periods of 1× milking may be required.

Our predicted BCS gain was consistent with the actual BCS gain, whereas our predicted BW gain was

slightly less than measured. Body condition score is a subjective, visual, and tactile evaluation of the amount of subcutaneous fat on a cow (Domecq et al., 1997; Roche et al., 2004). As such, it does not take into account any visceral fat increase (Roche et al., 2009), which may, in part, explain the lower actual BCS change relative to the BW increase. No difference was observed in plasma leptin concentrations, which indicates no effects on overall adipose storage. This is not consistent with the BW and BCS gains in cows milked 1× relative to 2×. However, leptin secretion is greater in subcutaneous rather than visceral adipose depots (Wronska and Kmiec 2012); therefore, the lack of milking frequency effects on leptin concentrations in the blood is consistent with increasing visceral adipose stores during late lactation.

Overall, 1× milking in pasture-based cows during late lactation tended to decrease DMI, increased EBAL, BW and BCS gains in cows milked 1× compared with 2×.

Milking Cows 1× in Late Lactation Reduced Milk Production and Altered Gluconeogenesis

Milking cows 1× during late lactation increased milk fat and protein concentrations, which is consistent with the literature (Lacy-Hulbert et al., 1999; O'Brien et al., 2002; Ferris et al., 2008). These differences only partially, however, offset the decrease in milk yield; ECM yield was 16% lower in cows milked 1×.

No effect of changing to 1× milking on lactation length was found. In pasture-based systems, cows can be dried off early to ensure adequate BCS gain occurs before calving; however, this strategy reduces annual milk production and milk revenue from the current lactation. It was previously reported from modeling studies that milking cows 1× in late lactation could enable them to be milked for longer due to the greater BCS gain while lactating and that this increase in DIM could partially offset the decrease in daily milk production (Douglas et al., 2011). However, in our study most cows milked 2× were dried off due to low BCS, whereas the cows milked 1× were dried off due to low milk yield. Overall, these trends meant that there were no positive effects of 1× milking on lactation length that could compensate, in part, for the reduced daily milk yields in late lactation.

The lower milk production in cows milked 1× was associated with a coordinated decrease in precursors for lactose production. Concentrations of plasma glucose and insulin were greater, and gluconeogenic mRNA expression (*G6PC* and *PCK1*) tended to be decreased in cows milked 1× compared with 2×, indicating a more positive EBAL in these animals. Ruminant gluconeogenesis

genesis occurs primarily in the liver and is a function of glucose demand by the mammary gland for lactose synthesis (Aschenbach et al., 2010). The hepatic transcription of both *G6PC* and *PCK1* is downregulated by insulin (Aschenbach et al., 2010), and insulin concentrations in the current study concentrations were greater in cows milked 1× at wk 1, 4, and 8. Decreased gluconeogenesis is also indicated by the tendency for lower transcription of *INSRB* in cows milked 1×, which may be occurring as part of increased insulin resistance in response to greater circulating insulin.

Additionally, increased hepatic expression of *ADIPOR1* in cows milked 1× may have contributed to the downregulation of *G6PC* and *PCK1*. The signaling pathway of *ADIPOR1* differs from that of *ADIPOR2*, with adiponectin signaling through *ADIPOR1* occurring via phosphorylation of the transcription factor 5' AMP-activated protein kinase (AMPK) rather than peroxisome proliferator-activated receptor α (PPARA), which is stimulated by *ADIPOR2* (Yamauchi et al., 2007). The AMPK pathway is inhibitory and its direct targets include the gluconeogenic genes *G6PC* and *PCK1* (Lochhead et al., 2000). In contrast, the metabolic functions of *RBP4* and *PPARGC1A* involve the stimulation of hepatic gluconeogenesis by increasing *PCK1* and *G6PC* transcription (Yoon et al., 2001; Yang et al., 2005). Therefore, reduced *RBP4* and *PPARGC1A* in cows milked 1× may also be contributing to the lower transcription of the gluconeogenic genes.

In contrast to *G6PC* and *PCK1*, expression of *PC* and *PCK2* did not differ between milking frequency treatments. Pyruvate carboxylase and *PCK2* are also coupled to the tricarboxylic acid cycle (Aschenbach et al., 2010) and this additional role could mask any effects of altered milking frequency on these genes. We have previously reported a similar pattern of gene expression changes in dairy cows in response to reduced milking frequency in early lactation, where *PCK1*, *PCK2*, and *G6PC* were lower in cows milked 1× relative to 2× and *PC* expression was unaffected (Grala et al., 2014b). Together, these observations indicate a coordinated downregulation of gluconeogenesis in response to lower milking frequency, probably due to reduced glucose demand by the mammary gland for lactose production.

Little residual effect was found of milking cows 1× during late lactation on performance during the subsequent lactation. Milk production from the cows previously milked 1× relative to 2× did not differ during the following early lactation period, indicating that milking cows 1× does not negatively affect the secretory potential of the mammary gland during the next lactation. This outcome is consistent with the results of Ferris et al. (2008) and was expected as, during the nonlactating period, senescent cells in the mammary

glands are replaced due to extensive cell turnover (Capuco et al., 2003). The only variable that was different in early lactation was glucose concentration, which was lower in cows previously milked 1×. Despite an effect on glucose, no effects were found on insulin concentrations or milk production; therefore, we believe this is not a physiologically important result. It is also important to note that during the nonlactating period all cows were managed to target a BCS of 5.0 at calving, which meant that any flow-on effects of a higher BCS at dry-off between treatments and individual cows were mitigated. Calving BCS affects milk production, BCS loss, and blood hormone and metabolite profiles (Roche et al., 2013), so it was crucial that this was consistent across cows to investigate the effect of 1× milking in late lactation on the ability of the mammary gland to produce milk during the subsequent lactation. Nevertheless, in practice, the greater BCS at dry-off of cows milked 1× means that they require less feed during the dry period to reach the BCS target at calving and are, therefore, more likely to reach the target in a pasture-based system. This effect can confer advantage if cows previously milked 2× have not been able to reach the target BCS at calving.

Milking Cows 1× in Late Lactation Reduced Transcription of Genes Involved in FA Oxidation

Plasma NEFA concentrations were lower in cows milked 1× at wk 4, indicating that release of FA from adipose stores was reduced in cows milked 1× at this time. Lower concentrations of NEFA in plasma cause reduced demand for β -oxidation in the liver, consistent with the decrease in expression of the FA oxidative gene *CPT2*, in cows milked 1× relative to cows milked 2×. Interestingly, hepatic *ADIPOR2* signaling leads to upregulation of *CPT2* through the actions of PPARA (Kersten et al., 2000; Yamauchi et al., 2007; Rakhshandehroo et al., 2010). The downregulation of *CPT2* we report may, therefore, be mediated via *ADIPOR2* signaling, because *ADIPOR2* mRNA was also lower in cows milked 1× compared with those milked 2×. We measured levels of *PPARA* mRNA, but no difference was detected between milking frequency treatments to support a link between *ADIPOR2* with *CPT2*. This is not surprising because the activity of *PPARA* as a signaling molecule is primarily controlled by posttranslational mechanisms (e.g., phosphorylation) rather than at the transcriptional level (Burns and Vanden Heuvel, 2007). The effects of adiponectin are mainly mediated through *ADIPOR2* in the liver (Yamauchi et al., 2003). We did not measure adiponectin levels, although its secretion from adipose tissue is known to correlate positively with plasma insulin and negatively

with plasma NEFA in dairy cattle (Singh et al., 2014), which were greater and lower, respectively, in the cows milked 1× in this study. Downregulation of *ADIPOR2* transcription in the cows milked 1× may, therefore, reflect receptor desensitization, possibly in response to elevated adiponectin levels.

Reduced expression of *ADIPOR2* and *CPT2* indicates reduced capacity for FA oxidation in response to reduced milking frequency. These effects are small, but support the greater EBAL and BCS gain in cows milked 1×.

Milking Cows 1× in Late Lactation Down-Regulated Hepatic Adipokine Signaling

In addition to an improved metabolic state in late lactation, the altered hepatic expression of genes involved in adipokine signaling (*ANGPTL4* and *RBP4*) indicates that the cows milked 1× had a reduced inflammatory state. The transcription of hepatic *ANGPTL4* was reduced in cows milked 1× relative to 2× and is reported to be sensitive to levels of free FA in dairy cattle (Koltes and Spurlock, 2012; Wang et al., 2015), which, in turn, are associated with inflammation (Sordillo et al., 2009). In addition, activation of toll-like receptors results in an increase in *ANGPTL4* mRNA in the liver and *ANGPTL4* has been suggested to be a positive acute phase protein (Lu et al., 2010), increasing during inflammation. Expression of *RBP4* was also reduced in cows milked 1×. A strong correlation of *RBP4* expression with tumor necrosis factor- α (TNF- α) blood concentrations in cows has been previously reported (Rezamand et al., 2012). As adiponectin has been reported to normalize toll-like signaling (Tao et al., 2014), and if cows milked 1× have increased adiponectin, as is hypothesized here, then this would be consistent with lower TNF- α or other pro-inflammatory mediators. These hypotheses require further investigation to determine the inflammatory state in response to lower milking frequency in late lactation.

CONCLUSIONS

We have found that milking cows 1× during late lactation reduced daily milk production but had no positive effect on lactation length, thus reducing total milk production. Other effects were a small decrease in DMI, and greater gains in BCS and BW in cows milked 1×. The hepatic gene expression differences we observed indicate attenuation of glucose production and adipokine signaling in the liver in response to the decreased milking frequency. These changes reflect the reduced milk production, and improved BCS at dry-off of cows milked 1×. No negative carry-over effects were

found on milk production in the subsequent lactation, indicating the secretory potential of the mammary gland was not affected by milking cows 1× during late lactation. Reducing milking frequency from 2× to 1× daily in late lactation may be an effective strategy to help achieve body condition targets for the next calving; however, BCS gain was small and milk production was reduced. Thus, these factors need to be considered in line with other management practices, such as a longer dry period or using more feed during the nonlactating period, to determine the most appropriate management strategy.

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