Bioinformatics and Biology Insights

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ORIGINAL RESEARCH

Expression of Metabolic, Tissue Remodeling, Oxidative Stress, and Inflammatory Pathways in Mammary Tissue During Involution in Lactating Dairy Cows

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Abstract: Histological and functional changes associated with involution in the mammary gland are partly regulated by changes in gene expression. At 42 d postpartum, Holstein cows underwent a period of 5 d during which they were milked 1X daily until complete cessation of milking. Percutaneous mammary biopsies (n = 5/time point) were obtained on d 1, 5, 14, and 21 relative to the start of 1X milking for transcript profiling via qPCR of 57 genes associated with metabolism, apoptosis/proliferation, immune response/inflammation, oxidative stress, and tissue remodeling. Not surprisingly, there was clear downregulation of genes associated with milk fat synthesis (*FASN*, *ACACA*, *CD36*, *FABP3*, *SCD*) and lipid-related transcription regulation (*SREBF1*, *SREBF2*). Similar to milk fat synthesis-related genes, those encoding proteins required for glucose uptake (*SLC2A1*), casein synthesis (*CSN2*, *CSN3*), and lactose synthesis (*LALBA*) decreased during involution. Unlike metabolic genes, those associated with immune response and inflammation (*C3*, *LTF*, *SAA3*), oxidative stress (*GPX1*, *SOD2*), and pro-inflammatory cytokine signaling (*SPP1*, *TNF*) increased to peak levels by d 14 from the start of 1X milking. These adaptations appeared to be related with tissue remodeling as indicated by upregulation of proteins encoding matrix proteinases (*MMP2*), *IGFBP3*, and transcriptional regulation of apoptosis/cell proliferation (*MYC*). In contrast, the concerted upregulation of *STAT3*, *TGFB1*, and *TGFB1R* during the first 14 d was suggestive of an activation of these signaling pathways probably as an acute response to regulate differentiation and/or mammary cell survival upon the onset of a marked pro-inflammatory and oxidative stress response induced by the gradual reduction in milk removal. Results suggest a central role of *STAT3*, *MYC*, *PPARG*, *SREBF1*, and *SREBF2* in regulating concerted alterations in metabolic and cell survival mechanisms, which were induced partly via oxidative stressed-triggered inflammation and the decline in metabolic activity.

Keywords: transcriptomics, lactation, involution, dairy

Bioinformatics and Biology Insights 2010:4 85–97

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Introduction

Involution is the normal process by which the mammary gland returns to its non-lactating state.[1,2] Proposed mechanisms of involution include local chemical feedback by milk constituents, formation of other substances in stored milk, milk stasis and udder distention, and stretching of the cells that lead to the loss of secretory activity.[3,4] At the tissue level, the process of mammary gland involution involves invasion of leukocytes, increased epithelial cell death (through apoptosis or autophagy), and/or proliferation of connective tissue.[5–7] Unlike the mouse, relatively little research in the cow has employed gene expression technologies such as microarray and/or quantitative RT-PCR (qPCR)[8,9] to study the molecular events associated with involution. Those studies conducted to date in cows undergoing normal or forced involution (ie, after sudden cessation of milking) have shown downregulation of mRNA abundance and concentration of enzymes related to the synthetic capacity of epithelial cells,[7] downregulation of milk protein gene expression, upregulation of mRNA and synthesis of lactotransferrin,[10,11] and upregulation of pro-apoptotic proteins (BAX) coupled with downregulation of beta integrins.[8]

Several transcription regulators coordinating the process of involution, namely in non-ruminants, have been identified. For example, signal transducer and activator of transcription 3 (acute-phase response factor) (*STAT3*) was postulated as a death factor in differentiated mouse mammary epithelium.[12] Upregulation of *STAT3* has been observed during involution in bovine[9] and ovine[13] mammary tissue. Transgenic mouse research demonstrated that v-myc myelocytomatosis viral oncogene homolog (avian) (*MYC*) functions to enhance apoptosis and involution in the mammary gland.[14] Transforming growth factor, beta 1 (TGFB1) stimulates apoptosis (programmed cell death type I) as well as autophagy (programmed cell death type II) in bovine mammary cells.[6] Its synthesis increases during the dry period in cows[15] and it is controlled partly by somatotropic-axis hormones, eg, *in vitro* studies in mammary epithelial cells demonstrated that *TGFB1* expression is increased by estradiol and progesterone, while it is decreased by GH and IGF-1.[16]

A recent microarray study of the involution process in the short-term following cessation of milking revealed a strong upregulation of immune and antioxidant-related genes.[9] Although these results were indicative of novel features of the involution process, statistical analysis of microarray data did not seem to include a multiple testing correction of the raw *P*-values.[17] Furthermore, there was no evaluation of suitable internal control genes (ICG) for normalization of qPCR data. The primary objective of the present study was to specifically evaluate the temporal expression of gene networks associated with metabolism, transcriptional control of apoptosis and cell proliferation, mammary tissue remodeling, and immune/inflammatory response both in the short-term after shifting from 2X to 1X daily milking (5 d) and 9 and 16 d (long-term) after complete cessation of milking. To that end, we first performed a search of suitable ICG[18] that could be used to normalize mRNA abundance data from this type of studies. Finally, milk production and composition during 1X milking and blood profiles of metabolites and insulin before and after cessation of milking were measured and related to gene expression patterns.

Materials and Methods

Animals and sampling

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee. Twelve Holstein cows were milked twice daily (0600 and 1700 h) until 42 d postpartum. For a period of 5 d cows were milked once daily (0600 h) and then dried-off (complete cessation of milking). To ensure that quarters to be biopsied were healthy, several measurements were considered: 1) a California Mastitis Test (CMT) was conducted before the last milking to rule out subclinical mastitis; 2) a milk sample from each quarter on the last milking was collected for bacteriological culture (McConkey agar and Blood Agar) to assure bacteriological health; and 3) during the last 8 d of milking, somatic cell count (SCC, cells/mL; DHIA Laboratory, Dairy One, Ithaca, NY) was measured and evaluated as an indicator of udder health. As a prophylactic measure, each quarter was infused with intramammary cephapirin benzathine (Cefa-Dri®, Fort Dodge Animal Health, Division of Wyeth) following the last milking. Individual body weights were recorded at the beginning and at the end of the study.

Milk production was electronically recorded at each milking. Milk samples for composition were collected the last 3 d of twice-a-day milking and the 5 d of once-a-day milking. A composite sample based on milk yield was obtained when cows were being milked twice a day (at 0600 and 1700 h). Samples were stored with Bronopol® preservative at 4 °C prior to analysis of fat, protein, lactose, urea-N (MUN), and somatic cell count (SCC) (cells/mL) (DHIA Laboratory, Dairy One, Ithaca, NY).

Blood was collected from the coccygeal vein to isolate serum for metabolite analysis. Samples were taken daily from the last day of twice-a-day milking (d -1) until d 21, and centrifuged for 20 min at 2,000 rpm after allowing the clot to form. Samples at d 1, 5, 14, and 21 were used to determine concentrations of total protein, glucose, hydroxybutyrate (BHBA), and non-esterified fatty acids (NEFA) using commercial kits at the Clinical Pathology Laboratory of the School of Veterinary Medicine, University of Illinois. Samples were taken prior to biopsies to avoid any confounding effect of the procedure on variables of interest. Insulin was measured at d 1 and 14 using a commercial bovine insulin ELISA kit (Mercodia, Uppsala, Sweden).

Mammary gland biopsies

Mammary gland tissue was harvested on d 1, 5, 14, and 21 (n = 5/time point) from the initiation of 1X daily milking via percutaneous biopsy.[18] The procedure was conducted under mild general anesthesia with Xylazine (Phoenix Pharmaceutical, Inc., St. Josephs, MO; 0.75 mL total intravenously; 20% solution) and local anesthesia with lidocaine HCl (5 mL total subcutaneously; 2% solution). The midpoint area to either side of the udder was clipped and surgically scrubbed before biopsy. A 3-cm incision was made through the skin and subcutaneous tissue, which was afterwards detached from the mammary capsule at the site of incision. A clear image of the capsule was always necessary to introduce the biopsy instrument to prevent it from tangling with the subcutaneous tissue. A core of mammary tissue was removed from rotating a trocar that was attached to a cordless drill at high speed and pushing into the udder.[19] Once the core was cut, the retractable blade was extended and rotated for 3–4 seconds to sever the core of tissue. The capsule was not cut at any time prior to introducing the trocar. Pressure was applied with sterile gauze to the biopsy point until bleeding ceased. Eleven-mm Michel wound clips (#9534503, Henry Stein, Inc., Melville, NY) were used to close the skin incision, which was then covered with iodine ointment (#1048023, Povidone Ointment, Henry Schein, Inc., Melvine, NY). Portions of extracted tissue were weighed for immediate homogenization in 5 mL ice-cold TRIzol reagent (Invitrogen, Carlsbad, CA), snap-freezing in liquid-N_2 and storage at −80 °C until use. Homogenized samples were stored at −80 °C until RNA extraction.

Extraction and purification of total RNA, cDNA synthesis, evaluation of ICG, and qPCR

Total RNA was extracted from mammary gland samples and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Residual DNA was removed with the RNase-Free DNase Set (Qiagen, Valencia, CA, USA). RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA (A260/A280) was above 2.0. RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies). Samples had a median RNA integrity value of 8.06 ± 0.15.

Primers were designed with Primer Express software version 3.0 (Applied Biosystems, Foster City, CA, USA) using default settings, except for amplicon length (100–150 bp). Primers were designed across exon/exon junctions when possible and were aligned against public databases using BLAST software available at National Center for Biotechnology Information (NCBI) (Suppl. Table 1). When designing primers, primer specificity and absence of primer-dimers were evaluated. Evaluation of primers was performed using 3 separate tests: 1) PCR products were visualized in a 2% agarose gel (Invitrogen) stained with ethidium bromide to check for the absence of primer-dimers and the presence of bands at the respective amplicon size; 2) PCR products were sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana-Champaign, and resulting data were aligned against the NCBI nucleotide database (Suppl. Table 2); and 3) the presence of a single PCR product was verified by the occurrence of a single peak in the dissociation

step after qPCR. Primers were used only when they passed all required tests.

Target genes

Fifty-seven genes encompassing networks with central biological functions during involution in non-ruminants were evaluated (Fig. 1). Several of these genes have known relationships in, pathways which coordinate metabolism in mammary cells as well as tissue remodeling. A portion of the assessed RNA was diluted to 100 mg/L using DNase/RNase free water prior to cDNA synthesis. Sufficient cDNA was prepared at the outset to run all selected genes. Each cDNA was synthesized by RT using 100 ng RNA, 1 µg dT18 (Operon Biotechnologies, AL), 1 µL 10 mmol/L dNTP mix (Invitrogen), 1 µL Random Primers (Invitrogen), and 7 µL DNase/RNase free water. The mixture was incubated at 65 °C for 5 min and kept on ice for 3 min. A total of 9 µL of Master Mix composed of 4.5 µL 5X First-Strand Buffer, 1 µL 0.1 M DTT, 0.25 µL (100 U) of SuperScriptTM III RT (Invitrogen), 0.25 µL of RNase Inhibitor (Promega), 3 µL DNase/RNase free water was added. The reaction was performed in an Eppendorf Mastercycler® Gradient using the following temperature program: 25 °C for 5 min, 50 °C for 60 min and 70 °C for 15 min. cDNA was then diluted 1:3 with DNase/RNase free water.

For qPCR analysis, 4 µL of diluted cDNA were combined with 6 µL of a mixture composed of 5 µL 1x SYBR Green master mix (Applied Biosystems), 0.4 µL each of 10 µM forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA), and 0.2 µL DNase/RNase free water in a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate to control reproducibility of the essay and a 6 point relative standard curve (4-fold dilution) plus the non-template control were used (User Bulletin #2, Applied Biosystems, CA). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) using the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15s at 95 °C, and 1 min at 60 °C. The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95 °C for 15s plus 65 °C for 15s following the last cycle. Complete details regarding qPCR protocol

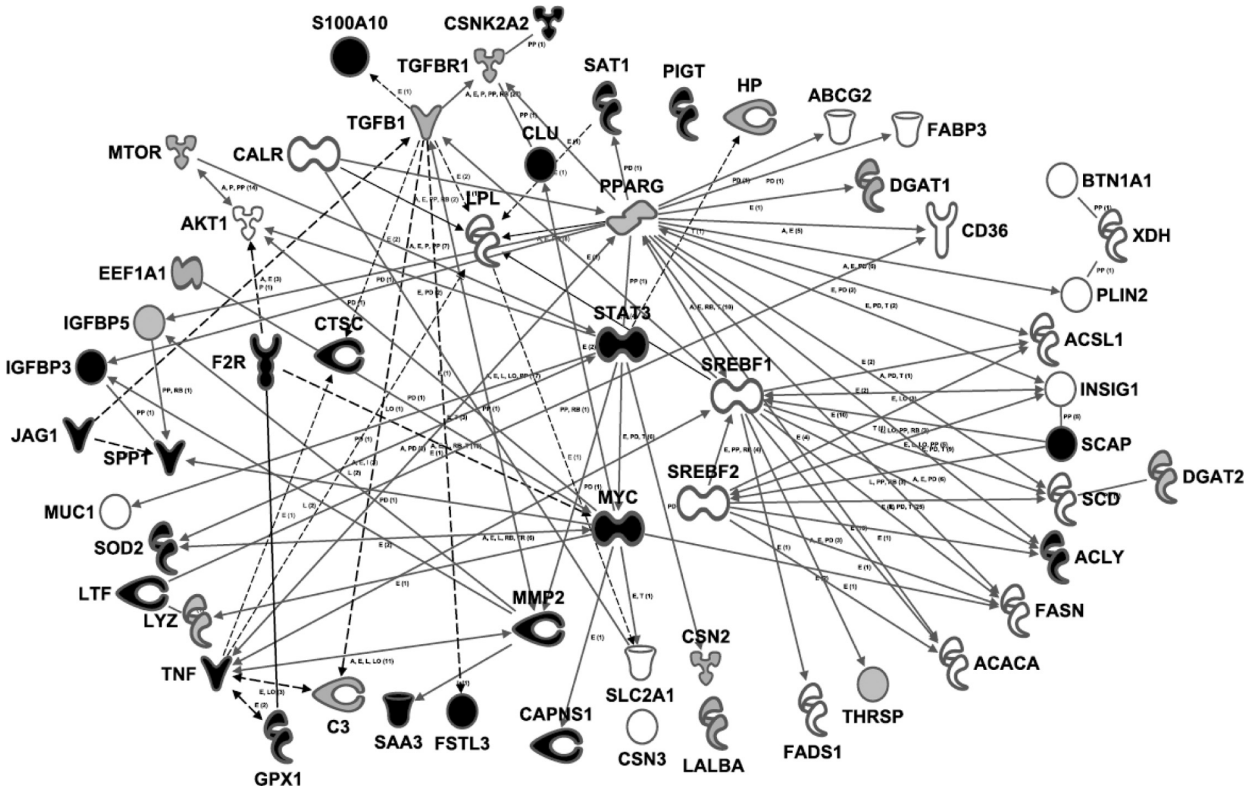

Figure 1. Currently-known relationships among genes analyzed based on manually curated examination of the published literature within the Ingenuity Pathway Analysis® Knowledge Base. Upregulated (black background) and downregulated (white background) genes at d 21 relative to the start of 1X d milking (d 1) are shown for comparison. Gray background denotes that expression was not different between the time points.

can be found at http://docs.appliedbiosystems.com/pebiodocs/04364014.pdf. Data were analyzed with the 7900 HT Sequence Detection Systems Software (version 2.2.3, Applied Biosystems).

Statistical analysis

Prior to statistical analysis all qPCR data were tested for normality using PROC Univariate of SAS (SAS Inst., Cary, NC, USA). The data were considered not normally distributed if the Shapiro-Wilk test had $P \leq 0.05$.[20] All genes had a Shapiro-Wilk test with $P < 0.05$ and were normalized by log-transformation. The normalized log-transformed data were subjected to PROC REG of SAS and data points with studentized residuals >2.5 were considered outliers and removed from final analysis. The final dataset was analyzed using PROC MIXED in SAS. The statistical model consisted of time (ie, day relative to start of 1X daily milking) as a fixed effect and cow as random effect. Blood metabolite and insulin concentration, milk production, composition, and yield also were analyzed using PROC MIXED.

Results and Discussion

Physiological context

The cessation of milk removal begins the involution process and initially results in distension of the mammary gland followed by a decline in the rate of milk secretion, an increase in intramammary pressure, a decrease in mammary blood flow, increased tight-junction permeability and lactose efflux, and an inflammatory response.[3,8,9] In dairy cattle, these changes occur within 16 to 18 h of milk accumulation, are followed by complete cessation of milk secretion by approximately 30 h, and culminate with an increase in mammary epithelial cell apoptosis by 3 to 8 d after the last milking.[8,21] Lactation can be fully restored in cows after 7 d without milking and partially restored after 11 d,[22] implying that significant cell loss through apoptosis does not begin until after 7 d.

Recent studies have used transcriptomics to examine genes and signaling pathways that are affected during the early stages (ie, within the first 8 days of cessation of milking) of mammary involution in non-pregnant lactating cows.[8,9] It is implicit that the use of that type of model would allow the discovery of tissue factors that regulate the process of involution without the confounding effects of pregnancy-related hormones. The present experiment utilized non-pregnant cows at peak lactation (42 d post-partum; Fig. 2) and focused on the coordinated adaptations of genes across metabolic pathways, immune response, oxidative stress, and markers of apoptosis and cell proliferation during a 21 d period (Tables 1 and 2).

Evaluation of ICG for normalization

We have previously published suitable approaches for evaluation of ICG in bovine tissues.[18,23] For the present study, we mined microarray data from these cows (P. Piantoni, S. L. Rodriguez-Zas, R. E. Everts, H. A. Lewin, W. L. Hurley, and J. J. Loor, unpublished results) using the approaches outlined in our previous publications to identify 8 putative ICG (see supplemental materials). Aquaporin 11 (*AQP11*), eukaryotic translation initiation factor 2B, subunit 2 beta (*EIF2B2*), SH3-domain GRB2-like endophilin B2 (*SH3GLB2*), S100 calcium binding protein A16 (*S100A16*), mitochondrial GTPase 1 homolog (*MTG1*), mitochondrial ribosomal protein L39 (*MRPL39*), ribosomal protein S15a (*RPS15A*), and protein phosphatase 1, regulatory (inhibitor) subunit 11 (*PPP1R11*) were initially selected for expression stability analysis using geNorm. *PPP1R11*, *MTG1*, *RPS15A*, and *MRPL39* were the most stable (see details of analysis in supplemental materials and Suppl. Figs. 2–4) using previously-published protocols[18,23] and were used as ICG to normalize qPCR data. In addition, these genes were found to be suitable ICG in mammary parenchyma and stroma of pre-weaned calves.[24]

Mammary gland metabolism

Milk production and yields of milk fat, protein, and lactose decreased when cows were shifted from 2X to 1X daily milking (Fig. 2 and supplemental material). These results and the numerical increase in milk SCC (supplemental material)[25] were expected responses. From a physiological standpoint, reduced milking frequency represented a cue to signal lower mammary metabolic rate, hence, triggering systemic homeorhetic adaptations favoring use of nutrients (eg, glucose) for anabolic processes in other tissues. For example, greater blood insulin and insulin sensitivity would promote glucose use by adipose tissue and would prevent lipolysis, leading to lower blood NEFA and their decreased availability for oxidation and ketogenesis in liver, in turn resulting in lower blood BHBA (Fig. 2).

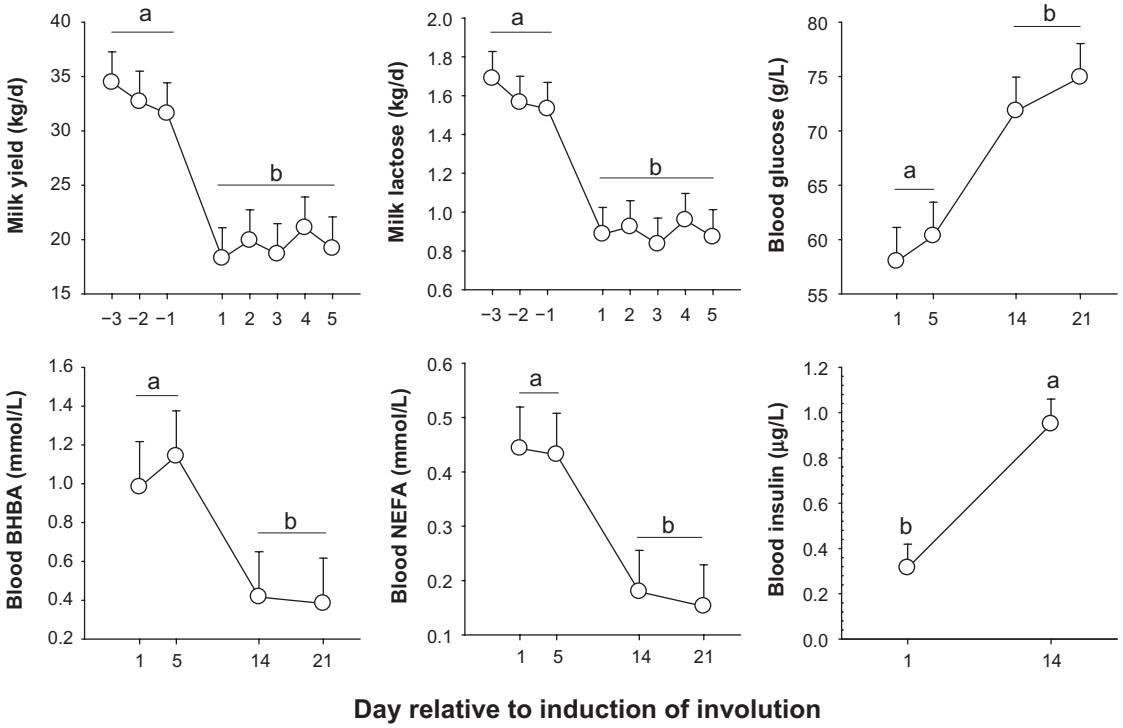

Figure 2. Milk production, lactose yield, and blood metabolites and insulin during 1X milking (d 1 to 5) or after cessation of milking (d 5 to 21). Different letters denote significant effects ($P < 0.05$) due to day relative to induction of involution.

The reductions in yield of fat, protein, and lactose due to 1X daily milking were at least in part related to the gradual downregulation of the cluster of genes associated with fatty acid uptake (CD36 molecule (thrombospondin receptor), *CD36*; lipoprotein lipase, *LPL*), glucose transport (solute carrier family 2 (facilitated glucose transporter), *SLC2A1*), lactose synthesis (alpha-lactalbumin, *LALBA*), *de novo* fatty acid synthesis (acetyl-CoA carboxylase-alpha, *ACACA*; fatty acid synthase, *FASN*), intracellular long-chain fatty acid transport (fatty acid binding protein 3, *FABP3*), esterification (diacylglycerol O-acyltransferase homolog 1, *DGAT1*), desaturation (stearoyl-CoA desaturase, SCD; delta-6 fatty acid desaturase, *FADS2*), and lipid droplet formation (adipophilin, *ADFP*; butyrophilin, subfamily 1, member A1, *BTN1A1*; xanthine dehydrogenase, *XDH*) (Table 1). The expression of the transporter ATP-binding cassette, sub-family G (WHITE), member 2 (*ABCG2*) also was gradually downregulated during cessation of milking, supporting its role as candidate gene for milk production traits.[26] The reduction in mRNA expression of lipogenic enzymes was likely associated with a decrease in activity as shown recently for *ACACA* and *FASN*.[7] Recent work with cows under a reduced milking frequency protocol[27] also found downregulation of beta casein (*CSN2*), kappa casein (*CSN3*), and *LALBA* as well as several lipogenic genes. Studies of mammary involution regulation in mice showed that downregulation of *CSN2* during milk stasis is a signature of increased locally-produced serotonin (via tryptophan hydroxylase), which acts as an autocrine-paracrine signal regulating early involution.[28] Recent data support a similar mechanism in the bovine mammary gland[29] and revealed that *LALBA* also is a target of serotonin *in vitro*.[30]

Several of the lipogenic genes are controlled, at least in non-ruminants, by a transcriptional network encompassing peroxisome proliferator-activated receptor gamma (*PPARG*), sterol regulatory element binding transcription factor 1 (*SREBF1*), and *SREBF2* (Fig. 1). We have previously shown that *PPARG* expression in bovine mammary increases at the onset and throughout lactation, and may partly account for the concomitant upregulation of both *SREBF1* and *SREBF2*.[31] The fact that *PPARG* expression was not affected during cessation of milking does not argue against a role for this nuclear receptor in activating the mammary lipogenic program

Table 1. Expression profiles in mammary tissue of genes associated with milk component synthesis during a shift from 2X to 1X daily milking (day 1 to 5) followed by complete cessation of milking (day 5 to 21).

Gene category	Cellular function	Day relative to induction of involution				SEM	*P* =
		1	5	14	21		
Milk fat synthesis		Log-transformed mRNA abundance					
ACACA	*de novo* FA synthesis	4.82^{a}	4.23^{ab}	3.78^{bc}	3.50^{c}	0.24	0.03
ACSL1	FA acid activation	4.58^{a}	4.50^{ab}	4.15^{ab}	3.97^{b}	0.17	0.09
ACLY	Cytosolic citrate synthesis	3.93^{a}	4.16^{a}	4.57^{b}	4.70^{b}	0.12	0.01
ADFP	Lipid droplet formation	4.92^{a}	4.45^{a}	3.69^{b}	3.44^{b}	0.24	0.02
BTN1A1	Lipid droplet formation	4.63^{a}	4.07^{ab}	2.81^{bc}	2.09^{c}	0.48	0.03
CD36	FA uptake	4.67^{a}	4.24^{ab}	3.55^{c}	3.37^{c}	0.26	0.03
DGAT1	Esterification	4.49^{a}	4.33^{ab}	4.23^{b}	4.31^{ab}	0.08	0.04
DGAT2	Esterification	4.03	4.41	4.48	4.48	0.21	0.37
FABP3	Intracellular FA transport	4.83^{a}	3.89^{ab}	3.01^{b}	2.59^{b}	0.42	0.03
FADS1	Delta-6 desaturation	4.40^{a}	3.63^{ab}	3.11^{b}	2.94^{b}	0.35	0.03
FASN	*de novo* FA synthesis	4.57^{a}	3.86^{ab}	3.19^{b}	2.90^{b}	0.36	0.05
GPAM	Esterification	4.11	4.12	3.57	3.04	0.39	0.19
INSIG1	Lipogenesis regulation	4.02	3.76	3.51	2.93	0.30	0.11
LPL	Blood triacylglycerol hydrolysis	4.78^{a}	4.03^{ab}	3.14^{b}	2.84^{b}	0.42	0.04
PPARG	Transcription regulation	3.79	3.88	3.89	4.02	0.11	0.39
SCAP	SREBF chaperone	4.07^{a}	4.14^{ab}	4.21^{b}	4.27^{b}	0.05	0.08
SCD	Delta-9 desaturation	4.16^{a}	3.57^{ab}	2.63^{b}	1.94^{b}	0.50	0.05
SLC2A1	Glucose transport	4.11^{a}	3.85^{a}	3.36^{b}	3.24^{b}	0.16	0.02
SREBF1	Transcription regulation	4.66^{a}	4.55^{a}	3.80^{b}	3.60^{b}	0.20	0.01
SREBF2	Transcription regulation	4.07^{a}	3.93^{ab}	3.82^{bc}	3.62^{c}	0.10	0.02
THRSP	Transcription regulation	4.37^{ab}	4.38^{a}	4.17^{b}	4.22^{ab}	0.10	0.09
XDH	Lipid droplet formation	4.49^{a}	4.25^{ab}	3.65^{bc}	3.17^{c}	0.24	0.04
Milk protein and lactose synthesis							
CSN2	Beta casein synthesis	2.23^{a}	1.91^{ab}	1.34^{b}	1.27^{b}	0.33	0.07
CSN3	Kappa casein synthesis	3.78^{a}	3.42^{ab}	2.97^{b}	2.61^{b}	0.25	0.06
LALBA	Alpha lactalbumin synthesis	3.55^{a}	3.18^{a}	1.96^{b}	1.99^{ab}	0.55	0.05
LTF	Lactotransferrin synthesis	3.19^{a}	3.79^{b}	4.93^{c}	4.79^{c}	0.17	0.0002
LYZ1	Lysozyme 1 synthesis	3.37	3.25	3.89	3.97	0.23	0.16
Milk production							
ABCG2	Transport	4.53^{a}	4.17^{ab}	3.34^{b}	3.16^{b}	0.32	0.03

because we demonstrated that the PPARγ-specific ligand rosiglitazone led to upregulation of *SREBF1* and a number of lipogenic genes without affecting *PPARG* expression.[32] The downregulation of the putative PPARγ targets *SREBF1*, *ACACA*, *FASN*, and *DGAT1* (Table 1) provides additional support for a role of this nuclear receptor in regulation of milk fat synthesis during lactation.[31] In rodents evidence indicates that SREBF2 can regulate expression of several lipogenic genes,[33] ie, it overlaps in that function with SREBF1 (eg, Fig. 1). Our results support the idea that both transcription factors may have overlapping functions during milk fat synthesis. Together with previous data,[31,32] we provide further evidence of the central role of PPARG, SREBF1 and SREBF2 in mammary epithelial cells during lactation.

The upregulation of ATP-citrate lyase (*ACLY*) after cessation of milking was unexpected because, although this enzyme is not the primary source of cytosolic acetyl-CoA in ruminant mammary cells,[34] in non-ruminants it furnishes acetyl-CoA from citrate during lipogenesis.[34] With the recent discovery that ACLY is required for increases in histone acetylation (a mechanism of transcription regulation) in response to growth factor stimulation and cellular differentiation,[35] *ACLY* upregulation may be a mechanism in the developing stromal tissue (eg,[15]) to generate acetyl-CoA from glucose carbon for histone acetylation, thus, participating in the underlying transcriptional regulation associated with involution. It remains to be determined which growth factor (eg, TGF-β), if any, controls

Table 2. Expression profiles in mammary tissue of genes associated with regulation of apoptosis, tissue remodeling, and immune response during a shift from 2X to 1X daily milking (day 1 to 5) followed by complete cessation of milking (day 5 to 21).

Gene category	Cellular function/biological process	Day relative to induction of involution				SEM	*P* =
		1	5	14	21		
Apoptosis/cell proliferation		Log-transformed mRNA abundance					
STAT3	Transcription regulation	3.90[a]	4.11[a]	4.40[b]	4.42[b]	0.06	0.01
MYC	Transcription regulation	3.75[a]	3.85[a]	4.36[b]	4.44[b]	0.12	0.01
AKT1	Protein kinase activity	3.67[a]	3.71[a]	3.52[b]	3.47[b]	0.06	0.01
MTOR	Protein kinase activity	4.17[a]	4.15[a]	4.27[b]	4.28[b]	0.05	0.02
TGFB1	Growth factor/transcription regulation	4.23[a]	4.40[b]	4.60[c]	4.36[ab]	0.07	0.009
TGFBR1	TGFB binding	4.28[a]	4.60[b]	4.65[b]	4.37[a]	0.07	0.001
JAG1	Notch binding/growth factor activity	4.03[a]	4.06[ab]	4.37[c]	4.30[bc]	0.08	0.02
CLU	Protein binding	2.79[a]	3.09[a]	4.28[b]	4.47[b]	0.18	0.002
IGFBP3	Insulin-like growth factor binding	4.14[a]	3.97[a]	4.56[b]	4.45[b]	0.09	0.002
IGFBP5	Insulin-like growth factor binding	3.79	3.98	3.89	3.71	0.12	0.30
MUC1	Apoptosis/anti-inflammatory response	4.59[a]	4.62[a]	3.63[b]	4.00[b]	0.22	0.007
S100A10	Calcium ion binding/actin filament organization	3.61[a]	3.93[b]	4.38[c]	4.39[c]	0.10	0.008
PIGT	Protein binding/attachment of GPI anchor	4.11[a]	4.20[a]	4.37[b]	4.45[b]	0.04	0.003
CALR	Calcium ion binding/transcription regulation	4.32[a]	4.15[c]	4.31[ab]	4.18[bc]	0.05	0.04
DNA damage and tissue remodeling							
CAPNS1	Calcium ion binding/protein binding	3.90[a]	4.20[b]	4.29[b]	4.25[b]	0.07	0.01
CSNK2A2	Protein binding/kinase activity	4.17[a]	4.30[b]	4.27[b]	4.30[b]	0.03	0.01
SAT1	Polyamine biosynthesis	3.56[a]	4.23[b]	4.30[b]	4.28[b]	0.09	0.005
EEF1A1	Nucleotide binding/translation elongation	4.21[a]	4.39[b]	4.31[ab]	4.35[ab]	0.05	0.06
MMP2	Peptidase activity	3.86[a]	4.14[ab]	4.37[b]	4.34[b]	0.11	0.03
CTSC	Peptidase activity	3.08[a]	3.35[b]	4.34[c]	4.72[c]	0.19	0.01
FSTL3	Activin binding/protein binding	3.92[a]	3.99[a]	4.47[b]	3.95[b]	0.16	0.009
F2R	Thrombin binding	4.40[a]	4.15[a]	3.73[b]	3.66[b]	0.11	0.01
Immune and oxidative stress response							
TNF	Cytokine activity/transcription regulation	3.50[a]	4.19[b]	4.08[b]	4.27[b]	0.17	0.05
SAA3	Neutrophil/macrophage chemotaxis	1.75[a]	2.45[a]	3.92[b]	4.58[b]	0.41	0.02
HP	Endopeptidase activity/defense response	4.22	4.30	4.84	4.59	0.33	0.28
C3	Complement activation/inflammation	3.22[a]	3.81[b]	4.12[b]	3.87[ab]	0.22	0.04
GPX1	Oxidoreductase activity/redox homeostasis	3.95[a]	4.18[ab]	4.42[c]	4.37[bc]	0.09	0.02
SOD2	Oxidoreductase activity/anti-apoptosis	3.45[a]	3.92[a]	4.74[b]	4.90[b]	0.19	0.01
SPP1	Cytokine activity/inflammation	2.88[a]	3.15[a]	4.40[b]	4.16[b]	0.17	0.001

ACLY transcription and function during mammary involution.

Transcription regulators of apoptosis and cell proliferation

It is well-accepted that during the process of involution there is an increase in epithelial cell death through apoptosis and autophagy.[6,7,21] Apoptosis of epithelial cells assessed by degree of DNA fragmentation can already be seen at 1 wk after cessation of milking.[21] Consistent with the fact that significant cell loss by apoptosis does not occur until approximately 7 d after cessation of milking, the trend for upregulation of both *STAT3* and *MYC* was evident at d 5 and continued through d 14 (Table 2). It has been speculated that STAT3[36] regulates expression of *MYC* (Fig. 1), which may have potentiated apoptosis in the present study. It also appears that the pro-apoptotic effect of STAT3 is blocked by activation of the PI-3 K/Akt signaling pathway, which would partly explain the opposite expression patterns of v-akt murine thymoma viral oncogene homolog 1 (*AKT1*) and both *STAT3* and *MYC*. *AKT1* has been associated with mammary cell proliferation.[37] It also seems possible that in the absence of a stimulus for milk protein synthesis upon cessation of milking, the degree of insulin sensitivity in mammary tissue was reduced and allowed for apoptosis and remodeling

to begin. The fact that MYC inhibits expression of *AKT1*,[38] supports the existence of a regulatory loop encompassing STAT3 and MYC in bovine mammary cells, which controls the process of apoptosis during milk stasis.

Both genomic and functional analyses of MYC targets suggest that while it behaves as a global regulator of transcription, groups of genes involved in cell cycle regulation, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial function are over-represented in the MYC target-gene network.[39] In a metabolic context, the well-established upregulation of glycolytic and lipogenic (eg, *FASN*) gene expression in rat and human cells induced by MYC overexpression would furnish the energy required for cellular growth provided that there is an appropriate supply of substrates (eg, glucose) and growth factors.[39] In the absence of nutrients or growth factors, the upregulation of *MYC* predisposes cells to apoptosis and would partly explain its role in mammary involution, ie, cessation of milking reduced mammary metabolic activity causing reduced glucose uptake (and hence greater blood glucose concentration, Fig. 2).

Although not a MYC target gene the expression of secreted phosphoprotein 1 (*SPP1*) is high in mammary of MYC-transgenic mice and also during lactation and involution vs. pregnancy.[40] Expression of *SPP1* seems to be associated with mammary gland morphogenesis in rodents, and it is a negative regulator of matrix metallopeptidase 2 (*MMP2*) expression and activity.[41] Our data (Table 2), however, showed that both *SPP1* and *MMP2* were gradually upregulated during cessation of milking, which indicates that their expression may be required for bovine mammary tissue remodeling during the course of involution. A recent study with mice also found gradual upregulation of *MMP2* in mammary tissue during the first 72 h after weaning.[42] The increase in *MMP2* mRNA is characteristic of the remodeling process that leads to destruction of the basement membrane, effectively allowing the tissue to return to a pre-pregnant state.[42] The "normal" course of murine mammary involution also was characterized by greater *STAT3* mRNA and activity, greater caspase-3 activity, and more importantly a robust pro-inflammatory and oxidative-stress response (see discussion below) triggered in part by pronounced nitric oxide synthesis.[42]

Among the large number of genes it regulates, there is evidence that mechanistic target of rapamycin (serine/threonine kinase) (*MTOR*) (formerly known as "mammalian target of rapamycin") also may be a MYC target[38] and our results seem to support that view. The *MTOR* gene encodes a nutrient-sensitive protein kinase, which under normal conditions regulates cell growth and mitotic cell division in response to glucose and insulin availability.[43,44] It also seems to play a role in the regulation of bovine mammary protein synthesis during lactation.[45] Based on the expression patterns and biological roles of STAT3, MYC, and MTOR (Table 2), the MTOR protein may have elicited effects on non-epithelial cellular proliferation (eg, stromal fibroblasts) through induction of STAT3 and MYC.[46] From the present data it seems possible that AKT1 in bovine mammary may not be responsive to MTOR (Table 2), but rather it may be associated with milk protein induction during lactation in response to insulin[47] or IGF-1.[48]

Transforming growth factor beta network

Transforming growth factor, beta 1 (*TGFB1*) signaling occurs by binding of the growth factor to transforming growth factor, beta receptor II (*TGFBR2*), which activates and binds to the structurally-similar TGFBR1.[49] Both *TGFB1* and *TGFBR1* expression were significantly upregulated by d 5 of 1X daily milking but *TGFB1* reached peak upregulation at d 14 (ie, 9 d from cessation of milking; Table 2), potentially coinciding with a gradual increase in stromal fibroblasts.[15] Unlike previously thought,[49] our data suggest that TGFB1 in mammary tissue of high-producing dairy cows plays an important role in the later stages of involution. The profile of expression of both *TGFB1* and *TGFBR1*, ie, a gradual increase followed by a return to basal expression, suggests a link with follistatin-like 3 (secreted glycoprotein) (*FSTL3*) and the control of TGFB1 ligand availability (see discussion below).

The expression of *TGFB1* and *TGFBR1* coincided with the expression of *STAT3* and *MYC* (Table 2). As previously reported in rodent mammary cells, marked elevation of jagged 1 (*JAG1*) and clusterin (*CLU*) by d 14 and 21 may have been induced by TGFB1.[50,51] A previous study of the early stages of bovine mammary involution reported an increase in *CLU*.[9] Jagged 1 is the ligand for the receptor Notch 1, which upon activation by MTOR signaling prevents cellular differentiation

in poorly-differentiated but not in well-differentiated human breast cancer cells.[52] Our data on *JAG1* and *MTOR* expression suggest that perturbation of cell differentiation by augmented Notch signaling might be a mechanism during involution to control cellular differentiation during mammary tissue remodelling. Recent studies have shown that TGFB1 activation in murine mammary cancer cells results in marked upregulation of *CLU* as well as secretion of the protein, which serves to mediate the epithelial-to-mesenchymal transition.[53] Such a close relationship likely explains the similar pattern of expression of *TGFB1* and *CLU* (Table 2). Our data would suggest that Notch and MTOR signaling and CLU synthesis allow the completion of extracellular mammary tissue remodeling during the final stages of involution when *TGFB1* and *TGFBR1* expression return to the basal levels (Table 2).

The parallel upregulation of *TGFB1* and *TGFBR1* expression supports the well-accepted view that mammary-derived signals in response to milk stasis activate programmed cell death during the first stage of mammary gland involution.[49] Interestingly, we did not observe an upregulation of insulin-like growth factor binding protein 5 (*IGFBP5*) (Table 2) which has been proposed as a potent apoptosis factor.[27] However, in the present study the upregulation of *IGFBP3*, partly via a pro-inflammatory state induced by cytokines[54] such as tumor necrosis factor (*TNF*) (see section below), might have compensated for the lack of change in *IGFBP5*.

Gene markers of cellular senescence, DNA damage, and tissue remodeling

In human fibroblasts, calpain, small subunit 1 (*CAPNS1*), which can initiate *MYC* expression, was linked to cellular senescence induced *in vitro* by DNA damage.[55] *CAPNS1* is an intracellular calcium-dependent cysteine protease that belongs to the calpain family and is involved in remodeling of cytoskeletal/membrane attachments, degradation of enzymes that control progression through the cell cycle, and apoptosis.[56] Casein kinase 2 (*CSNK2A2*) contributes to the radiation-induced senescence of human mesenchymal stem cells[57] and also forms part of the TGFB1 signaling network (Fig. 1) that elicits the dissolution of tight junctions during epithelial-to-mesenchymal transitions.[58] Spermidine/spermine N1-acetyltransferase (*SAT1*) can increase apoptosis through regulating cytosolic levels of spermidine and spermine[9,27] and its expression is markedly upregulated during murine involution.[59] In human cancer cells, eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*) has been identified as a novel marker of cellular senescence whose downregulation induces a novel, caspase-independent mechanism of apoptosis to eliminate abnormal tetraploid cells and inhibit tumorigenesis.[60] We observed peak upregulation of all of these genes by day 5 of 1X milking (Table 2) suggesting that temporary milk stasis was a strong stimulus potentially due to an increase in DNA fragmentation as has been previously observed in involuting bovine and murine mammary tissue.[21]

In the present study, marked changes in expression of genes involved in tissue remodeling were observed through each phase of involution, ie, peak upregulation of *CAPNS1* by d 5 followed by gradual upregulation of *MMP2*, cathepsin C (*CTSC*; a peptidase), and *FSTL3* by d 14 (Table 2). The observed upregulation of *MMP2* has been reported previously in involuting bovine mammary[61] and agrees with the demonstrated effect of the protein on mammary tissue structure during involution.[42] *CTSC* is an exopeptidase that can be found intracellularly in lysosomes or in exocytic vesicles and it is involved in the activation of proteinases in immune cells.[62] *FSTL3* acts through its binding to members of the TGFB superfamily such as activin and it also has an NF-kappaB response element that allows for TNF to upregulate its expression.[63] Furthermore, TGFB1 potentiates the effect of TNF on *FSTL3* expression through Smad proteins. Recent data have shown that *FSTL3* mRNA is particularly high in stromal-vascular tissue of adipose tissue, and can be secreted by this tissue at least *in vitro*.[64] Because of this novel property it has been classified as a new adipokine,[64] just as leptin and adiponectin. It could be possible that the upregulation of *FSTL3* we observed corresponded with an increase in stromal tissue development (eg,[15]). More importantly, the sustained upregulation of *FSTL3* may represent a mechanism whereby mammary cells secrete this protein as a mechanism to bind TGFB ligands, thus, exerting control over the activity of the growth factor. In fact, the pattern of *TGFB1* expression (Fig. 2) may have been a consequence of more FSTL3 protein sequestering TGFB1 ligand (eg, avidin), ie, there was no feed-forward regulator for sustained synthesis of TGFB1.

Immune and oxidative stress response genes

Studies with rodents have clearly shown an upregulation of acute-phase response genes involving STAT3, NF-kappaB, and nitric oxide production during the early phase of involution.[42,59] Thus, our finding of TNF upregulation during 1X milking and its sustained elevation through d 21 (Table 2) indicates that, just as in rodents, cytokines play a role in bovine mammary tissue remodeling, eg, through activation of MMP2.[42] The finding of sustained *TNF* and *SAA3* coupled with that of glutathione peroxidase 1 (*GPX1*) and superoxide dismutase 2, mitochondrial (*SOD2*) through d 21 (ie, ~17 days from cessation of milking; Table 2) are indicative of a pronounced state of local inflammation and oxidative stress even at the latter stages of involution. A pro-inflammatory state in bovine mammary epithelial cells either may contribute to or may be a consequence of lactotransferrrin (*LTF*) upregulation (Table 1) and synthesis after cessation of milking.[65] It is evident, however, that *LTF* can assist the involution process by reducing viability of bovine mammary epithelial cells (eg, through caspase activation) and by inhibiting synthesis of caseins (*CSN2*, *CSN3*) through a mechanism partly involving upregulation of IL-1β.[65] It appears likely that other cytokines such as TNF and SPP1 participate in the overall inhibition of mammary epithelial cell viability.

Just as hypothesized in rodents,[59] a key function of STAT3 during bovine mammary involution is to trigger an acute-phase response (ie, SAA3 is one direct target;)[66] not only to counteract the pro-inflammatory state but to promote phagocytosis of apoptotic cells that arise during the remodeling process.[59] Therefore, our data support the view that STAT3 activity during involution promotes apoptosis and guides tissue remodelling while protecting the tissue from excessive damage.[66] It remains to be determined whether an endogenous acute-phase protein feed-back activates STAT3 as has been proposed for orosomucoid in murine mammary.[59] In this context it is possible that CLU may play an anti-inflammatory role either by promoting synthesis of acute-phase proteins[67] or by directly inhibiting NF-kappaB signaling.[68] Because expression of HP was not affected significantly during the study (Table 2), it is unlikely that it participates in the acute-phase response during involution as it does in the rodent mammary gland.[66] It also is possible that upregulation of *CLU* may help reduce the production of reactive oxygen species[69] that trigger upregulation of *GPX1* and *SOD2* (Table 2). Because CLU, like STAT3, is a direct target of TGFB1,[53,70] this growth factor may have dual functions during involution, ie, promoting apoptosis and preventing excessive cellular damage by downregulating NF-kappaB signaling through CLU.

The presence of a sustained pro-inflammatory and oxidative stress state in bovine mammary differs substantially from the situation in rodents.[49] This may be indicative of inherent species differences potentially linked with mammary metabolic capacity. At the histological level, such a pronounced inflammatory response and the corresponding pattern of *TGFB1* would suggest a substantial influx of neutrophils and macrophages recruited to remove cellular debris during tissue remodeling.[49] Our data confirmed that genes related to the immune response are upregulated during early stages of bovine mammary involution[9] and extend those findings to show the presence of a robust pro-inflammatory and oxidative stress response even after ~3 weeks from the cessation of milking. We propose that such a response is partly driven by differences in mammary metabolic capacity and/or genetic merit, ie, cessation of milking at peak lactation would likely require a lengthier process of remodeling due to the presence of greater numbers of epithelial cells. The transcription factor STAT3, as in rodent mammary, apparently links cellular immune responses with growth and proliferation and strengthens its fundamental role in the process of involution.

Summary

Mammary gland involution is a complex process that is dependent on interactions between the mammary epithelium and the surrounding stromal tissues.[49] Our data demonstrated orchestrated upregulation of apoptosis, tissue remodeling, and inflammation in mammary tissue accompanied by reduced biosynthesis of milk components (ie, milk protein, fat and lactose) (Fig. 2) due to the reduction in metabolic activity induced by reduction and cessation of milking, which triggered mammary involution. As in rodents, the transcription factor STAT3 as well as TGFB1 appear to play central roles during mammary involution through temporal-spatial regulation of apoptosis as well as inflammation and oxidative stress. Coincident with the stage-dependent characteristics of involution,[8,22]

two distinct stages were implicated by the apparent upregulation of TGFB1 between d 1 and 14 followed by its downregulation between d 14 and 21. However, unlike rodents, inflammation and oxidative stress persisted well-beyond the point of milk stasis potentially due to the substantial amount of epithelial tissue in the mammary gland of these cows at peak lactation.

Acknowledgements

Support for the gene expression work was provided by funds from NIFA Section 1433 Animal Health and Disease funds appropriated to the University of Illinois under project ILLU-538-923 (to JJL).

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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