Effects of milking frequency and domperidone injections on milk production and prolactin signaling in the mammary gland of dairy cows

I. M. Toledo,^{1*} X. Zhao,² and P. Lacasse^{1†}

¹Sherbrooke Research and Development Centre, Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada J1M 0C8

²Department of Animal Science, McGill University, Sainte-Anne-de-Bellevue, QC, Canada H9X 3V9

ABSTRACT

Eleven mid-lactation Holstein cows were milked twice daily during the first 2 experimental weeks. During wk 3 to 10, the cows were differentially milked: right quarters were milked thrice daily (3×) and left quarters were milked once daily (1×). During wk 11 to 14, all quarters were milked twice daily. After 4 wk of differential milking, the cows received daily i.m. injections of the dopamine antagonist domperidone (DOMP; 300 mg; n = 6) or of dimethyl sulfoxide as the control (CTL; n = 5) for 8 wk (wk 7–14). During the differential milking period (wk 3–6), milk production was greater for quarters milked 3× than for those milked 1× but did not differ between DOMP and CTL cows. During the differential milking + injection period (wk 7–10), milk production continued to differ according to milking frequency. However, DOMP injection did not have an effect or interact with milking frequency on milk production. During the injection period (wk 11–14), milk production remained greater in the quarters previously milked 3× and milk production increased in DOMP injected cows but not in CTL cows. Injections of DOMP increased prolactin concentration, which was greater in the serum of DOMP cows than in that of CTL cows during the differential milking + injection and the injection periods. The expression of genes that are directly related to milk synthesis (*CSN2*, *LALBA*, and *ACACA*) was greater in the 3× quarters than in the 1× quarters. In addition, DOMP increased *CSN2* expression during the injection period. The expression of both isoforms of the *PRLR* gene was greater in the 3× quarters during the differential milking + injection and the injection periods. At the protein level, injections of DOMP tended to increase the number of long

PRLR isoform during the differential milking + injection period. The number of short PRLR isoform was greater in the 1× quarters than in the 3× quarters during the differential milking, the differential milking + injection, and the injection periods. The total amount of STAT3 protein was greater in the 1× quarters during the differential milking and the differential milking + injection periods. The amount of phosphorylated STAT3 protein was greater in the 1× quarters during the differential milking period. The total amount of phosphorylated STAT5 protein was greater in the 3× quarters during the differential milking and the differential milking + injection periods. The results of this experiment support the hypothesis that the responsiveness of the mammary gland to PRL is modulated by milking frequency, although the underlying mechanism remains to be determined.

Key words: prolactin, differential milking, serotonin, STAT5

INTRODUCTION

Prolactin (PRL), as its name implies, is the most important hormone for the control of lactation. In most mammals, suppression of PRL with bromocriptine strongly inhibits lactation (Taylor and Peaker, 1975; Flint and Gardner, 1994). Although the involvement of PRL in the control of ruminant lactation has been controversial (Cowie and Tindal, 1971; Knight, 1993), we previously demonstrated that administration of a specific and potent PRL-release inhibitor, quinagolide, decreases the milk production of dairy cows (Lacasse et al., 2011) and that PRL injection attenuates the inhibitor effect (Lacasse et al., 2012). Recently, we also showed that the injection of a PRL secretagogue, domperidone (DOMP), stimulates milk production (Lacasse and Ollier, 2015; Tong et al., 2018) and that the galactopoietic response is greater when preceded by 2 wk of moderate PRL inhibition (Tong et al., 2018). For a comprehensive review of the galactopoietic role of PRL in ruminants, readers are invited to consult the review paper by Lacasse et al. (2016).

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*Current address: IFAS Extension, University of Florida, Gainesville 32608.

†Corresponding author: pierre.lacasse@canada.ca

One reason why the galactopoietic effect of PRL on milk production has been difficult to demonstrate could be that milk secretion can be modulated by both systemic and local factors. A well-known local modulator of milk secretion is milking frequency (Elliott, 1961; Stelwagen, 2001). We found that milking frequency also affects the expression of the PRL receptors (**PRLR**; Bernier-Dodier et al., 2010) and the response to PRL inhibition (Lacasse et al., 2011). The binding of PRL to its cell surface receptors [long PRLR isoform (**PRL-R_{long}**)] initiates several signaling pathways, including phosphorylation of the signal transducer and activator of transcription (**STAT**) 5a and 5b, which are believed to mediate the physiological responses to PRL in the mammary gland (Teglund et al., 1998). The suppressors of cytokine signaling (**SOCS**) are induced in response to PRL and other cytokines. The SOCS proteins appear to act as inhibitors of cytokines using the JAK/STAT pathway and, therefore, constitute another mechanism that modulates PRL response. Wall et al. (2006) reported that frequent milking and administration of PRL increase milk production and decrease *SOCS3* gene expression. Reciprocally, milk stasis induced an increase in *SOCS3* gene expression that coincided with declines in activated STAT5 and milk protein gene expression and an increase in apoptosis. Factors controlling the expression of the PRLR and *SOCS* are not well characterized but are important for understanding how mammary cell activity and apoptosis and, therefore, lactation persistency are controlled.

A regulatory pathway of milk production that comprises the effects of local and systemic factors can be envisaged. In that model, hormones released at milking, especially PRL, would play a central role by providing the “lactogenic signal.” Factors affecting the magnitude of the lactogenic signal would, thereby, affect milk secretion and persistency. Accordingly, the weakening of the lactogenic signal as lactation advances (Koprowski and Tucker, 1973) leads to a continuous loss of secretory cells by apoptosis and a decrease in milk yield after the peak of lactation (Knight and Wilde, 1993; Capuco et al., 2001). Another key point in mammary gland local regulation is the gland’s sensitivity to the lactogenic signal. The accumulation of milk in a given gland, resulting from incomplete milking or the cessation of milking, would lead to reduced sensitivity of that gland to the lactogenic signal and to the reduction, or arrest, of milk synthesis. Some factors affecting milk production may affect both the lactogenic signal and the responsiveness of the gland. Indeed, milking frequency determines the number of times that the lactogenic signal is triggered but also appears to have a local effect, because the response to differential milk-

ing is not similar in all glands (Elliott, 1961) despite exposure to the same lactogenic signal. Even though a galactopoietic role for oxytocin has been proposed (Lollivier and Marnet, 2005), the objective of the present study was to determine whether the galactopoietic effect of milking frequency is related to a modification of mammary gland responsiveness to PRL.

MATERIALS AND METHODS

Animals and Experimental Design

The present study was conducted in compliance with the rules and guidelines of the Canadian Council on Animal Care (1993). Eleven mid-lactation (155 ± 5.5 DIM) Holstein cows from the herd at Agriculture and Agri-Food Canada’s Sherbrooke Research and Development Centre (Sherbrooke, QC, Canada) were assigned to treatments according to milk yield, SCC, and parity. Seven of them were gestating. Before the beginning of the study, the cows were tested and found to show no clinical signs of mastitis. The cows were milked twice daily (2 $\times$) during the first 2 experimental weeks (the pretreatment period). During wk 3 to 6, the cows were differentially milked (the differential milking period). The right quarters were milked thrice daily (3 $\times$) and the left quarters were milked once daily (1 $\times$). During wk 7 to 10, the cows were differentially milked and receiving daily i.m. injections of the dopamine antagonist DOMP (300 mg; Glentham Life Sciences Ltd., Corsham, UK; $n = 6$) or of dimethyl sulfoxide (**DMSO**) as the control (**CTL**; $n = 5$) (the differential milking + injection period). During wk 11 to 14, all quarters were milked 2 $\times$ but the cows continued to receive daily i.m. injections of DOMP or DMSO (the injection period).

The cows were kept in a tie-stall barn and had free access to water throughout the study. Every morning (0830 h), they were fed a TMR containing (on a DM basis) 34.4% grass silage, 22.8% corn silage, 24.1% corn grain, 9.0% soybean meal, 3.6% beet pulp, 2.6% chopped dry hay, 1.8% nonmineral supplement, and 1.6% mineral supplement. The TMR contained (on a DM basis) 17.6% CP, 20.6% ADF, 30.2% NDF, 1.0% of Ca, 0.4% of P, 1.5% of K, and provided 1.56 Mcal/kg of NE_L. Feed intake was recorded daily and individual BW were recorded in wk -1, 8, and 15.

Blood samples were collected from coccygeal vessels in uncoated Vacutainers (Becton, Dickinson and Co., Rutherford, NJ) before the morning milking (0700 h) once weekly. In addition, blood samples were collected from the caudal artery and 2 mammary veins simultaneously, 1 h before and 1 h after the morning milking, in wk 2, 6, 10, and 14 (once per week). After

collection, the blood tubes were kept for approximately 2 h at room temperature for clotting before they were centrifuged ($1,900 \times g$, 4°C , 15 min). The serum was then stored at -20°C for further determination of PRL concentrations.

Milk Composition

Milk samples were collected weekly at the morning milking. Milk components of whole milk were analyzed for lactose, protein, fat, and SCC concentrations in a commercial laboratory (Valacta, Sainte-Anne-de-Bellevue, QC, Canada). For measurements of PRL and serotonin, skim milk was prepared by centrifugation ($1,900 \times g$, 4°C , 15 min), aliquoted, and kept at -20°C .

Mammary Functional Capacity

In the present study, mammary functional capacity consisted of the maximum volume of fluid that the mammary gland can contain. Mammary functional capacity was determined by measuring the volume of milk harvested after a 26-h milking interval, which leads to complete filling of the gland. During the study, this measurement was taken the week before the experiment began and the week after the experiment ended. The cows were milked at 0800 h, and then the milking at 2000 h was skipped. At 1030 h the next day, the cows were milked until milk flow stopped and then injected with 20 IU of oxytocin to collect residual milk.

Milk Samples: RNA Extraction

Additional front-quarter milk samples were manually collected in wk 2, 4, 6, 8, 10, 12, and 14. Each sample was individually collected in a sterile 50-mL tube and kept on ice. Immediately after collection, milk (10 mL) from each quarter was mixed in a new tube and centrifuged ($2,000 \times g$, 4°C , 10 min). Subsequently, milk fat was added to Trizol LS reagent (Life Technologies, Grand Island, NY) to obtain a concentration of 0.5 g of fat/mL. The samples were stored at -80°C until further RNA extraction. Before extraction, the milk samples were thawed and transferred into 2.0-mL tubes for centrifugation ($12,000 \times g$, 4°C , 10 min). The Trizol content in the middle phase (between fat and pellet) was used for milk fat RNA extraction. The RNA was extracted by means of the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After extraction, RNA amounts were quantified by means of a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). After quantification, all the samples were frozen

at -80°C for further real-time reverse-transcription PCR (RT-PCR) analysis.

Mammary Biopsies

Mammary biopsies were obtained from rear quarters in experimental wk 2, 6, 10, and 14, as previously described by Tong et al. (2018). After collection, the mammary tissue was rinsed in sterile saline solution to remove all traces of blood and then cut into small pieces. The mammary tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for further analyses.

Quantitative Reverse Transcription-PCR

The abundance of genes related to PRL signaling [*PRLR*_{long}, short *PRLR* isoform (*PRLR*_{short}), and *SOCS3*], to serotonin synthesis (*TPH1*), to mammary activity (*LALBA*, *CSN2*, and *ACACA*), and to apoptosis (*BCL2* and *BAX*) in milk fat mRNA was determined by quantitative reverse-transcription PCR. Gene-specific primer sets (Table 1) for all genes either were designed using a platform developed at the Université de Sherbrooke (Brosseau et al., 2010) or were described in previous studies (Bernier-Dodier et al., 2011; Thompson et al., 2011; Dudemaine et al., 2014) and synthesized by Integrated DNA Technologies Inc. (Coralville, IA). After milk RNA extraction, processing, and storage, all samples were incubated with DNase I (New England Biolabs, Ipswich, MA) for 15 min at 37°C to remove genomic DNA and subsequently heat-denatured at 75°C for 10 min. Total RNA (750 ng/reaction) was reverse-transcribed to cDNA using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) following the manufacturer's instructions. Quantitative PCR was completed with SYBR Green as the indicator through an initial activation-denaturation step (50°C for 2 min and 95°C for 2 min), followed by 40 cycles of a 2-step amplification protocol (95°C for 3 s and 60°C for 30 s) using a Step-One Plus real-time PCR system (Applied Biosystems Inc.). Specific genes were amplified by running a mixture of 3.0 μL of the diluted cDNA product for each specific primer in a final reaction volume of 10 μL . In addition, a dissociation curve analysis (60 to 95°C) was used to verify the amplification of a single product. Triplicate reactions were completed for each sample and were run in parallel with a relative standard curve formed by pools of all samples. This pool was initially diluted 1/7.5 followed by a 2-fold factor to reach a 1/480 dilution. The first 1/7.5 dilution received the arbitrary quantity value of 1,000.

Table 1. Primers (F, forward; R, reverse) used during real-time PCR

Gene ¹	GenBank No.	Primer (5'-3')	Amplicon size (bp)
<i>ACTB</i>	NM_173979.3	F: TGGCACCCAGCACAATGA R: CCTGCTTGCTGATCCACATCT	123
<i>UXT</i>	NM_001037471.2	F: TGGCAGAAGCTCTCAAGTTCATT R: CATGTGGATATGGGCCCTTGAT	104
<i>GAPDH</i>	NM_001034034.2	F: GCGAGATCCTGCCAACATCAAG R: TCAAGTGAGCCCCRCGCTTC	101
<i>RPS24</i>	NM_001025339.2	F: CTCCTTTCTCCCTGGCTGC R: TCCGCTGAAGCAGTCGGTTG	100
<i>BAX</i>	NM_173894.1	F: GGGTTGTGCGCCCTTTTCTACTT R: CGCTCTCGAAGGAAGTCCAATG	115
<i>BCL2</i>	NM_001166486.1	F: ACAGCATCGCCCTGTGGATG R: GTGCCTTCAGAGACAGCCAG	147
<i>SOCS3</i>	NM_174466.2	F: AGAAGATCCCTCTGGTGTGAGC R: GTGACTTTCTCGTAGGAGTCCAGG	109
<i>CSN2</i>	XM_010806178.1	F: AGCAAACAGAGGATGAACCTCCAGG R: AGGGATGTTTTGTGGGAGGCTG	110
<i>LALBA</i>	NM_174378.2	F: CTCTGCTCCTGGTAGGCATC R: ACAGACCCATTTCAGGCAAAC	125
<i>ACACA</i>	NM_174224.2	F: CATGTATGGACACCACTTCTGCAT R: GTTTGGTAGGACATCAAAAATCGA	82
<i>PRLR_{long}</i>	NM_001039726.2	F: TTGATGTTTCATCTGCTGGAGAAG R: GCAAGTCCTCGCAGTCAGAA	96
<i>PRLR_{short}</i>	NM_174155.3	F: GCCTTCTCGCCTGTGTCTATG R: GCAAGTCCTCGCAGTCAGAA	107
<i>TPH1</i>	XM_005216077	F: AACTTTTCAGGATGTCTACTTTGTATCTG R: CACTCCAAATGGACGCTTGATTG	98

¹*ACTB* = β -actin; *UXT* = ubiquitously expressed transcript; *RPS24* = ribosomal protein S24; *BAX* = BCL-2-associated X protein; *BCL2* = B-cell CLL/lymphoma 2; *SOCS3* = suppressors of cytokine signaling 3; *CSN2* = β -casein; *LALBA* = α -lactalbumin; *ACACA* = acetyl-CoA carboxylase α ; *PRLR_{long}* = prolactin receptor, long isoform; *PRLR_{short}* = prolactin receptor, short isoform; *TPH1* = tryptophan hydroxylase-1.

The *ACTB*, *GAPDH*, *UXT*, and *RPS24* genes were quantified to select reference genes. The NormFinder program (<http://moma.dk/normfinder-software>) was used to analyze the expression stability of candidate reference genes. The genes with the most stable expression were *GAPDH* and *RPS24*, and they were therefore used as the reference genes in this study. The abundance of the target genes is determined as the relative quantity ratio by dividing the relative quantity of the targets by the geometric mean of the relative quantity of the reference genes (*GAPDH* and *RPS24*).

Hormone Analysis

The concentrations of PRL in both serum and skim milk were determined by RIA, as previously described by Bernier-Dodier et al. (2011). Bovine PRL, rabbit antiserum specific for bovine PRL, and goat anti-rabbit gamma globulin were purchased from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). The intra- and interassay coefficients of variation were 4.8 and 8.6%, respectively.

The milk concentration of serotonin was determined using a commercial 5-HT ELISA kit (IM1749; Beckman Coulter GmbH, Sinsheim, Germany). Milk was centri-

fuged at $15,000 \times g$ for 15 min at 4°C before the assay was performed.

Determination of Prolactin Receptor and of STAT Protein and Activation Levels

The abundance of short and long isoforms of PRLR was determined in mammary tissue as described by Tong et al. (2018). The determination of STAT protein and activation levels was performed as described in Tong et al. (2018).

Statistical Analysis

Data were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Time was used as a repeated effect. For DMI and PRL blood concentration, cow was used as the subject, whereas cow(half gland) was used for variables measured at the mammary gland level. Except for mammary functional capacity, data were analyzed separately for the pre-treatment period (wk 1 and 2), the differential milking period (wk 3–6), the differential milking + injection period (wk 7–10), and the injection period (wk 11–14). Means of the data from the pretreatment period were

Figure 1. Effect of daily injections of domperidone (dashed lines; $n = 6$) or dimethyl sulfoxide (solid lines; $n = 5$) and differential milking [once daily (black) vs. thrice daily (gray)] on milk composition of mid-lactation cows. Cows were differentially milked from wk 3 to 10 and injected from wk 7 to 14. Data are presented as LSM $\pm$ SEM.

used as covariates. Differences were considered statistically significant when $P < 0.05$ and were considered a trend when $P < 0.10$.

RESULTS

DMI, Milk Yield, and Milk Composition

No differences in DMI ($P > 0.15$) due to treatment were observed throughout the study; DMI averaged 25.2 and 24.6 $\pm$ 0.4 kg/d for CTL and DOMP cows, respectively.

Milk production did not differ between DOMP and CTL cows or between the half glands during the pretreatment period ($P > 0.40$; Figure 1). During the differential milking period (wk 3–6), milk production was greater for quarters milked 3 $\times$ than for quarters milked 1 $\times$ ($P < 0.001$) but did not differ between DOMP and CTL cows ($P = 0.37$). During the differential milking + injection period (wk 7–10), milk production continued to differ according to milking frequency ($P < 0.001$). In addition, the effect of milking frequency increased with time (milking frequency $\times$ time; $P < 0.05$), with milk production declining in the 1 $\times$ half glands but remaining stable in the 3 $\times$ half glands. Injection of DOMP did not have an effect or interact with milking

frequency on milk production during the differential milking + injection period. Although all quarters were milked 2 $\times$ during the injection period (wk 11–14), milk production remained greater in the quarters previously milked 3 $\times$ ($P < 0.001$). There was a treatment $\times$ time interaction ($P = 0.03$), with milk production increasing in the DOMP-injected cows but remaining stable in the CTL cows.

During the pretreatment period, the milk contents of protein ($P > 0.30$), fat ($P = 0.7$), lactose ($P > 0.13$), and SCC ($P > 0.50$) did not differ between DOMP and CTL cows or between the half glands (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-17330>). Milk composition was not affected by the DOMP treatment ($P > 0.15$, Table 2). Milk protein was not affected by milking frequency throughout the experiment. During the differential milking period, there was a milking frequency $\times$ time interaction ($P < 0.001$) on milk fat content, which was decreasing in the 3 $\times$ quarters but increasing in the 1 $\times$ quarters. Milk from quarters milked 3 $\times$ had a greater ($P < 0.001$) lactose content than milk from quarters milked 1 $\times$. This effect declined over time (milking frequency $\times$ time; $P < 0.001$) and was no longer significant ($P > 0.05$) after wk 12. During the differential milking period, there was a milking frequency $\times$ time interaction ($P = 0.02$)

on SCC, which was decreasing in the 3× quarters but increasing in the 1× quarters. The SCC remained lower in the 3× quarters during the differential milking + injection period ($P < 0.01$) and tended to be lower ($P = 0.06$) in the injection period. Energy-corrected milk was not affected by DOMP injection but was greater in the quarters milked 3× than in those milked 1× ($P < 0.001$); this effect persisted even after differential milking was discontinued ($P < 0.001$; Table 2)

Hormone Concentrations

Basal serum PRL concentration was similar for all cows before the injections began ($P > 0.5$; Figure 2A). Domperidone injections increased PRL concentration, which was greater in the serum of DOMP cows than in that of CTL cows during the differential milking + injection period ($P = 0.02$) and the injection period ($P < 0.01$).

Milk PRL concentration was similar between DOMP and CTL cows and between the half glands during the pretreatment period ($P > 0.5$; Figure 2B). Milk PRL concentration was greater in the DOMP cows than in the CTL cows during the differential milking + injection period and the injection period ($P < 0.001$). In addition, PRL concentration was greater in the milk from 1× quarters than in that from 3× quarters during the differential milking period ($P < 0.01$) but not during the differential milking + injection period ($P = 0.12$) and the injection period ($P = 0.7$).

Milk serotonin concentration was similar between DOMP and CTL cows or between the half glands during the pretreatment period ($P > 0.30$; Supplemental Table S1). In none of the treatment periods was serotonin concentration affected by treatment or milking frequency ($P > 0.10$; Table 2).

Gene Expression, PRLR and STAT Protein and Activation Levels, and Functional Capacity

As expected, all genes were expressed similarly among the treatments and quarters during the pretreatment period ($P > 0.10$; Supplemental Table S2; <https://doi.org/10.3168/jds.2019-17330>). The expression of genes directly related to milk synthesis (*CSN2*, *LALBA*, and *ACACA*) was greater ($P < 0.05$) in quarters milked 3× than in those milked 1× (Table 3). This effect persisted during the injection period ($P < 0.05$), even though all quarters were milked 2× during that period. The only effect of DOMP was increased *CSN2* expression ($P = 0.05$) during the injection period. The expression of both the proapoptotic gene *BAX* and the antiapoptotic gene *BCL2* was greater in the 1× quarters during the differential milking + injection period and the injection

Table 2. Effect of daily injections (INJ) of domperidone (DOMP; n = 6) or of dimethyl sulfoxide as the control (CTL; n = 5) and differential milking [DIF; once daily (1×) vs. thrice daily (3×)] on milk composition of mid-lactation cows¹

Variable	DIF ²						DIF + INJ ³						INJ ⁴					
	CTL			DOMP			CTL			DOMP			CTL			DOMP		
	1×	3×		1×	3×		1×	3×		1×	3×		1×	3×		1×	3×	SEM ⁵
Protein (%)	3.36	3.42		3.43	3.44		3.39	3.52		3.38	3.38		3.52	3.55		3.50	3.50	0.05
Fat (%)	3.99	3.74		4.09	3.90		4.02	3.58		3.84	3.89		4.02	4.38		3.97	4.40	0.30
Lactose (%)	4.29	4.56		4.34	4.58		3.84	4.53		4.14	4.54		4.18	4.45		4.57	4.23	0.13
SCC (log ₁₀)	1.70	1.63		1.95	1.69		2.25	1.73		2.26	1.59		2.35	1.98		2.47	1.91	0.25
ECM (kg/d)	11.6	19.6		10.4	19.4		9.3	19.1		8.1	18.4		10.8	18.9		11.2	17.9	1.0
Serotonin (ng/mL)	1.70	1.96		1.29	1.73		1.58	1.80		1.17	1.48		1.44	1.24		1.31	1.86	0.30

¹Data are presented as LSM. Cows were differentially milked from wk 3 to 10 and injected from wk 7 to 14. Data from the pretreatment period (wk 1 and 2) were used as covariates.

²Within the differential milking period (wk 3 to 6), effect of milking frequency for lactose ($P < 0.01$) and ECM ($P < 0.001$).

³Within the differential milking + injection period (wk 7 to 10), effect of milking frequency for lactose ($P < 0.001$), SCC ($P < 0.01$) and ECM ($P < 0.001$).

⁴Within the injection period (wk 11 to 14), effect of milking frequency for lactose ($P < 0.01$) and ECM ($P < 0.001$).

⁵The SEM reported is the highest among all periods.

period ($P < 0.05$), such that the *BAX:BCL2* ratio was not affected. The expression of both isoforms (long and short) of the *PRLR* gene was greater in the quarters

milked 3× during the differential milking + injection period and the injection period ($P < 0.05$). The expression of *SOCS3* was not affected by milking frequency

Figure 2. Effect of daily injections of domperidone (dashed lines; $n = 6$) or dimethyl sulfoxide (solid lines; $n = 5$) and differential milking [once daily (black) vs. thrice daily (gray)] on serum prolactin (A) and milk prolactin (B) concentrations of mid-lactation cows. Cows were differentially milked from wk 3 to 10 and injected from wk 7 to 14. Data are presented as LSM $\pm$ SEM.

Table 3. Effect of daily injections (INJ) of domperidone (DOMP; n = 6) or of dimethyl sulfoxide as the control (CTL; n = 5) and differential milking [DIF; once daily (1×) vs. thrice daily (3×)] on milk-fat-extracted mRNA levels¹

Variable ²	DIF ³				DIF + INJ ⁴				INJ ⁵				SEM ⁶
	CTL		DOMP		CTL		DOMP		CTL		DOMP		
	1×	3×	1×	3×	1×	3×	1×	3×	1×	3×	1×	3×	
<i>CSN2</i>	3.90	4.46	2.01	7.78	0.38	2.91	0.85	4.28	0.14	1.92	1.76	3.58	1.40
<i>LALBA</i>	3.90	4.75	2.03	7.28	0.42	3.85	0.68	4.14	0.11	2.45	1.74	3.57	1.42
<i>ACACA</i>	4.80	4.50	1.75	5.74	0.82	3.70	1.20	4.45	0.26	2.51	1.55	3.15	1.40
<i>BAX</i>	0.79	0.68	0.92	0.58	1.30	0.93	1.23	0.62	1.20	0.86	0.95	0.70	0.16
<i>BCL2</i>	0.73	0.81	0.83	0.47	1.24	0.87	1.05	0.66	1.22	0.69	0.93	0.83	0.16
<i>BAX:BCL2</i>	1.05	0.94	1.06	1.29	1.07	1.02	1.23	1.03	1.00	1.26	1.12	0.92	0.14
<i>PRLR</i> _{long}	4.67	4.43	2.12	7.15	0.55	3.38	0.91	4.60	0.12	2.29	1.49	3.19	1.50
<i>PRLR</i> _{short}	4.79	4.67	2.19	6.97	0.68	4.15	0.99	4.85	0.15	2.89	1.36	3.13	1.50
<i>SOCS3</i>	0.18	0.58	0.34	0.23	0.15	2.71	0.46	0.26	0.49	0.51	1.20	0.42	1.40

¹Data are presented as LSM of the ratio of each gene relative to the geometric mean of *GAPDH* and ribosomal protein S24 (*RPS24*). Mid-lactation cows were differentially milked from wk 3 to 10 and injected from wk 7 to 14.

²*CSN2* = β -casein; *LALBA* = α -lactalbumin; *ACACA* = acetyl-CoA carboxylase α ; *BAX* = BCL-2-associated X protein; *BCL2* = B-cell CLL/lymphoma 2; *BAX:BCL2* = ratio of *BAX* to *BCL2*; *PRLR_{long}* = prolactin receptor, long isoform; *PRLR_{short}* = prolactin receptor, short isoform; *SOCS3* = suppressors of cytokine signaling 3.

³Within the differential milking period (wk 3 to 6), effect of milking frequency for *CSN2* ($P < 0.01$).

⁴Within the differential milking + injection period (wk 7 to 10), effect of milking frequency for *CSN2* ($P < 0.001$), *LALBA* ($P < 0.01$), *ACACA* ($P < 0.01$), *BAX* ($P < 0.01$), *BCL2* ($P < 0.05$), *PRLR_{long}* ($P < 0.01$), and *PRLR_{short}* ($P < 0.01$).

⁵Within the injection period (wk 11 to 14), effect of milking frequency for *CSN2* ($P < 0.05$), *LALBA* ($P < 0.05$), *ACACA* ($P < 0.05$), *BAX* ($P < 0.05$), *BCL2* ($P < 0.05$), *PRLR_{long}* ($P < 0.05$), and *PRLR_{short}* ($P < 0.05$); effect of injections for *CSN2* ($P < 0.05$).

⁶The SEM reported is the highest among all periods.

or DOMP injections. The expression of *TPH1* was too low (no detectable amplification after 40 cycles) to be quantified (data not shown).

The number of both short and long isoforms of PRLR did not differ among treatments and quarters during the pretreatment period ($P > 0.50$; Supplemental Table S3; <https://doi.org/10.3168/jds.2019-17330>). Domperidone injections tended to increase the number of PRLR_{long} during the differential milking + injection

period ($P = 0.06$; Table 4). The number of PRLR_{short} was greater in the 1× quarters than in the 3× quarters during the differential milking period ($P < 0.01$), the differential milking + injection period ($P = 0.02$), and the injection period ($P = 0.05$).

The amount and activation of both STAT3 and STAT5 were similar among treatments and quarters during the pretreatment period ($P > 0.10$; Supplemental Table S4; <https://doi.org/10.3168/jds.2019-17330>).

Table 4. Effect of daily injections (INJ) of domperidone (DOMP; n = 6) or of dimethyl sulfoxide as the control (CTL; n = 5) and differential milking [DIF; once daily (1×) vs. thrice daily (3×)] on prolactin receptor (PRLR) short and long isoform levels in mammary gland biopsies collected at the end of each treatment period¹

Variable ²	DIF ³				DIF + INJ ⁴				INJ ⁵				SEM ⁶
	CTL		DOMP		CTL		DOMP		CTL		DOMP		
	1×	3×	1×	3×	1×	3×	1×	3×	1×	3×	1×	3×	
PRLR _{long} ⁷	6.14	5.85	6.14	5.98	5.87	5.57	5.43	5.38	ND ⁸	ND	ND	ND	0.16
PRLR _{short}	6.60	5.78	6.96	6.27	6.92	6.36	6.87	6.31	6.34	5.86	6.53	6.03	0.24

¹Data are presented as LSM. Mid-lactation cows were differentially milked from wk 3 to 10 and injected from wk 7 to 14.

²PRLR_{long} = prolactin receptor, long isoform; PRLR_{short} = prolactin receptor, short isoform.

³Within the differential milking period (wk 3 to 6), effect of milking frequency for PRLR_{short} ($P < 0.01$).

⁴Within the differential milking + injection period (wk 7 to 10), effect of milking frequency for PRLR_{short} ($P < 0.01$); effect of injections for PRLR_{long} ($P < 0.05$).

⁵Within the injection period (wk 11 to 14), effect of milking frequency for PRLR_{short} ($P < 0.05$).

⁶The SEM reported is the highest among all periods.

⁷Relative PRLR protein level ($\log_{10}$).

⁸Not detected.

The total amount of STAT3 protein was greater in the 1× quarters during the differential milking period ($P < 0.01$) and the differential milking + injection period ($P = 0.02$; Table 5). The amount of phosphorylated STAT3 protein was greater in the 1× quarters during the differential milking period ($P < 0.001$). The total amount of STAT5 protein was lower in the cows subsequently injected with DOMP during the differential milking period ($P < 0.01$; Table 5). The total amount of phosphorylated STAT5 protein was greater in the 3× quarters during the differential milking period ($P < 0.01$) and the differential milking + injection period ($P = 0.02$) and tended to be greater in the injection period ($P = 0.10$). The percentage of activated STAT5 was greater ($P < 0.01$) in the 3× quarters during all the treatment periods.

Mammary functional capacity was evaluated before and at the end of the experiment. At the start of the experiment, functional capacity was greater ($P = 0.05$) in the CTL cows than in the DOMP cows, averaging, respectively, 15.1 ± 0.8 and 12.5 ± 0.9 kg/half gland. At the end of the experiment, functional capacity remained greater in the CTL cows ($P = 0.02$) but was also greater in the half glands that were milked 3× than in those milked 1× ($P < 0.001$), averaging 10.6 ± 1.0 , 16.0 ± 1.0 , 8.9 ± 0.9 , and 12.6 ± 0.9 kg/half gland for the 1× CTL, 3× CTL, 1× DOMP, and 3× DOMP, respectively.

DISCUSSION

This study represents a first published attempt to test the hypothesis that the responsiveness of the mammary gland to PRL is modulated by milking frequency in dairy cows. Our results provide convincing evidence that milking frequency modulated the response of the mammary gland to PRL.

Domperidone injection induced a moderate increase in serum PRL concentration, which reached about 25 ng/mL. In previous studies, the same dose of DOMP induced a gradual increase in PRL concentration, to about 45 ng/mL (Lacasse and Ollier, 2015; Tong et al., 2018). In those studies, because of its low solubility in water, DOMP was administered s.c. as an oil suspension, which resulted in slow release of the compound. In the present study, DOMP was injected i.m. as a solution in DMSO. The latter is unlikely to have reduced the activity of DOMP, given that the i.v. infusion of DOMP in a solution of water and DMSO was found to induce a sharp increase in PRL concentration, although PRL concentration was already decreasing 30 min after the infusion (Lacasse et al., 2019). Intramuscular injection also induces rapid absorption of the product. Although we did not analyze the kinetics of PRL con-

Table 5. Effect of daily injections (INJ) of domperidone (DOMP; n = 6) or of dimethyl sulfoxide as the control (CTL; n = 5) and differential milking [DIF; once daily (1×) vs. thrice daily (3×)] on signal transducer and activator of transcription 3 (STAT3) and signal transducer and activator of transcription 5 (STAT5) levels in mammary gland biopsies collected at the end of each treatment period¹

Variable	DIF ²				DIF + INJ ³				INJ ⁴			
	CTL		DOMP		CTL		DOMP		CTL		DOMP	
	1×	3×	1×	3×	1×	3×	1×	3×	1×	3×	1×	3×
Phosphorylated STAT3 (AU) ⁶	7.85***	4.30	8.2	6.8	6.1	5.5	5.3	4.9	5.4	4.4	4.4	3.9
Total STAT3 (AU)	22.0**	17.1	23.9	15.7	16.9*	13.2	21.0	13.6	13.1	10.4	10.4	8.2
STAT3 activation (%) ⁷	37.3	25.0	39.0	45.5	38.0	45.1	31.0	35.7	54.1	46.2	51.8	8.6
Phosphorylated STAT5 (AU)	4.37***	6.59	3.37	7.03	3.33*	5.16	3.04	6.29	2.30†	3.87	2.94	4.68
Total STAT5 (AU)	39.0##	34.0	25.3	30.7	20.0	22.7	17.8	21.1	20.3	17.3	14.7	16.5
STAT5 activation (%) ⁸	10.5**	20.1	11.1	24.1	16.2**	25.4	12.8	29.4	12.2**	25.2	17.3	29.0

¹Data are presented as LSM. Mid-lactation cows were differentially milked from wk 3 to 10 and injected from wk 7 to 14.

²Differential milking period (wk 3 to 6).

³Differential milking + injection period (wk 7 to 10).

⁴Injection period (wk 11 to 14).

⁵The SEM reported is the highest among all periods.

⁶AU = arbitrary units.

⁷Calculated as (phosphorylated STAT3/total STAT3) × 100.

⁸Calculated as (phosphorylated STAT5/total STAT5) × 100.

Within a period, effect of milking frequency: † $P < 0.1$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; within a period, effect of injections: ## $P < 0.01$.

centration after the DOMP i.m. injection, it is likely that the injection resulted in a rapid increase in PRL concentration followed by a decline.

The response of milk production to DOMP was lower in the present study than in previous studies by our group (Lacasse and Ollier, 2015; Tong et al., 2018). A possible explanation is that the level of PRL concentration reached in those studies was greater. However, another difference between those studies and the present one is the season when the studies were performed. The previous studies were performed during the spring, whereas the present study was performed in the fall. Although the cows were kept indoors and exposed to the same lighting regimen, the air temperature fluctuates with outdoor air temperature. It is well established that the blood concentration of PRL increases with air temperature (Tucker and Wettemann, 1976). Consequently, in our previous experiments, DOMP injections were performed after a period of low PRL concentration (the winter), whereas in the present study, the injections followed a period of high PRL concentration (the summer). This difference is illustrated by the fact that PRL concentration during the pretreatment period was similar to the concentrations measured during the DOMP injection periods. In Tong et al. (2018), we showed that the milk response to DOMP is greater when it is preceded by a period of low PRL concentration. Furthermore, a short-day photoperiod during the dry period reduces PRL concentration during the dry period and increases milk production in the subsequent lactation (Auchtung et al., 2005; Lacasse et al., 2014), and this effect is reduced by the infusion of recombinant PRL (Crawford et al., 2015). Similarly, cooling dry cows during the summer was found to enhance milk production in the next lactation (Tao and Dahl, 2013). These results suggest that the galactopoietic action of PRL is reduced after a period of high circulating PRL levels and that may have contributed to the lower milk response to DOMP in this experiment.

As expected, milk production varied with milking frequency. Because of lower persistency in the 1× half glands, this difference increased with time, with the 3× half glands producing 1.8- and 2.2-fold more milk than the 1× half glands during the differential milking period and the differential milking + injection period, respectively. Also using differential milking at 1× versus 3×, Bernier-Dodier et al. (2010) and Boutinaud et al. (2003) similarly observed that half glands milked 1× had lower persistency and that the effect increased with time. Although the effect on milk production of differential milking at 2× versus 3× was less important, higher milking frequency has also been associated with better lactation persistency (Sorensen et al., 2008).

Milk production depends on the number of secretory cells and their activity. The gene expression of *CSN2*, *LALBA*, and *ACACA*, which encode β -CN, α -LA, and acetyl-CoA carboxylase, respectively, was lower in the 1× half glands than in the 3× half glands, indicating a modulation of cell activity by milking frequency. This result was expected, given that milk production is rapidly modulated by milking frequency and that most studies in which milking frequency was modulated showed changes in the expression of genes involved in the synthesis of milk (Boutinaud et al., 2019).

The level of STAT5 activation parallels the expression of genes involved in milk synthesis. In mammary epithelial cells, once it is activated, STAT5 dimerizes and is translocated to the nucleus, where it induces the expression of several genes involved in milk synthesis (Hynes et al., 1997). The activation level of STAT5 is decreased by milk stasis (Schmitt-Ney et al., 1992). Murney et al. (2015), using differential milking at 1× versus 4× daily, reported that STAT5 activation increased with milking frequency and correlated strongly with milk protein mRNA level. Therefore, it is likely that the modulation of STAT5 activation mediates at least some of the effect of milking frequency on milk synthesis.

Although STAT5 can be activated by several signaling pathways, activation is predominantly the result of PRL binding to the PRLR_{long} isoform in mammary epithelial cells (Furth et al., 2011). Although Western blotting did not show an effect on this receptor, real-time RT-PCR showed a reduction in gene expression in the half glands milked 1×. Similarly, Bernier-Dodier et al. (2010) reported that milking 1× reduced PRLR_{long} expression. In contrast, Murney et al. (2015) reported greater expression of PRLR_{long} in quarters milked 1× despite a reduction in STAT5 activation. Western blots also showed an increase in the PRLR_{short} isoform in the half glands milked 1×. The PRLR_{short} isoform is unable to activate STAT5 and has been shown to inhibit the function of the PRLR_{long} isoform (Binart et al., 2010). Nevertheless, gene expression of both isoforms were reduced by milking 1× and were strongly correlated ($R^2 = 0.96$, $P < 0.001$). The primers used in the present study were the same as those used by Bernier-Dodier et al. (2010), who found similar results. To determine whether the assay was distinguishing between the isoforms, the real-time RT-PCR was repeated using a more stringent approach (Supplemental Material S1; <https://doi.org/10.3168/jds.2019-17330>). The new assay gave virtually the same results (Supplemental Table S5; <https://doi.org/10.3168/jds.2019-17330>). Although some results suggest that milking frequency affects milk production by modulating the isoforms of PRLR on the

mammary epithelial cells, the lack of correspondence between the mRNA results and the protein results is difficult to explain. The fact that the RNA extracted from milk fat is essentially from mammary epithelial cells, whereas that extracted from mammary tissue biopsies also contains RNA from other cell types, which may have contributed to these results. Furthermore, Cánovas et al. (2014) reported that gene expression in RNA extracted from mammary tissue and milk fat was less correlated for lowly expressed genes than for highly expressed genes.

It is also possible that milking frequency modulated PRL signaling downstream of its receptor. The SOCS-3 protein has been shown to abrogate transactivation of a STAT5-responsive gene and has been proposed to act in a feedback manner to regulate sensitivity to PRL (Tam et al., 2001). Accordingly, Wall et al. (2006) reported the downregulation of *SOCS3* gene expression with frequent milking in early lactation. However, neither our results nor those of Alex et al. (2015) support the mediation of the effect of milking frequency by SOCS-3. Recently, serotonin has been shown to suppress β -CN expression by inhibiting STAT5 activation in human mammary epithelial cells (Chiba et al., 2014). Stretching of the alveoli has been shown to induce the expression of the limiting enzyme (TPH-1) for the synthesis of serotonin in mice (Matsuda et al., 2004). Several observations suggest that serotonin acts as a local feedback regulator of milk synthesis (Horseman and Collier, 2014). Nevertheless, in the present experiment, milk serotonin concentration was low and was not affected by milking frequency. Accordingly, *TPH1* gene expression was not detected in most of the samples. Therefore, this experiment provides no evidence that milking frequency affects PRL signaling downstream of the PRLR.

After 8 wk of differential milking, 2 $\times$ milking was resumed, and an important, but partial, difference in yield remained between the gland pairs that had been milked at different frequencies. Although not all studies reported a carryover effect of milking frequency on milk yield, our result is in agreement with those of Elliott (1961), Bernier-Dodier et al. (2010), and Nguyen et al. (2014). Furthermore, Phyn et al. (2014) reported that 1 $\times$ milking for 3 or 6 wk postcalving had lactation-long negative effects on milk production. A carryover effect on milk production can be the result of a change in mammary epithelial cell number. Mammary functional capacity, which is an indirect measurement of the size of the gland (Davis et al., 1998), was greater at the end of the present experiment in the half glands milked 3 $\times$, indicating that cell number was affected by milking frequency. Comparing functional capacities measured before and at the end of the experiment showed that the difference was due to a loss of tissue in the half

glands milked 1 $\times$. Accordingly, Bernier-Dodier et al. (2010) reported that visual inspection of the quarters after milking revealed an obvious reduction in the size of the 1 $\times$ quarters. In the present study, although the *BAX:BCL2* ratio was not affected by milking frequency, STAT3 abundance and activation were enhanced by the lowest milking frequency. The activation of STAT3 is proapoptotic and has been shown to occur during mammary gland involution in mice (Kritikou et al., 2003) and cows (Singh et al., 2009) and during 1 $\times$ milking in cows (Murney et al., 2015). In addition, Bernier-Dodier et al. (2010) reported more apoptosis in half glands milked 1 $\times$. As mentioned previously, STAT5 activation was decreased by 1 $\times$ milking in the present study. Besides enhancing the expression of genes involved in milk synthesis, STAT5 also affects mammary epithelial cell survival and proliferation (Furth et al., 2011). Therefore, the application of 1 $\times$ milking for several weeks reduces the number of epithelial cells, most likely by apoptosis.

A carryover effect on milk production can also result from the permanent modulation of gene expression (metabolic activity) by epigenetic modifications (Boutinaud et al., 2019). Nguyen et al. (2014) reported that the reduction of milk yield of half glands milked 1 $\times$ was associated with greater CpG methylation levels upstream of the *CSN1S1* gene and with a lower RNA level of this gene in 1 $\times$ half glands than in 2 $\times$ half glands. In the present study, we did not look at epigenetic modifications, but the gene expression of *CSN2*, *LALBA*, and *ACACA* was still downregulated during the injection period. Similarly, Grala et al. (2011) reported persistent reductions in *CSN1S1*, *LALBA*, and *ACACA* gene expression after 1 $\times$ milking. Nevertheless, changes in PRLR protein and gene expression and downregulation of STAT5 activation were still present at that time, suggesting that PRL signaling was still impaired. In conclusion, the results of this experiment support the hypothesis that the responsiveness of the mammary gland to PRL is modulated by milking frequency, although the underlying mechanism remains to be determined.

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ORCIDS

- X. Zhao <https://orcid.org/0000-0002-1078-7332>
 P. Lacasse <https://orcid.org/0000-0002-3579-7991>