Temporary alterations to milking frequency, immediately post-calving, modified the expression of genes regulating milk synthesis and apoptosis in the bovine mammary gland

T.M. GRALA[1,2]*, C.V.C. PHYN[1], J.K. KAY[1], A.G. RIUS[1], M.D. LITTLEJOHN[2], R.G. SNELL[3] and J.R. ROCHE[1]

[1]DairyNZ, Private Bag 3221, Hamilton 3240, New Zealand
[2]ViaLactia Biosciences (NZ) Ltd., P.O. Box 109-185, Auckland 1149, New Zealand
[3]School of Biological Sciences, The University of Auckland, Private Bag 92-019, Auckland 1142, New Zealand
*Corresponding author: talia.grala@vialactia.com

ABSTRACT

Temporary changes to milking frequency during early lactation can elicit lactation-long changes in milk production. This study tested whether altered milking frequency, immediately post-calving, would affect mammary gene expression during and/or following treatment. Multiparous, grazing dairy cows (n = 150) were allocated to one of five treatments post-calving: milking thrice-daily (3X) for three or six weeks, and twice-daily (2X) thereafter; milking once-daily (1X) for three or six weeks, and 2X thereafter; or milking 2X for the entire lactation. Mammary tissue was biopsied from 12 cows per treatment at three, six and nine weeks post-calving and expression of milk synthesis and apoptosis genes measured using quantitative PCR. The milk fat, lactose and protein genes were down-regulated ($P < 0.05$) in cows milked 1X compared with cows milked 2X. At nine weeks post-calving, cows milked 1X for three weeks had reduced mRNA levels of milk synthesis genes compared with cows milked 1X for six weeks ($P < 0.05$). The expression of apoptosis genes was up-regulated ($P < 0.05$) in cows milked 1X relative to 2X, during and post-treatment. There were no gene expression changes during 3X milking compared with cows milked 2X. Changes in gene expression observed in cows temporarily milked 1X reflect physiological changes consistent with longer-term reductions in milksolids yield.

Keywords: once-a-day; early lactation; carry-over effects.

INTRODUCTION

Increasing milking frequency from twice-daily (2X) to thrice (3X) or four-times (4X) daily increases milk yield (Soberon *et al*., 2010; Wall & McFadden, 2010; Phyn *et al*., 2011). Additionally, increased milking frequency can evoke a positive carry-over effect when cows are switched to 2X milking (Hale *et al*., 2003; Wall *et al*., 2006). Conversely, decreasing milking frequency to once-daily (1X) reduces both milk and milksolids yields (Farr *et al*., 1995; Rémond e*t al*., 1999; Boutinaud *et al*., 2008). Although there appears to be a negative carry-over effect on milk production after cows are switched from 1X to 2X milking, the length and severity of this carry-over effect are unclear. Rémond *et al*. (1999) reported negative carry-over effects in cows milked 1X for six weeks during early lactation, but not in cows milked 1X for three weeks. However, Phyn *et al*. (2011) reported negative carry-over effects in cows milked 1X for three or six weeks.

The negative carry-over effect following reduced milking frequency may be due to a reduction in mammary cell activity, increased cell death or decreased proliferation (Hale *et al*., 2003; Nørgaard *et al.,* 2005; Wall *et al*., 2006) or a combination of the three. Evidence for reduced cell activity is supported by reductions in enzyme activity (Farr *et al*., 1995) and decreased α-lactalbumin and κ-casein mRNA (Boutinaud *et al.,* 2008) in mammary epithelial cells of cows milked 1X. Additionally, a recent microarray study (Littlejohn *et al*., 2010) highlighted down-regulated milk synthesis, and differential mechanical stress and apoptosis signalling between cows milked 1X compared with 2X. Changes in apoptosis gene expression indicate that milking 1X may lead to increased mammary epithelial cell death. However, Bernier-Dodier *et al.* (2010) reported that both mammary epithelial cell apoptosis and proliferation were increased; thus increased cell turnover rather than increased cell death may be responsible for the decrease in milk production. In contrast, there are no consistent reports of changes to mammary enzyme activity, nor cell number when milking frequency is increased (Wall & McFadden, 2010); however, increased extracellular matrix remodelling and neovascularation have been observed (Connor *et al*., 2008).

To our knowledge, there are no published data on gene expression changes following a period of 1X milking and only limited data on mammary gene expression from cows with altered milking frequency in a New Zealand pasture-based dairy system. We hypothesised that the milk yield response to altered milking frequency, both immediate and long-term, would correspond with gene expression changes associated with mammary cell secretory activity and/or number.

TABLE 1: Characteristics of gene-specific quantitative polymerase chain reaction (PCR) assays. UPL = Universal probe library.

Gene[1]	Genbank accession number	Primer direction	Primer sequence[2]	UPL Probe number	Amplicon size (bp)	Average PCR efficiency[3]
Endogenous control genes						
EIF3K	NM_001034489	Forward Reverse	AAGTTGCTCAAGGGGATCG TTGGCCTGTGTCTCCACATA	1	77	1.71
RPS15A	NM_001037443	Forward Reverse	TCAGCCCTAGATTTGATGTGC GCCAGCTGAGGTTGTCAGTA	32	104	1.57
Genes of interest						
ACACA	NM_174224	Forward Reverse	AACATCCCCACGCTAAACAG GAGTCATGCCGTAGTGGTTG	25	61	1.72
B4GALT1	NM_177512	Forward Reverse	CCCAATGAACGACCATAACA CTCCAAAATACTGCACGTAAGGT	76	107	1.68
BAX	NM_173894	Forward Reverse	CCCTTTTGCTTCAGGGTTT TCAGCTTCTTGGTGGATGC	125	108	1.88
BCL2	NM_001166486	Forward Reverse	GCACCTGCACACCTGGAT CTAGGGCCATACAGCTCCAC	75	66	1.80
BECN1	NM_001033627	Forward Reverse	CAAGATTCTGGACCGTGTCA TGGGCTGTGGCAAGTAATG	128	60	1.76
CSN1S1	NM_181029.2	Forward Reverse	TACCCCAGCTGGAAATTGTT ATTCCCTCTTTCATACTGTGAAGTC	73	64	1.55
CSN2	NM_181008.2	Forward Reverse	ATGAAGGTCCTCATCCTTGC CCAGGTACATTGAGTTCTTCCA	21	74	1.54
FAS	NM_174662	Forward Reverse	TCCAGATCTCACGCAAACAG TCCTCTCTCGACTTTTCACCA	101	94	1.66
FASN	NM_001012669.1	Forward Reverse	CCTATGGCCTACAGTGTACAGGA TGATGCACTCGATGTAGTAGGTG	89	78	1.63
LALBA	NM_174378.2	Forward Reverse	CCCAGGCTGAACAGTTAACAA CACTGGTATGAAACGTGGTACAG	24	111	1.71
LTF	NM_180998.2	Forward Reverse	TCGGTTATTCTGGTGCCTTC CACTGTCGTCTCTTTAACAAAAGC	19	74	1.77
PYCARD	NM_174730	Forward Reverse	GTACGGTGCCGAGCTCAC CCTCGGCTAGCACGTTTCT	10	110	1.79

[1]EIF3K = Eukaryotic translation initiation factor 3K; RPS15A= Ribosomal protein S15a; ACACA = Acetyl-CoA carboxylase-α; B4GALT1 = UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1; BAX = Bcl2-associated X protein; BCL2 = B-cell CLL/lymphoma 2; BECN1 = Beclin 1, autophagy related; CSN1S1 = αS1-casein; CSN2 = β-casein; FAS = Fas (TNF receptor superfamily, member 6); FASN = Fatty acid synthase; LALBA = α-lactalbumin; LTF = Lactotransferrin; PYCARD = PYD and CARD domain containing.
[2]Primer sequences are shown 5' to 3'.
[3]PCR efficiency was averaged over two qPCR reactions.

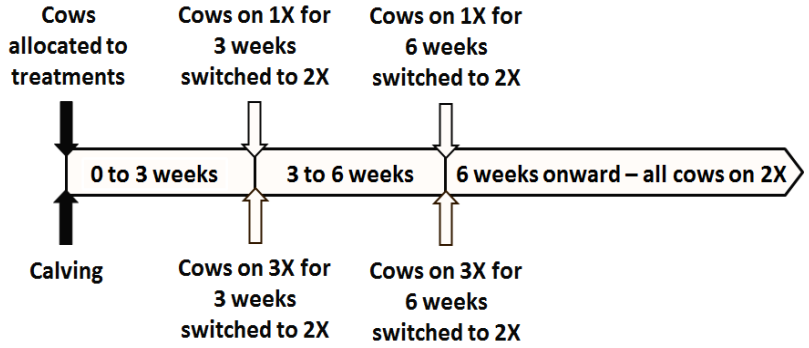
MATERIALS AND METHODS

Experimental design, treatments and tissue sampling

This study was conducted at Lye Farm, DairyNZ (Hamilton; 37°46' S 175°18' E) from July to October 2009. All treatments and measurements were approved by the Ruakura Animal Ethics Committee, Hamilton.

Multiparous, Holstein-Friesian dairy cows (n = 150) grazing pasture were randomly allocated to one of five treatments on the day of calving. Cows were milked 1X for three or six weeks post-calving and 2X thereafter, 3X for three or six weeks and 2X thereafter, or milked 2X for the entire lactation as displayed in Figure 1. Daily milking times were 07:00 h for 1X (24 h milking interval), 07:00 h and 15:00 h for 2X (16/8 h milking interval), and 07:00 h., 15:00 h and 22:00 h for 3X (9/8/7 h milking interval). Milk and milk component yields were measured daily and weekly, respectively, and data reported by Phyn *et al*. (2011). Mammary tissue was collected at three, six and nine weeks post-calving from 12 cows within each treatment using the method described by Farr

FIGURE 1: Experimental timeline. Cows were allocated to treatments immediately post-calving. The four treatment groups detailed were compared to cows milked twice-daily for the entire lactation.



et al. (1996). Resulting biopsies of 70 x 4 mm were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Extraction of RNA and cDNA synthesis

Total cellular RNA was extracted using an RNeasy kit (Qiagen, Hilden, Germany) then treated with DNase (DNA-free kit; Ambion, TX, USA) as per the manufacturer's instructions. RNA quantity and quality were assessed using a NanoDrop ND-1000 (NanoDrop, DE, USA) and a Bioanalyzer 2100 (Agilent, CA, USA). Two micrograms of each sample were used for cDNA synthesis using the Superscript III Supermix kit (Invitrogen, CA, USA). Total RNA was reverse transcribed using 27 µM of random pentadecamer primers. Reverse transcriptase (RT) negative controls (to detect genomic DNA contamination) were generated by excluding the reverse transcriptase enzyme.

Quantitative PCR, mRNA and statistical analysis

Transcripts representing the immune protein lactotransferrin (LTF), milk fat synthesis (fatty acid synthase (FASN); Acetyl Co-A carboxylase-α (ACACA)), lactose synthesis (UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1 (B4GALT1); α-lactalbumin (LALBA)), major milk proteins (β-casein (CSN2); αS1-casein (CSN1S1)), apoptosis (PYD and CARD domain containing (PYCARD); FAS (fas, TNF receptor superfamily, member 6); Bcl2-associated X protein (BAX); B-cell CLL/lymphoma 2 (BCL2)) and autophagy (Beclin 1 (BECN1)) were analysed using RT-quantitative PCR (RT-qPCR). Assays were designed to span an intron-exon boundary of each gene using publicly available gene sequences (NCBI; http://www.ncbi.nlm.nih.gov/gene) and Roche Universal Probe Library design software. Primer sequences and probe numbers are presented in Table 1. RT-qPCR was performed using LightCycler 480 Probes Master reaction mix and assays were analysed on a LightCycler 480 (Roche Diagnostics Mannheim, Germany). Each RT-qPCR reaction consisted of 0.5 µM of each primer and 0.05 µM of probe. Standard cycling conditions were used (95°C for 10 minutes, (95°C for 10 seconds, 60°C for 30 seconds) x 50 cycles, 40°C for 40 seconds).

Each RT-qPCR run included a no-template control, a reverse transcriptase negative control, and three inter-assay calibrators. Triplicate measurements were performed for all samples and standard curves. The Roche LightCycler 480 software was used to analyse gene expression using relative standard curve second-derivative maximum analysis. A five-point standard curve of serial cDNA dilutions was used. Endogenous control genes were tested across all samples and their suitability determined using Normfinder and GeNorm (Andersen *et al.,* 2004; Vandesompele *et al*., 2002). Gene expression of eukaryotic translation initiation factor (EIF3K) and ribosomal protein S15a (RPS15A) did not change with the different treatments. The Roche LightCycler 480 Software was then used to perform advanced relative quantification analysis of gene expression using the normalisation factor of EIF3K and RPS15A. The geometric mean of the inter-run calibrators was calculated for each plate and applied to each sample.

Of the 174 successful mammary biopsies, RNA was extracted from 168 (six samples were either too small or degraded). Final numbers for each milking frequency were 65 1X (three weeks, n = 32; six weeks, n = 33), 34 2X, and 69 3X (three weeks, n = 29; six weeks, n = 40). Samples were evenly spread across the time-points (three weeks, n = 56; six weeks, n = 57; and nine weeks n = 55). Data collected were analysed after $\log_{10}$ transformation with a repeated measures analysis using the GenStat procedure AREPMEASURES (Payne *et al*., 2009). The data were then analysed at each time-point as a mixed model with treatment as a fixed effect and cow as a random effect. Data are presented as least squares means and standard error of the difference between the means.

RESULTS

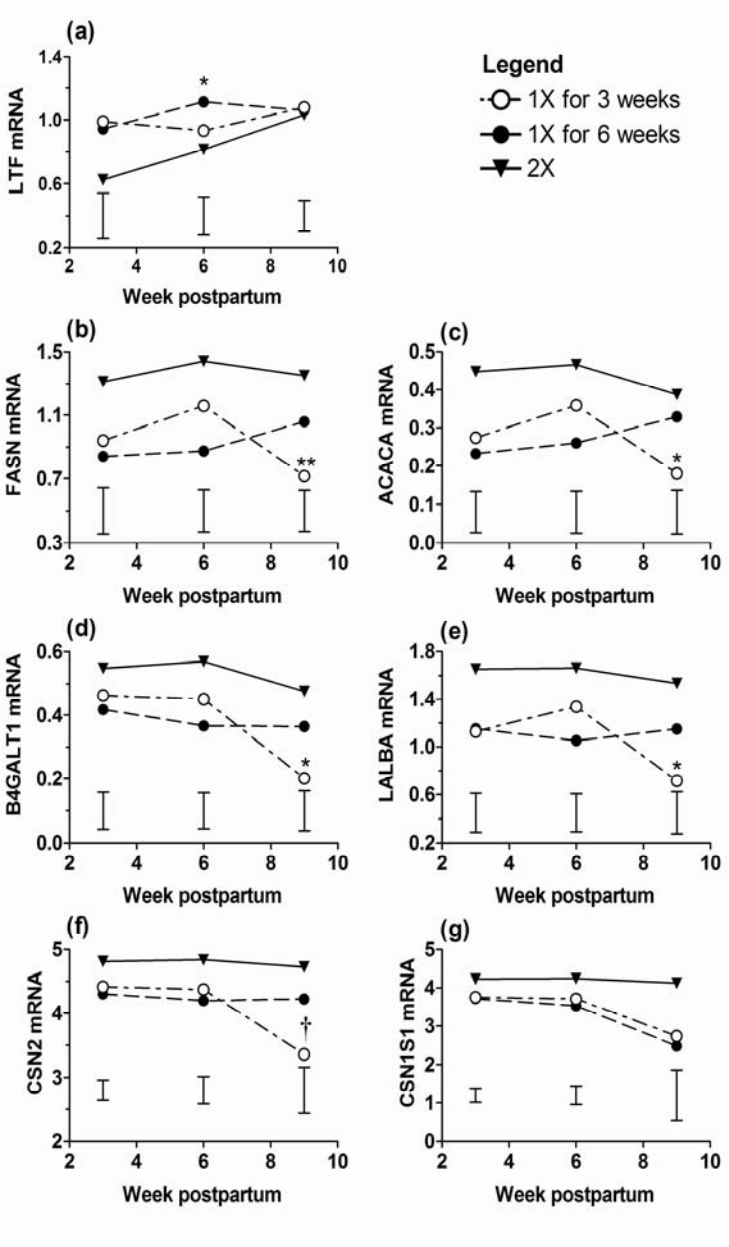
Transcription for all the mammary genes measured was different in cows milked 1X compared with those milked 2X. Expression of LTF was greater (2.2-fold; $P < 0.01$) at three weeks post-calving in cows milked 1X (Figure 2a). At six weeks LTF expression was greater ($P < 0.05$) in cows milked 1X for six weeks compared with those milked 1X for three weeks. Expression of FASN,

ACACA, B4GALT1, LALBA, CSN2 and CSN1S1 (Figure 2b-g) was reduced (≥1.3-fold; P <0.01) at three and six weeks in cows milked 1X post-calving. At nine weeks post-calving, cows milked 1X maintained decreased (33-fold; P <0.01) CSN1S1 expression relative to cows milked 2X. At nine weeks cows milked 1X for three weeks had decreased FASN, ACACA, B4GALT1, and LALBA expression (all P <0.05), and tended to have decreased CSN2 (P = 0.06) expression, compared with cows milked 1X for six weeks.

Expression of FAS and PYCARD was greater (≥1.5-fold; P <0.05) in cows milked 1X compared with those milked 2X (Figure 3a and 3b). At nine weeks expression tended to be greatest in cows milked 1X for three weeks (PYCARD, P = 0.07). Cows milked 1X had a greater (P <0.01) ratio of BAX:BCL2 at three weeks post-calving. At nine weeks, a trend was present (P = 0.08) as only cows milked 1X for three weeks had an increased BAX:BCL2 ratio relative to cows milked 2X (Figure 3c).

The mRNA levels of milk fat, protein and lactose synthesis genes were not altered in cows milked 3X relative to 2X (T.M. Grala, Unpublished data). However, at nine weeks post-calving, ratios of BAX:BCL2 (1.5-fold; P = 0.06; Figure 3c) and BECN1:BCL2 (1.6-fold; P <0.05; Figure 3d) were lower in cows milked 3X compared with cows milked 2X. Additionally, cows milked 3X for six weeks had greater FAS expression (P <0.05; Figure 3a) compared with cows milked 2X.

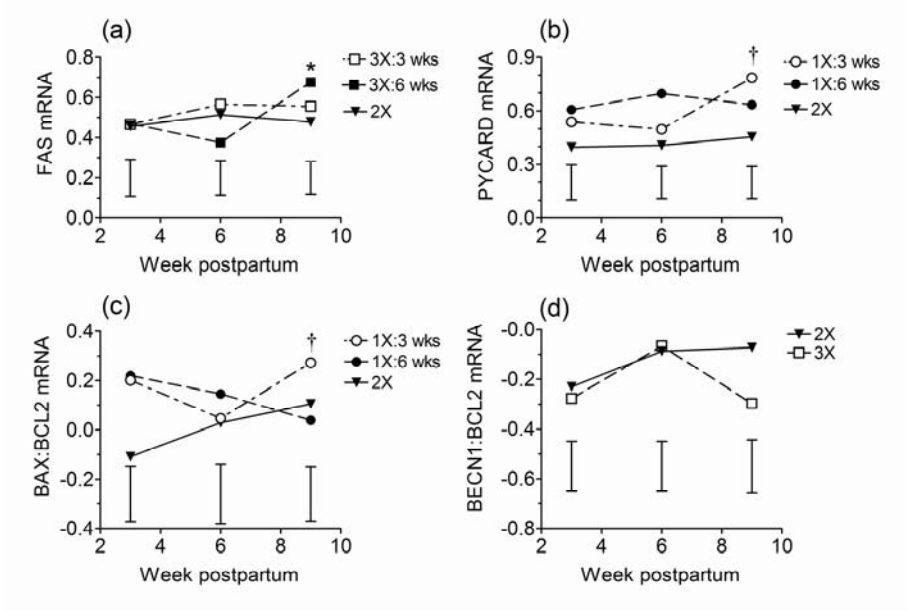
FIGURE 2: Relative mRNA expression of milk fat, lactose and protein synthesis genes in mammary at three, six and nine weeks post-calving in Holstein-Friesian dairy cows milked either twice daily (2X; control) or once daily for the first three or six weeks post-calving (1X for three weeks and 1X for six weeks, respectively). (a) LTF (lactotransferrin); (b) FASN (fatty acid synthase); (c) ACACA (acetyl-CoA carboxylase-α); (d) B4GALT1 (UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1); (e) LALBA (α lactalbumin); (f) CSN2 (β-casein); and (g) CSN1S1 (α -casein). Data were $\log_{10}$ transformed and are presented as least squares means with the standard error of the difference between the means, n = 12 per treatment. Milking frequency by duration interactions are noted on the graphs. ** = P <0.01; * = P <0.05; † = P <0.1.



DISCUSSION

The expression of milk fat, lactose and protein genes was reduced in cows milked 1X compared with 2X. This result is consistent with decreased activity of key mammary enzymes, ACACA, galactosyltransferase (GT) and FASN, previously reported in cows milked 1X (Farr *et al*., 1995). Additionally, Singh *et al.* (2008) found decreased expression of LALBA and CSN1S1 mRNA and LALBA protein by 24 hours post-milking. In the present study, milk synthesis gene expression remained lower after the cows were switched from 1X to 2X milking, indicating long-term negative effects on milk production (Phyn *et al.,* 2011). Furthermore LTF expression was increased in cows milked 1X compared with cows milked 2X during milking frequency treatment. LTF is a major component of the innate immune system and increases in LTF expression in response to involution and 1X milking have previously been reported (Nuijens *et al*., 1996; Littlejohn *et al*., 2010). LTF also promotes apoptosis, through

FIGURE 3: Relative mammary expression of apoptosis genes at three, six and nine weeks post-calving in Holstein-Friesian dairy cows milked either twice daily (2X; control;); once daily for the first three or six weeks post-calving (1X for three weeks, and 1X for six weeks, respectively) or thrice daily for the first three or six weeks post-calving (3X for three weeks and 3X for six weeks, respectively). (a) FAS (Fas; TNF receptor superfamily, member 6); (b) PYCARD (PYD and CARD domain containing); (c) Ratio of BAX:BCL2; and (d) Ratio of BECN1:BCL2. Data were $\log_{10}$ transformed and are presented as least squares means with the standard error of the difference between the means, n = 12 per treatment. Milking frequency by duration interactions are noted on the graphs. * = P <0.05; † = P <0.1.



activation of FAS death receptor (Legrand *et al.,*2008). In the current study, FAS and PYCARD expression was up-regulated in cows milked 1X both during and after treatment, indicating activation of the death receptor-initiated cell death pathway, extrinsic apoptosis. The increased BAX:BCL2 expression indicates that intrinsic apoptosis, initiated by cell stress, was also stimulated at three weeks. Together these results indicate that while cell stress may play a role in the immediate cell loss, long-term continued apoptosis may be caused by extracellular factors. To summarise, sustained milk yield reductions following a period of 1X milking (Phyn *et al*., 2011) may result from both a decrease in mammary cell activity and number.

The absence of significant gene expression changes in cows milked 3X indicates that short-term increases in milk yield (Phyn *et al*., 2011) were not the result of increased mammary epithelial cell activity or reduced cell death. Results from enzyme activity and apoptosis assays have indicated no consistent effect of increased milking frequency relative to cows milked 2X (Hale *et al*., 2003; Nørgaard *et al*., 2005). However, the milk yield response may result from increases in mammary cell proliferation or extracellular matrix remodelling (Connor *et al*., 2008). The magnitude of the milk yield change in cows milked 3X was smaller than in cows milked 1X (Phyn *et al*., 2011) so it is possible that that this study lacked the statistical power to observe small transcriptional changes in the subset of 3X cows that were biopsied. Interestingly, at nine weeks, FAS expression was increased in cows milked 3X compared with cows milked 2X (P <0.05), indicating that once cows were switched to 2X milking, extrinsic apoptosis increased. Increased apoptosis may explain the absence of a significant long-term increase in milk yield in cows milked 3X in this study (Phyn *et al*., 2011).

Gene expression of ACACA, FASN, LALBA and B4GALT exhibited milking frequency by duration effects at nine weeks. These effects were the result of reduced expression in cows milked 1X for three weeks relative to those milked 1X for six weeks. This result is surprising given a similar negative carry-over effect on milk and milksolids yields was reported for cows milked 1X for three weeks or six weeks post-calving (Phyn *et al*., 2011). Interestingly, the inverse occurs in PYCARD gene expression, indicating greater apoptosis in cows milked 1X for three weeks compared with cows milked 1X for six weeks. Therefore, in cows milked 1X for three weeks, greater apoptosis and reduced milk synthesis occurred at nine weeks compared with cows milked 1X for six weeks and those milked 2X from calving. An additional biopsy at 12 weeks would have been beneficial to determine whether these effects are long-lasting and whether gene expression in the cows milked 1X for six weeks exhibited a similar pattern. Further study will be necessary to fully elucidate the effect of the duration of a decrease in milking frequency.

In conclusion, increases in milk yield during 3X milking were not reflected in gene expression measurements; however other mechanisms, such as cell proliferation, may be the cause of the short-term increase. Sustained milk yield reductions as a result of only three weeks of 1X milking may be caused by both a decrease in mammary cell activity and in

cell number, as indicated by the changes in milk synthesis and apoptosis gene expression. These effects need to be considered before using alternative milking frequencies during early lactation.

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