Local regulation of increased milk yield due to early lactation increased milking frequency

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Dairy Science

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June 23, 2015

Blacksburg, VA

Keywords: Lactation, Increased Milking Frequency

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ABSTRACT

Increased milking frequency (IMF) during early lactation increases milk yield not only during the IMF period, but also after the cow is returned to twice daily milking (2X). The increase in yield is locally regulated within the gland; however the mechanism for the increase in yield is unknown. The objective of this study was to demonstrate a difference in milk and component yield, both during the IMF period and throughout the remainder of lactation, and examine potential local mechanisms driving the increase in production. Eight multiparous dairy cows were assigned to unilateral frequent milking [UFM ; 2X left udder half and 4-times-daily milking (4X) right udder half] for the first 21 days of lactation. Both udder halves were milked 2X for the remainder of lactation. Early lactation IMF significantly increased milk, fat, and protein yields in the right udder halves by 2.27 kg/d, 73.5 g/d, and 68 g/d respectively through the first 210 DIM ($P < 0.001$). At d 21, the right udder halves had a significant increase in activated signal transducer and activator of transcription 5 (STAT5), as well as a reduction in activated Akt ($P \leq 0.05$). There was no difference in STAT3 expression at d 21. There was no significant difference in gene expression of prolactin, insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 5 (IGFPB5), or chitinase 3-like-1 (CHI3L1) in mammary tissue at d 21 or 60; and no difference in protein expression of STAT5, Akt, or STAT3 in mammary tissue at d 60.

Acknowledgements

I would like to thank Dr.Corl for giving me the opportunity to study and work at Virginia Tech, and for all the guidance and knowledge he has so graciously bestowed upon me throughout my time as a graduate student (and especially the assistance in distinguishing real and made-up words). I would also like to thanks Dr. Akers and Dr. Rhoads for serving on my committee; I've learned a great deal in both of your journal clubs and classes. I would also like to thank Andrea Lengi, without whom this thesis would be completely void of any actual results, for helping me learn new lab methods, stressing over the immanent Ebola outbreak, and making time spent in the lab fun and enjoyable.

I would like to say a big thank you to all the graduate students in the Dairy Science Department that made these two years so much fun. From lunch club, wine nights, Game of Thrones, and so much more I have had a blast getting to hang out with all of you. (Special shout out to Alyssa and Kirsten for teaching me about things like ice-scrapes and basic winter survival skills.)

Thank you so much to my Mom and Dad for loving and encouraging me every chance you had, I wouldn't be here today without your support. Thanks so much to all my friends and family that make life great, and to Jake for pretending to be interested in things like western blots, chitinase 3-like 1, and other things I've rambled about while working on my thesis. All of you mean so much to me, and I can't say thank you enough.

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List of Abbreviations

1 X	Once daily milking
2 X	Twice daily milking
3 X	Three times daily milking
4 X	Four times daily milking
Akt	Protein kinase B
CHI3L1	Chitinase-3 Like 1
DHIA	Dairy Herd Improvement Association
DIM	Days in milk
IGF-1	Insulin like growth factor-1
IGFBP5	Insulin like growth factor binding protein-5
IMF	Increased milking frequency
PRL	Prolactin
r-bST	Recombinant bovine somatotropin
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator of transcription 5
UFM	Unilateral frequent milking

Chapter 1: Introduction

The world population is expected to grow to over 9 billion people by 2050 (FAO, 2011). This makes it imperative for the agricultural community to meet the demands of the growing population in an efficient and profitable manner. In addition to meeting the increased demand of a growing population, dairy producers are faced with several unique obstacles that further compound the challenge of increasing production.

The use of biotechnology, such as the administration of exogenous recombinant bovine somatotropin (r-bST) and the supplementation of ionophores, such as monensin, increase milk production and feed efficiency in lactating cows (Bauman, 1992). However, unfounded growing consumer concern over the safety of biotechnology in food production has reduced administration of these products by dairy farmers. For these reasons producers must utilize management strategies that maximize production while simultaneously controlling costs.

There are many options available to producers to increase milk yield throughout their herds. These include practices such as improving genetic selection, optimizing herd nutrition, or increasing voluntary cull rates. These management practices, if implemented correctly, lead to increased production; however, genetic improvement takes time before marked improvements are realized, in some cases optimizing herd nutrition is restricted by forage quality and availability, and some producers don't have the opportunity to increase voluntary cull rates. In these scenarios, increased milking frequency (IMF) is a viable management practice for producers to increase production immediately upon implementation.

Increased milking frequency as a management practice has been used to increase milk production for nearly 100 years. Depending on certain environmental factors and parity of the cows, three times daily milking (3X) throughout the entire lactation increases milk production by

approximately 13-14% compared to cows milked twice daily (2X) (VanRaden et al., 1999). Though 3X milking significantly increases milk yield, the producer must consider if the increase in production outweighs the added costs of an extra milking session. In addition to increased milk yield, there are also significant increases in cost associated with production. Additional milking sessions increase the cost of labor, parlor supplies, and other operating expenditures.

An alternative to 3X milking throughout the entire lactation, is IMF during early lactation. Early lactation IMF significantly increases milk yield, not only during the IMF period, but also once milking is returned to 2X later in lactation (BarPeled et al., 1995, Hale et al., 2003, Wall and McFadden, 2007a, Soberon et al., 2011, Wright et al., 2013). A study conducted by Hale et al. (2003) demonstrated that IMF during the first 21 days in milk (DIM) increased milk production from 34.5 kg/d in 2X cows to 37.8 kg/d in cows milked four times a day (4X). One of the most advantageous aspects of early lactation IMF, with regards to the producer, is the ability to see the increase in milk yield without the need to implement additional milking sessions for the whole herd. In studies investigating early lactation IMF, the milking times are not spaced in equal milking intervals. Milking intervals separated by 3, 9, 3, and 9 hours will elicit an acute and persistent increase in milk yield (Wall and McFadden, 2007b). This enables the producer to milk his early lactation cows at the beginning and end of the milking session; thereby, eliminating the need for a third milking session, decreasing the cost of labor, and gaining efficiency with parlor resources.

Though the increase in milk yield due to early lactation IMF is well documented, the mechanisms responsible for the increase in production is still undetermined. It is the objective of this study to demonstrate the increase in milk, milk fat, and milk protein yield due to early

lactation IMF, as well as examine the local regulators responsible for the acute, and the persistent increase in yields.

Chapter 2: Literature Review

Increased Milking Frequency

Milking frequency is defined as the number of milkings a cow receives in a 24 hour period. Increased milking frequency is used to describe anything greater than twice daily milking, and has been used as a management tool to increase milk production for decades. One of the first investigations into IMF was a study conducted in 1922 that indicated 3X milking increased milk production 4.6 kg/d in both Holstein and Guernsey multiparous cows (as reviewed by Wall et al., 2008) . Though the study into the effects of IMF began in the 1920s it wasn't widely scrutinized and implemented on farms until the 1980s and 1990s. It was during this time period that research efforts into the benefits and regulators of IMF, and its effects on production, were intensely studied.

Increasing milkings from 2X to 3X throughout the entire lactation will increase production by approximately 13%, while increasing to 4X can increase production by an additional 7% (VanRaden and Wiggans, 1995, Wall and McFadden, 2008). In a recent study comparing the effects of 1X versus 4X for the entirety of a lactation, udder halves milked 4X produced 137% more than udder halves milked 1X (Alex et al., 2015).

Though the increase in milk yield due to IMF is well characterized, the response to IMF has varied among experiments. In studies comparing 2X to 3X for the entire length of lactation, there have been differing responses depending on the parity of the animals in the study. DePeters et al. (1985) found multiparous Holsteins milked 3X for the duration of lactation saw a 17% increase in production relative to 2X, while primiparous cows only increased by 6%. Conversely, in three additional studies, multiparous Holstein cows increased milk yield 14.6% relative to 2X,

while primiparous cows increased 19.5% (Amos et al., 1985, Allen et al., 1986, Gisi et al., 1986). Though the relationship between parity and response to IMF is still inconclusive, one explanation for the potential increased milk yield response in primiparous cows, is their reduced udder capacity, and subsequent increase in mammary pressure between milkings (Wall and McFadden, 2008).

In addition to parity, breed may also affect milk yield response due to IMF. A study analyzing data from the Dairy Herd Improvement Association (DHIA) records of 2,143 first lactation Jersey cows and 4,293 first lactation Holstein cows indicated that Jerseys increased production by 6.3% when milked 3X, while Holsteins increased 17.3% (Campos et al., 1994). These findings led the authors to suggest it might not be as lucrative to increase milking frequency from 2X to 3X on farms milking predominately Jersey cows.

Though IMF increases milk yield, its effects on milk components aren't as consistent. Many studies have shown that as milking frequency increases, milk fat and protein concentrations decrease. However, this decrease in milk solid concentrations is often compensated for by an increase in milk yield (Klei et al., 1997, Dahl et al., 2004). A study comparing DHIA records from 14 Holstein herds in California indicated that switching to 3X decreased milk fat percentage from 3.6% to 3.5% . Allen et al. (1986) reported a reduction in milk fat percentage, but an overall increase in milk fat yield from 282 kg/lactation to 292 kg/lactation, due to the increase in milk production. Smith et al. (2002) evaluated DHIA records from 10,754 Holstein herds to compare the effects of 3X vs. 2X on production. Thrice daily milking significantly decreased milk fat percent from 3.7% in cows milked 2X, to 3.6 %; and decreased milk protein percentage from 3.7% in cows milked 2X to 3.1% in cows milked 4X.

IMF increases milk yield regardless of parity or breed, however the response to IMF is variable. Additionally, IMF reduces milk fat and milk protein concentrations, however the fat and protein yield is increased due to the overall increase in milk production.

Early Lactation Increased Milking Frequency

In addition to IMF for the duration of lactation, there is also an opportunity to increase milk yield by increasing milking frequency for a short time during early lactation. Early lactation IMF not only increases milk production acutely, but also persistently. This response is not seen when short intervals of IMF are used during mid- or late lactation, as they don't elicit the carryover effect consistent with early lactation IMF (Wall and McFadden, 2008). The carryover effect of early lactation IMF on milk production makes it a very advantageous management tool for dairy producers.

Many of the first studies that demonstrated the carryover effect of milk yield due to IMF were conducted on cows that were suckled, in addition to being milked, for a time period during early lactation. Thomas et al. (1978) compared milk yield effects of cows machine milked 2X to cows machine milked 2X and suckled by her calf, for the first 56 days of lactation. At day 57, the calves were weaned and all cows were milked 2X for the remainder of the lactation. Cows that were suckled, in addition to being machine milked, produced 1.68 kg/d more than the control group during the IMF period, and 1.77 kg/d more than the control through 196 DIM (Thomas et al., 1978). It is interesting to note that the persistent effect was greater than the acute effect, but may be accounted for by errors in measuring the volume of milk suckled by the calves during the IMF period. This was the only study where the persistent increase was greater than the acute increase in milk production.

To further investigate the effects of early lactation IMF, Hale et al. (2003) conducted an experiment to determine the milk yield effects due to early lactation IMF in multiparous Holstein cows, and if a shorter period of IMF would elicit the persistent effect seen with longer periods of IMF. Cows were assigned to one of three groups; Control, IMF1, or IMF4. Control cows were milked 2X throughout the entire lactation, IMF1 cows were milked 4X from 1-21 DIM, and IMF4 cows were milked 2X from 1-3 DIM, and 4X from 4-21 DIM. All groups were milk 2X from d 21 throughout the remainder of lactation. During the acute, frequent milking period, control, IMF1, and IMF4 cows produced 33.5 kg/d, 42.3 kg/d, and 38.3 kg/d, respectively. After the cessation of IMF, cows produced 34.8 kg/d, 37.4 kg/d, and 37.5 kg/d, respectively. The milk yield difference between the cows in the control group and both IMF1 and IMF4 was significant, however there was no significant difference between the two IMF groups. The authors concluded that a shorter period of 21 d was sufficient to elicit the carryover effect of increased yield due to IMF. (Hale et al., 2003). This was in opposition to many of the previous studies that had utilized longer periods of 41-140 d of IMF during early lactation (Everitt and Phillips, 1971, Thomas et al., 1978, BarPeled et al., 1995). In addition to determining that a shorter period of IMF would still cause an acute and persistent increase in milk yield, they also determined that there was no significant difference between beginning the IMF at 3 days post partum, versus beginning 1 day post partum.

Regulation of Milk Yield Response due to Early Lactation IMF

Increased milk yield is mediated in one of three ways; increased secretory cell activity, increased mammary cell proliferation, or decreased mammary cell apoptosis (Capuco et al., 2003). It is still unclear what mechanism mediates the increase in milk yield due to IMF, especially IMF during early lactation; but there have been many hypotheses regarding the potential mediators.

As previously reported, Hale et al. (2003) demonstrated 4X for a period of 21 d during early lactation was sufficient to increase milk production relative to 2X milking. They also investigated possible mechanisms responsible for the increase in milk yield. Biopsies were obtained from 4 cows from each treatment group at 7 and 14 DIM. Tritiated-thymidine incorporation and immunohistochemical localization of the Ki-67 antigen was used to evaluate proliferation. There was no significant difference between treatment groups for mammary stromal or mammary epithelial cell proliferation at either 7 or 14 DIM. Terminal deoxynucleotidyl transferases dUTP nick end labeling (TUNEL) was utilized to detect apoptotic cells, but there was no significant difference between treatment groups for apoptotic mammary stromal or mammary epithelial cells. Blood samples were taken three times a week for the first 2 weeks of lactation, and subsequently at week 3, 4, 5, 6, 8, and 10. Serum IGF-1, bST, and prolactin (PRL) were measured, but there was no treatment effect on serum concentrations of the three hormones (Hale et al., 2003). The authors concluded early lactation IMF did not significantly affect mammary cell turnover, or serum concentration of IGF-1, bST, or PRL.

Circulating PRL increases due to milking stimulus, and is at its highest concentration at 8 weeks of lactation. However, the production of PRL declines throughout the remainder of lactation, and isn't released after 32 weeks of lactation (Koprowski and Tucker, 1973). PRL promotes proliferation and differentiation of mammary epithelial cells and promotes lactogenesis

(Hennighausen et al., 1997). The correlating relationship with circulating PRL and milk production, as well as its lactogenic effects, prompted investigators to explore the possibility that PRL secretion mediated the increase in milk yield due to IMF in early lactation.

Wall et al. (2006) divided 15 multiparous Holstein cows into three different treatment groups for the first three weeks of lactation: 2X milking with the administration of exogenous PRL, 2X milking without exogenous PRL, or 4X milking. Cows milked 2X and administered PRL or milked 4X produced significantly more milk than cows milked 2X (46.9 kg/d, 45.7 kg/d, and 40.5 kg/d, respectively; Crawford et al., 2004). However, there was no effect on plasma PRL concentration, mammary cell proliferation, or mammary cell apoptosis between the three treatment groups. There were differences between the relative abundance of several transcription factors associated with PRL signaling. Expression of suppressor of cytokine signaling (SOCS) 3 was significantly down regulated in both the 2X cows with the administration of PRL and the 4X cows, relative to the 2X cows (Wall et al., 2006). SOCS3 acts as a negative regulator of PRL signaling and is increased during periods of milk stasis in lactating rats (Tam et al., 2001). This down regulation of SOCS3 in the mammary gland of 2X cows that were also administered PRL and 4X cows could indicate an increase in PRL signaling (Wall et al., 2006). Wall et al. concluded both 2X plus PRL, as well as 4X significantly increased milk production, reduced the expression of SOCS3 in the mammary gland, and had no effect on mammary cell proliferation, mammary cell apoptosis, or plasma PRL concentration, relative to 2X cows. Plasma PRL concentrations may not have been altered due to the timing of the blood sampling.

Regulation of Milk Yield Response due to Early Lactation IMF is Locally Regulated

The increase in milk yield due to IMF is locally regulated within the mammary gland. Wall and McFadden (2007a) assigned ten multiparous cows to unilateral frequent milking (UFM) for the first 21 days of lactation. The UFM consisted of the left udder half being milked 2X while the right udder half was milked 4X for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. This model allowed the investigators to determine if the mediator of the IMF effect on milk yield was locally regulated. During the UFM period the right udder half produced 3.5±0.2 kg/d more than the left udder half. After both udder halves were returned to 2X at 22 DIM, the right udder half continued to produce 1.8±0.2 kg/d more milk than the left udder half for the remainder of lactation (Wall and McFadden, 2007a). The UFM model was able to elicit the acute and persistent increase in milk yield observed in previous studies. These results indicated the increase was locally regulated and not a product of increased systemic hormone circulation.

To further evaluate the potential local regulator of IMF mediated increase in milk yield Wall et al. (2013) conducted an experiment to examine the physiological and transcriptional changes associated with the acute and persistent increases in milk yield. Six multiparous Holstein cows were assigned to UFM with the left udder half milked 2X and the right udder half milked 4X for the first 21 days of lactation. The cows were subsequently milked 2X for the remainder of lactation. In order to fully investigate the physiological and transcriptional changes associated with the acute and persistent changes in milk yield associated with UFM, mammary biopsies were taken on d 21, 23, and 40 of lactation. These time points were chosen to correspond with the immediate increase in yield seen at the initiation of 4X, the acute decline in yield upon cessation of 4X milking, and the persistent increase in yield for the remainder of lactation.

Cellular proliferation and apoptosis, as well as mammary cell population dynamics and transcriptional responses were evaluated.

As with previous studies, milk yield was increased in the 4X udder half relative to the 2X udder half (Figure 2.1). There was no significant difference in cellular proliferation between the udder halves, as measured by tritiated-thymidine incorporation; as well as no difference in either epithelial or stromal cell apoptosis between the udder halves, as measured by TUNEL (Wall et al., 2013).

In addition to changes in milk yield and cell turnover, the authors wanted to investigate the transcriptional changes associated with IMF during early lactation. The objective was to identify clusters of genes that altered expression in response to the three different phases of milk yield response due to IMF. Changes in transcription were assessed using Affymetric GeneChip bovine genome microarray. Of the 1637 genes identified, 75 were considered for further evaluation based on their fold change of equal to, or greater than, a magnitude of two. A total of 64 genes had a reversal of differential expression of genes at the three different biopsy periods (Wall et al., 2013). The authors concluded the reversal of gene expression indicated a potential autocrine or paracrine regulation of milk yield in response to early lactation IMF.

Potential Local Regulators of Increased Milk Yield due to Early Lactation Increased Milking Frequency

Increased milk yield response due to IMF is regulated within the mammary gland, and is not a product of change in mammary cell number; therefore, it is likely mediated by an increase in mammary epithelial cell activity. The coordinated transcriptional response identified by Wall et al. (2013) indicates the potential for the autocrine or paracrine regulation of increased cell signaling pathways within the mammary glands, in response to early lactation IMF. Local

production of PRL, IGF-1, IGFBP5, and CHI3L1 are potential regulators of increased milk yield due to early lactation IMF.

The importance PRL plays in regulating milk synthesis is well documented, having mammogenic, lactogenic, and galactopoietic effects (Flint and Knight, 1997, Freeman et al., 2000, Capuco and Akers, 2011). Though it is primarily secreted from the anterior pituitary, there is evidence of autocrine PRL production in the mammary gland of mice and rats (Kurtz et al., 1993, Steinmetz et al., 1993, Chen et al., 2010). In the mammary gland, PRL binds its receptor causing the dimerization of PRL receptors on the mammary epithelial cell membrane. Receptor dimerization causes cross phosphorylation of the receptor-associated Janus kinases (JAKs) as well as the intracellular domain of the PRL receptor (Freeman et al., 2000). The Src homology 2 (SH2) domains of the signal transducer and activator of transcription 5 (STAT5) are recruited to the phosphorylated receptor and phosphorylated by JAK2. Following phosphorylation, two STAT5 proteins form a dimer and are translocated into the nucleus where they bind to the γ-interferon activation site (GAS) on the gene promoters (Hennighausen et al., 1997). This binding leads to transcription of several milk proteins, mainly casein and α- lactalbumin (Freeman et al., 2000). Levels of activated STAT5 are elevated in udder halves milked 4X the first 14 DIM, relative to udder halves milked 1X, and the difference in expression correlated with major milk protein mRNA abundance (Murney et al., 2015). However, the difference between expression of activated STAT5 has not been investigated in cows undergoing UFM of 4X and 2X for the first 21 DIM. Increased production of local PRL and subsequent activation of STAT5 could mediate the increase in milk yield due to early lactation IMF.

In addition to local PRL, IGF-1 production and its signaling through Akt in the mammary gland could be a potential mediator of increased milk and component yield. Though the majority

of circulating IGF-1 is secreted from the liver, it often elicits physiological changes in an autocrine or paracrine manner in many different tissues; furthermore, IGF-1 mRNA has been identified in bovine mammary tissue (Holly and Wass, 1989 , Glimm et al., 1990) . Binding of IGF-1 to the IGF-1 receptor stimulates the phosphorylation and activation of Akt. Akt activation can directly increase cell activity by increasing lipid synthesis, and delays mammary gland involution in mice (Schwertfeger et al., 2001, Schwertfeger et al., 2003). In a recent study utilizing ingenuity pathway analysis (IPA), Wall et al., (2013) indicated early lactation IMF for the first 21 DIM was associated with weak activation of IGF-1 signaling at 40 DIM. Increased production of local IGF-1 and Akt activation could increase the production of milk and milk component yield. Additionally, IGF-1 activity is modulated by IGF-1 binding proteins (IGFBP). IGFBPs have the ability to both increase or decrease the activity of IGF-1, depending on the environment (Jones and Clemmons, 1995). IGFBP5 is one of the primary IGFBP found in the bovine mammary gland, and is indicated as an involution marker. In rats, increased circulating IGFBP5 is observed 48 h after removal of the pups (Tonner et al., 1997). This action indicates that, in the mammary gland, it binds and sequesters IGF-1, thereby preventing its anti-apoptotic actions on the mammary epithelial cells. Additionally, IGFBP5 inhibits IGF-1 mediated Akt phosphorylation and increased cell activity (Sakamoto et al., 2007). Changes in production of local IGF-1 and IGFBP5 could mediate the increase in yield as a result of early lactation IMF through Akt signaling in the mammary gland.

Though previous studies have shown cell turnover isn't effect by early lactation IMF, there is also the possibility that there is a change in cell number that is undetectable with current procedures. In addition to altering pathways that increase cell activity, early lactation IMF might also alter the regulation and expression of proteins typically active at the onset of involution, and

responsible for the increase in involution associated apoptosis. Wall et al. (2013) indicated that early lactation IMF significantly decreased the expression of CHI3L1, both acutely and persistently, in udder halves milked 4X for the first 21 DIM. CHI3L1 is a downstream gene product of STAT3, and is characterized as a protein that mediates inflammation and tissue remodeling (Scully et al., 2011). STAT3 is a known marker of involution, and deletion of STAT3 in mammary tissue delays involution in mice by suppressing mammary epithelial cell apoptosis (Chapman et al., 1999). With the onset of involution, CHI3L1 is up-regulated in a STAT3 dependent manner, and in human and mice models CHI3L1 stimulates tumor angiogenesis and reduces survival rate in breast cancer patients (Jensen et al., 2003, Hughes et al., 2012). However, it is also up-regulated in mammary epithelial cells at the onset of involution, suggesting its role in apoptosis, remodeling, and gland regression (Scully et al., 2011). Additionally, secreted CHI3L1 is present in mammary secretions collected from involuting cows (Aslam and Hurley, 1997). This alteration in expression of proteins associated with involution could be responsible for the persistent increase in milk yield after the cessation of IMF.

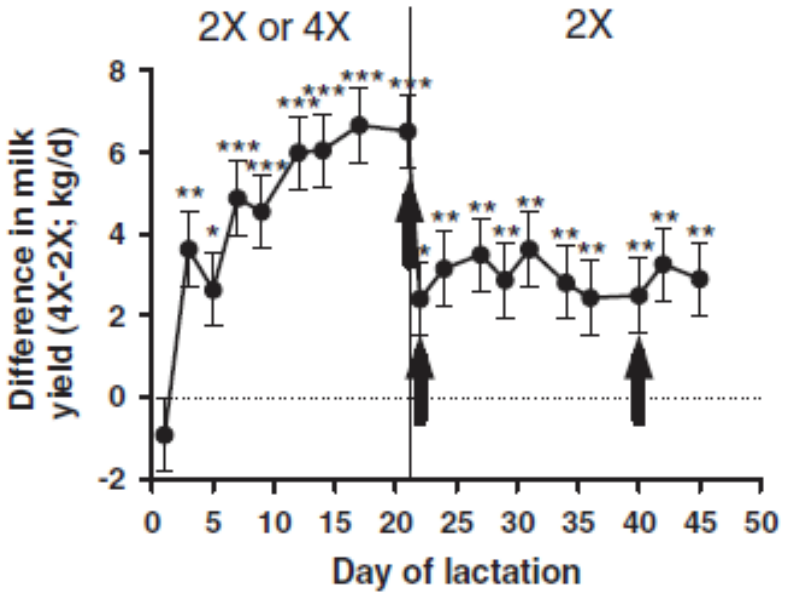

Figure 2.1 Changes in milk production in response to IMF Difference in milk yield between udder halves, of 6 cows assigned to UFM for the first 21days of lactation. Milk production from the 2X udder half was subtracted from that of the 4X udder half to obtain differential yield. There was no difference between udder halves in milk production on day 1 of lactation ($P > 0.30$). Milk production of the 4× udder half increased dramatically ($P < 0.001$) during UFM and was greater than that of the 2× udder half through day 45 of lactation ($P < 0.01$). Black vertical arrows indicate timing of biopsy sampling. (Wall et al., 2013)

Chapter 3: Effects and Regulation of Increased Milking Frequency during Early Lactation

INTRODUCTION

Increasing milking frequency from 2X to 3X increases milk yield approximately 12-14%, regardless of stage of lactation (VanRaden et al., 1999, Stelwagen, 2001). However, increased milking frequency (IMF) during early lactation increases milk yield acutely as well as persistently after cows are switched back to 2X milkings (Thomas et al., 1978, Hale et al., 2003, Wall et al., 2006). Hale et al. (2003) demonstrated an increase in milk yield of 9.6% in cows milked 4X the first 21 days in milk (DIM), compared to those milked 2X. This milking management approach provides producers the opportunity to attain increased milk yields without investing the resources required to increase milking frequency for the duration of a cow's lactation.

The increase in milk yield due to IMF appears to be locally regulated within the mammary gland. Wall et al., (2007a) utilized a unilateral milking frequency model [UFM; 2X for the left udder half and 4-times-daily milking (4X) for the right udder half] for the first 21 DIM to investigate the regulation of the increase in milk yield. During the IMF period, udder halves milked 4X for the first 21 DIM produced 3.9 kg/d more than the udder halves milked 2X the first 21 DIM. Upon the cessation of IMF, the udder halves milked 4X produced 1.8 kg/d more than the udder halves milked 2X the first 21 DIM (Wall and McFadden, 2007a). These results confirmed the increase in milk yield is locally regulated and not a result of changes in systemic hormones such as prolactin (PRL).

Milk production is determined by the number of mammary epithelial cells, and the secretory activity of those cells (Capuco et al., 2003). Several experiments have examined

mammary epithelial cell proliferation, as well as apoptosis and found no change in response to early lactation IMF (Wall et al., 2006, Wall and McFadden, 2010, Wall et al., 2013). The increase in milk yield due to early lactation IMF is not regulated by changes in cell turnover, or alternatively, the change in cell turnover is undetectable. Therefore, the increase in milk yield might be mediated by an increase in mammary epithelial cell activity, possibly driven by hormone changes.

PRL has mammogenic, lactogenic and galactopoietic effects, and though it is primarily secreted from the anterior pituitary, there is evidence of local PRL production within the mammary gland of mice and rats (Kurtz et al., 1993, Steinmetz et al., 1993, Freeman et al., 2000, Chen et al., 2010). PRL activates STAT5 via the Janus kinase 2 (JAK2)/STAT5 pathway, and increases transcription of STAT5 target genes, such as casein and α-lactalbumin (Freeman et al., 2000).

Though the majority of circulating insulin-like growth factor-1 (IGF-1) is secreted from the liver, it often elicits physiological changes in an autocrine or paracrine manner, and IGF-1 mRNA has been identified in bovine mammary tissue (Holly and Wass, 1989 , Glimm et al., 1990). IGF-1 can stimulate the phosphorylation of Akt, which regulates lipid and protein synthesis as well as cell cycle progression (Hadsell and Bonnette, 2000). In a recent study, Wall et al., (2013) found early lactation IMF was associated with weak activation of IGF-1 signaling at 40 DIM. The increased production of local IGF-1 and Akt activation could increase the production of milk and milk component yield. Additionally, IGF-1 activity is modulated by IGF-1 binding proteins (IGFBP). IGFBPs have the ability to both increase or decrease the activity of IGF-1, depending on the environment (Jones and Clemmons, 1995). Tonner et al. (1997)

implicated IGFBP5 as an involution marker, in rats. Additionally, IGFBP5 inhibits IGF-1 mediated Akt phosphorylation and subsequent increases in cell activity (Sakamoto et al., 2007).

Though studies have not been able to detect a change in mammary epithelial cell turnover due to early lactation IMF, it is possible there is a level of change in cell number that is undetectable by previously used approaches. In addition to altering cell signaling activities, we hypothesize that early lactation IMF alters the regulation and expression of proteins typically active at the onset of involution. Wall et al. (2013) found that early lactation IMF significantly decreased the expression of CHI3L1, both acutely and persistently, in udder halves milked 4X for the first 21 DIM. This alteration in expression could be responsible for the persistent increase in milk yield after the cessation of IMF. STAT3 is an involution marker that plays a key role in the initiation of involution after the removal of pups in lactating mice, and chitinase 3-Like-1 (CHI3L1) is a downstream target of activated STAT3 (Chapman et al., 1999, Scully et al., 2011). Early lactation IMF could increase milk yield by decreasing the expression of proteins associated with involution such as STAT3 and CHI3L1.

Our objective was to demonstrate the increase in milk, milk fat, and milk protein yield due to early lactation IMF, and examine the local regulators responsible for the acute, and the persistent increases in yields. We hypothesized this increase in the milk yield is mediated by the local production of factors that increase cell activity and reduce cell death.

MATERIALS AND METHODS

Animals and Treatments

The Virginia Tech Institutional Animal Care and Use Committee approved all procedures performed on animals. Eight multiparous cows (2.4±0.7 lactations), seven Holstein and one Jersey, were used for the experiment. One animal developed a case of Coliform mastitis in the left rear quarter and was removed from the study at d 21. At the initiation of lactation, each cow was assigned to unilateral frequent milking [UFM; 2X for the left udder half and 4-times-daily milking (4X) for the right udder half] for the first 21 days of lactation. Both udder halves were milked at 3 AM and 3PM, and the right udder halves were milked additionally at 6 AM and 6 PM (Figure 3.1). Beginning at 22 DIM both udder halves were milked 2X for the remainder of lactation.

Cows were housed in free stalls at the Virginia Tech Dairy Center and fed a total mixed ration (TMR) formulated to meet or exceed nutrient requirements with ad libitum access to clean water. The TMR was mixed daily and delivered at 9:30 AM.

Milk Sampling

Milk yields from each udder half were measured using a Surge RX quarter milking unit (Galesville, WI). Milk samples were taken at the second milking post partum to confirm that cows produced similar amounts of milk from each udder half prior to the initiation of the UFM. Cows that were unbalanced by greater than 0.68 kg were excluded from the study. A total of two cows were excluded from the study. During UFM, milk samples were taken from each udder half on d 7, 14, and 21 at each udder halves' milkings, in order to obtain half udder milk weights and samples for milk composition. Milk yield and samples for composition analysis were also collected from each udder half on d 60, 120, 180, and 210 of lactation at the 3 AM milking. Milk

composition was analyzed by United DHIA (Radford, VA). In addition to sampling for milk and component yield, milk samples were collected for RNA extraction from milk fat on d 21, 60, 120, and 180.

Mammary Biopsies

Mammary biopsies were obtained from each rear quarter on d 21 and 60 of lactation. Cows were sedated (standing sedation) with xylazine prior to the procedure. The cows were prepped for a caudal epidural, between the sacral-coccygeal vertebrae or the first and second coccygeal vertebrae, for pain management prior to the biopsy. The area was prepped by clipping and scrubbing with betadine and alcohol to provide a sterile field prior to the epidural. An 18 gauge needle was used to inject 1 mL of Lidocaine per 45 kg body weight (5-8 mL) into the epidural space. To prevent infection after the mammary biopsies, the cows were given antibiotics for three days following the procedure.

For mammary biopsy, a 2 to 3 cm incision was made through the skin using a size 20 scalpel blade. The underlying connective tissue capsule was dissected down to the mammary parenchyma avoiding any large subcutaneous blood vessels. A small piece (~1 g) of secretory tissue was removed using a scalpel blade and scissors. The incision was closed with cruciate sutures using 1-3 polyamide suture materials.

Tissue Preparation

Immediately following biopsy, secretory tissue was sectioned into small pieces (50-100mg) . Tissue pieces were processed differently for subsequent analyses. Tissue pieces used for immunoblotting were immediately placed in lysis buffer (50 mM Tris pH 7.4, 0.5% Triton X-100, 0.3 M NaCl, 2 mM EDTA pH 8.0, 1mM sodium orthovanadate, and protease inhibitor

cocktail), transported to the lab on ice (less than 5 min), and homogenized using a PRO 200 homogenizer (PRO Scientific Inc., Oxford, CT) . Following homogenization, samples were aliquoted and stored at -80°C until used for immunoblotting. Tissue pieces for RNA extraction were placed in liquid nitrogen and snap frozen. Frozen pieces were transferred to cryotubes, and stored in liquid nitrogen until they were transported to the lab and subsequently stored at -80°C until used for RNA extraction.

Immunoblotting

Immunoblotting was conducted on tissue samples to quantify protein abundance in mammary tissue. Tissue was homogenized in lysis buffer, transferred to a micro-centrifuge tube, and stored on ice for 30 minutes. Samples were then centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatant was transferred to a clean tube and 50 µL of the supernatant was saved for total protein quantification (Bradford Assay; Bio-Rad, Hercules, CA). An equal volume of 2X Laemmli Sample Buffer (Sigma Chemical Co., St. Louis, MO) was added to the protein supernatant. Samples were then incubated on the heat block at 95°C for 10 minutes. Proteins were separated via gel electrophoresis using 12% PAGEr Gold PlusPreCast SDS polyacrylamide gels (Lonza, Rockland, ME). A total of 80 µg of protein was loaded into each lane, and samples were standardized to 20µl. Following electrophoresis, the proteins were transferred to a PVDF membrane (GE Healthcare Life Sciences, Piscataway, NJ), using a Bio-Rad Trans-Blot SD semi-dry transfer apparatus (Bio-Rad, Hercules, CA). The apparatus was run on constant current for 80 minutes at 70 mA per mini gel. Membranes were rinsed in blocking buffer, either TBST-2% BSA (0.05 M Tris pH 7.4, 0.2 M NaCl, 0.1% Tween, and 2% Bovine Serum Abumin) for phospho-specific antibodies, or TBST-5% milk for non-phospho-specific antibodies (0.05 M Tris

pH 7.4, 0.2 M NaCl, 0.1% Tween, and 5% dried non-fat milk). Membranes were then blocked in blocking buffer for 1 hour at room temperature on a rocking platform. Primary antibody (Table 3.1) was diluted in 20 ml of blocking buffer and added to the membrane and was incubated overnight at 4°C on a rocking platform. After incubation, the membrane was washed in TBST twice for a period of 10 minutes at room temperature on a rocking platform. The secondary antibody was diluted in 20 ml of blocking buffer and added to the membrane for an incubation period of 1 hour, at room temperature on a rocking platform. Following incubation the membrane was washed four times for a period of 10 minutes each wash, in TBST at room temperature. ECL Prime (GE, Pittsburg, PA) reagents equilibrated to room temperature were used to detect protein using a Bio-Rad Chemidoc (Bio-Rad).

For d 60 western blot analysis, one cow was removed from analysis (n=6) due to sample degradation.

RNA Extraction

Milk Fat

Total RNA was extracted from milk fat. A total of 45 ml of milk were centrifuged for 10 minutes at 2,000 x g at 4°C. A total of 1,250 mg of fat from the fat pad was homogenized with 5 ml of Tri Reagent LS (Molecular Research Center, Cincinnati, OH). The homogenate was supplemented with 20 µl of Polyacryl Carrier 152 (Molecular Research Center, Cincinnati, OH) and stored at room temperature for five minutes to allow complete dissociation of nucleoprotein complexes. Following homogenization, the insoluble material was removed from the homogenate by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was transferred to a clean tube and supplemented with 0.5 ml of bromo-chloropropane (Molecular

Research Center), vortexed for 15 s and stored at room temperature for 15 min. Following incubation, the resulting mixture was centrifuged at 12,000 x g for 15 minutes at 4°C. Centrifugation separates the mixture into three phases, the upper aqueous phase was removed to a clean tube and mixed with 2.5 ml of isopropanol. The mixture was incubated at room temperature for 10 minutes, and subsequently centrifuged at 12,000 x g for 8 min at 4°C. The resulting RNA pellet was washed in 75% ethanol by centrifugation at 7,500 x g for 5 minutes at 4°C. The ethanol was removed, and pellet allowed to air dry for five minutes. After drying, 100 µl of RNase free water was added to the pellet to resuspended the RNA and placed on the heating block for 10 min at 55°C. Following resuspension, an additional ethanol precipitation was performed, and RNA pellets resuspended in 50 µl of RNAse free water. RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

Tissue

Total RNA was extracted from mammary gland tissue using 1.2 ml of RNAzol®RT (Molecular Research Center Inc., Cincinnati, OH) per 100 mg of tissue according to manufacturer's instructions. RNA pellets were resuspended in RNAse free water and concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

Real Time Quantitative PCR

Real time PCR (qPCR) was used to measure transcripts in milk fat RNA and tissue RNA. The Omniscript RT kit (Qiagen, Valencia, CA) was used to reverse transcribe a total of 2.8 µg of

RNA in a 60 µl cDNA reaction for milk fat, and 2.5 µg of RNA in a 50 µl cDNA reaction for mammary tissue , according to the manufacturer's instructions using oligo-dT (Eurofins MWG, Operon, Huntsville, AL). Real-time quantitative PCR was performed using the GoTaq® qPCR Master Mix (Promega, Madison, WI) according to the manufacturer's instructions. Real-time was performed in an Applied Biosystems 7300 Real Time PCR System (Foster City, CA). Each reaction was performed in duplicate wells as follows: one cycle of 95°C for 10 minutes, 40 replications of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for one minute, followed by 1 replication of 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Relative abundance of target gene transcripts were determined by using the geometric mean of three endogenous control genes (Vandesompele et al., 2002). Fold changes were calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001). Primers for both target and endogenous control genes are included in Table 3.2.

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS 9.4; SAS Institute, Inc., Cary NC). Three separate models were used to analyze data. For western blot analysis of tissue samples the model included cow, treatment and blot. For real-time PCR analysis of tissue sample the model included cow and treatment. For milk and component yield, as well as milk fat real-time analysis the model included treatment, day, and treatment X day interaction. Fixed effects included treatment and day; random effects included cow and cow by day. For milk and component yield, as well as milk fat real-time PCR data, day was treated as a repeated measure. The subject for the REPEATED statement was cow and compound symmetry was used as the

covariance structure. Significance was determined at $P \leq 0.05$, and trends were determined at $P \leq 0.10$. Data are expressed as least square means ± standard error of the means.

RESULTS

Half Udder Milk and Component Yield

Prior to initiation of UFM, both udder halves produced similar amounts of milk. Early lactation IMF for the first 21 DIM increased milk and component yield both acutely, and persistently (Table 3.3). The right udder halves produced 2.27 ± 0.44 kg/d more than the left udder halves from 1-210 DIM ($P < 0.0001$; Fig 3.2). There were no significant day*treatment interactions.

Milk fat concentration was reduced in the right udder halves by 0.27% from 1-210 DIM ($P \leq 0.01$). Even with the reduced milk fat concentration, the right udder halves produced 73.5 ± 25.91 g/d more than the left udder halves from 1-210 DIM ($P < 0.0001$; Fig. 3.3).

Milk protein concentrations were not significantly affected by early lactation UFM ($P > 0.6$). The right udder halves produced 68.0 ± 21.9 g/d more protein than the left udder halves from d 1 to 210 ($P < 0.0001$; Fig 3.4).

Protein Expression

There were no significant differences between the total or phosphorylated forms of STAT5 at d 21 between the udder halves. Images of the western blots for total and phosphorylated STAT5 at d 21 are shown in Figures 3.5. However, there was an increase in activated STAT5 at d 21 (phospho/total STAT5) in the right udder halves relative to the left udder halves. ($P \leq 0.055$, Fig. 3.5). There were no significant differences between total, phosphorylated or activated STAT5 expression between the udder halves at d 60 (See Fig 1 in Appendix A).

For Akt, the right udder halves had a significant reduction in phosphorylated Akt ($P \leq 0.01$) as well as activated Akt (phospho/total Akt) at d 21 relative to the left udder halves ($P \leq 0.02$; Fig 3.6). Images of the western blots for total and phosphorylated Akt at d 21 are shown in Figures 3.6. There were no significant differences between total, phosphorylated, or activated Akt at d 60 (See Fig 2 in Appendix A).

For STAT3 there were no significant difference between total, phosphorylated, or activated STAT3 at either d 21 or d 60 (See Fig 3 in App A).

Gene Expression

Real time quantitative PCR was used to quantify relative mRNA abundance of IGF-1, IGFBP5, PRL, and Chitinase 3-like-1 (CHI3L1) in milk fat and mammary gland tissue. Milk fat mRNA comes exclusively from mammary epithelial cells; samples were taken on d 21, 60, 120, and 180 of lactation in order to identify potential temporal patterns of gene expression in mammary epithelial cells in response to early lactation IMF. Mammary biopsies were taken on d 21 and 60 to correspond with the acute increase in milk yield associated with IMF, as well as the persistent carryover effect that remains after the cessation of IMF at d 21.

There were no significant differences between mRNA expression of IGF-1, IGFBP5, or CHI3L1 at d 21 (Table 3.4). The right udder halves tended to have a higher abundance of PRL mRNA relative to the left udder halves at d 21 ($P \leq 0.09$; Table 3.4). There were no significant differences between mRNA expression of IGF-1, IGFBP5, PRL, or CHI3L1 at d 60.

There was no significant treatment effect on the expression of target genes in the milk fat mRNA from cows milked unilaterally the first 21 days of lactation (Fig.3.7). There was a significant day effect on the relative abundance of IGF-1 in milk fat ($P \leq 0.02$; Fig.3.7A) indicating a temporal pattern of IGF-1 mRNA expression in the mammary epithelial tissue of both udder halves.

DISCUSSION

The increase in milk yield in the right udder halves in response to IMF was seen both acutely and persistently through 210 DIM. This is in agreement with previous studies that also elicited an acute and persistent increase in production with early lactation IMF (Hale et al., 2003, Wall and McFadden, 2007a, b, Wall et al., 2013). In the present study, at the cessation of UFM, the right udder halves produced 3 kg/d, or 35%, more milk than the left udder halves. Wall et al. (2013) also showed an increase of 3 kg/d more in the udder halves milked 4X for the first 21 DIM, which accounted for a 30% increase in production. In the present study, the increase relative to the left udder halves was reduced to 18% more milk for d 22-210, and averaged 24% for d 1-210. This increase in yield is higher than others have reported using UFM during early lactation. Wall and McFadden (2007a) reported an 18% increase in milk yield in udder halves milked 4X for the first 21 DIM. This 24.4% increase is also much higher than the 12 to 14% increase VanRaden et al. (1999) reported in response to 3X milking over the course of an entire lactation indicating early lactation IMF may be a more effective and profitable management practice for producers.

Few early lactation IMF studies have reported milk fat and protein yields, and for these reasons we examined if the increase in milk yield was due primarily to an increase in the water component of the milk. This was not the case, as early lactation IMF significantly increased both milk fat and milk protein yields. The 73.5 g/d increase in milk fat in the right udder halves of cows under UFM, was much higher than the 30 g/d increase observed by Wright et al., (2013). This difference could be attributed to primiparous cows used by Wright et al., (2013), whereas the cows on the present study were multiparous. A study using cows from four commercial dairies comparing the effects of 2X versus 4X milking for the first 21

d of lactation, showed a 3% increase in milk fat yield of cows milked 4X for the first 21 d of lactation (Soberon et al., 2011). This is much lower than the 21% increase in milk fat production observed in the present study. The cows in the present study also had a higher milk fat concentration, which may explain the higher response of milk fat in the present study. As with milk fat, there are little data in the literature regarding milk protein yield in response to early lactation IMF. Wright et al. (2013) reported a protein increase of 30 g/d in the gland milked 4X for the first 21 DIM, while the present study indicated a protein increase of 67.9 g/d for 1-210 DIM. The 23% increase in milk protein in the current study was much higher than the 4% indicated by Soberon et al. (2011).

In addition to demonstrating the increase in milk yield due to early lactation IMF, we investigated the mechanisms responsible for this increase in yield. An increase in milk yield is a product of increased mammary cell number and activity (Capuco et al., 2003). Previous studies have indicated there is no detectable change in cell turnover due to early lactation IMF (Wall et al., 2013). Additionally, Wall and McFadden confirmed that the increase in milk yield is locally regulated in the mammary gland (2007a); therefore, we hypothesized this local regulation might be due to autocrine or paracrine production of factors that increase cell activity in the mammary gland.

Prolactin has lactogenic, as well as, galactopoietic effects in the bovine mammary gland (Freeman et al., 2000, Capuco and Akers, 2011). Though it is primarily secreted from the anterior pituitary, there is evidence of autocrine PRL production within the mammary glands of mice and rats (Kurtz et al., 1993, Steinmetz et al., 1993, Chen et al., 2010). Our findings confirm the presence of PRL mRNA in the mammary glands of cows; this had not been previously investigated. We hypothesized that early lactation IMF would increase PRL

mRNA, as well as activated STAT5, in the right udder halves of cows undergoing UFM the first 21 DIM. At d 21, there was a significant increase in the abundance of activated STAT5 in the right udder halves, but no significant difference in the abundance of PRL mRNA. The increase in activated STAT5 might indicate a higher sensitivity to PRL signaling in the right udder halves, even in the absence of an increase in PRL mRNA. The increase in STAT5 activation at d 21 would indicate a direct mechanism for the increase in milk protein yield observed in the right udder halves. The fact that there was no significant difference in activated STAT5 at d 60 could indicate it is not the mechanism responsible for the persistent increase in milk yield, or that significance was lost with the reduced sample size (n=6) for d 60 western blot analysis.

In addition to autocrine PRL, we were interested in the response of autocrine IGF-1 production and signaling through Akt in the mammary gland, in response to UFM. IGF-1 commonly works in an autocrine or paracrine manner in many different organs (Holly and Wass, 1989). Additionally, IGF-1 mRNA is present in the bovine mammary gland (Glimm et al., 1990). Binding of IGF-1 to the IGF-1 receptor can stimulate the phosphorylation and activation of Akt, which then stimulates cell cycle progression, lipid synthesis, and protein synthesis (Hadsell and Bonnette, 2000). Akt activation can directly increase lipid synthesis (Schwertfeger et al., 2003), and delays mammary gland involution in mice (Schwertfeger et al., 2001). We hypothesized early lactation IMF would increase the abundance of IGF-1 mRNA present in the mammary glands and milk fat of cows, and that increase in IGF-1 would stimulate an increase in activated Akt in the mammary glands. Contrary to our hypothesis, there was no significant difference in the expression of IGF-1 between the udder halves. Interestingly, there was a significant increase in the activation of Akt at d 21 in the

left udder halves when compared to the right udder halves. Though there was no treatment effect on the expression of IGF-1 in the milk fat, there was a day effect. The mRNA from the milk fat comes exclusively from actively secreting mammary epithelial cells, therefore this day effect on expression is seen only in the mammary epithelial cells. In the present study, expression of IGF-1 was constant from d 21-120, and increased from d 120-180. This is contradictory to what Plath-Gabler et al. (2001) reported; they found that IGF-1 expression decreased throughout lactation, and didn't increase until the early on-set of involution. These findings indicate early lactation IMF does not affect local production of IGF-1, but does decrease Akt activation.

In addition to evaluating pathways that increase cell activity, we also hypothesized that the exposure of the gland to 4X milking the first 21 DIM would reduce the expression of proteins associated with involution. Changes in some transcripts associated with involution, including CHI3L1, have been observed in response to early lactation IMF (Wall et al., 2013). STAT3 is a known marker of involution, and deletion of STAT3 in mammary tissue delays involution in mice by suppressing mammary epithelial cell apoptosis (Chapman et al., 1999). With the onset of involution, CHI3L1 is up-regulated in a STAT3 dependent manner (Hughes et al., 2012). CHI3L1 is a downstream gene product of STAT3, and is characterized as a protein that mediates inflammation and tissue remodeling (Scully et al., 2011). In human and mice models, CHI3L1 stimulates tumor angiogenesis and reduces survival rate in breast cancer patients (Jensen et al., 2003). However, it is also up-regulated in mammary epithelial cells at the onset of involution, suggesting its role in apoptosis, remodeling, and gland regression (Scully et al., 2011). Additionally, CHI3L1 is present in mammary secretions collected from involuting cows (Aslam and Hurley, 1997). In addition to measuring CHI3L1

gene expression, we also evaluated IGFBP5 gene expression. IGFBP5 is also a marker of involution, and in the mammary gland binds IGF-1, preventing Akt activation and its downstream effects such as lipid synthesis or cell progression (Tonner et al., 1997). In the present study, we saw no effect of IMF on STAT3 expression or activation. Interestingly, we did detect activated STAT3 at both d21 and d60 in both udder halves. Classically, STAT3 is considered a marker of involution, and isn't expressed in mice mammary glands until the onset of involution (Hughes et al., 2012). In addition to no difference in STAT3 expression or activation level, we also observed no difference in the abundance of CHI3L1 or IGFBP5 mRNA in either d21 or d 60 tissue samples, or the expression in milk fat. This indicates that the increase in milk yield due to early lactation IMF does not appear to be regulated by STAT3 activity or its downstream gene products.

In conclusion early lactation IMF significantly increased milk, milk fat, and milk protein yield both acutely and persistently. Additionally, STAT5 activation was significantly increased in the udder halves milked 4X for the first 21 DIM, indicating its role in the increase in milk yield. Contrary to our hypothesis, early lactation IMF decreased activation of Akt at d 21, and had no significant effect on PRL, IGF-1, IGFBP5, or CHI3L1 expression.

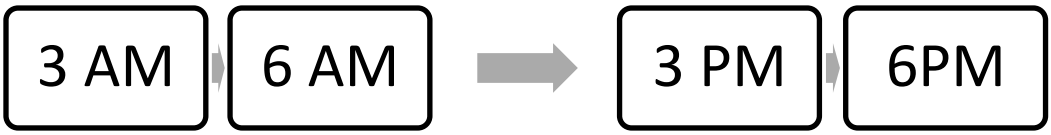

Figure 3.1 Milking Schedule for the UFM period (1-21 DIM). The right udder halves were milked at every milking, while the left udder halves milked only at 3AM and 3PM. Beginning at 22 DIM both udder halves were milked 2X.

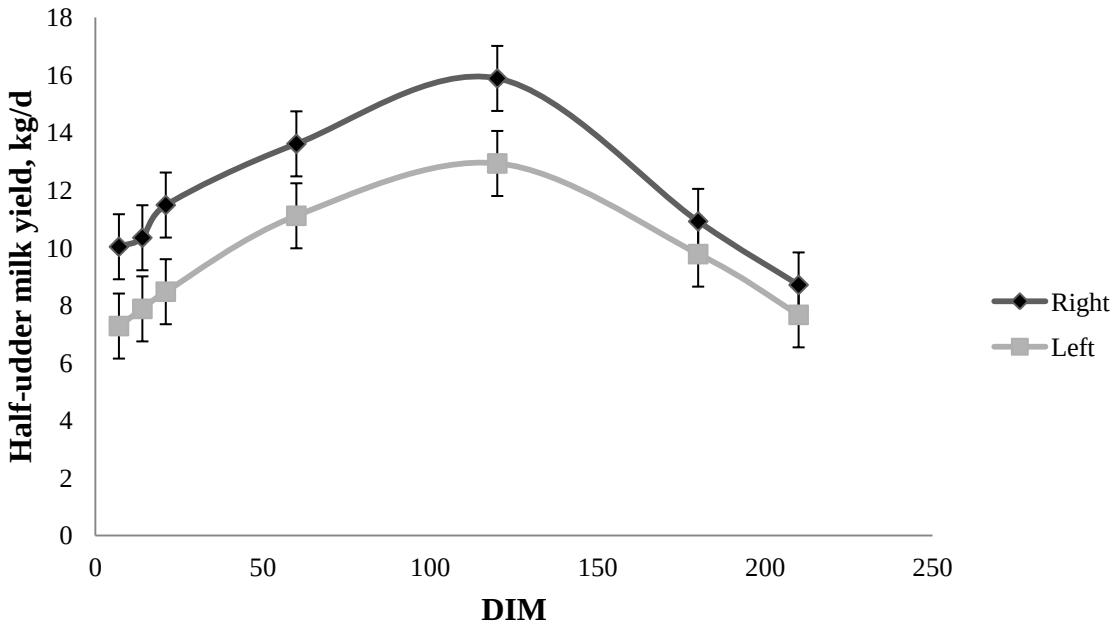

Figure 3.2. Mean milk yield of udder halves for cows unilaterally milked for the first 21 DIM. The right udder was milked 4X (black line) for the first 21 DIM, and the left udder halves were milked 2X (grey line) for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as lsmeans ± SEM; (Treatment $P < 0.0001$, Day $P < 0.001$, Day X Treatment $P < 0.12$).

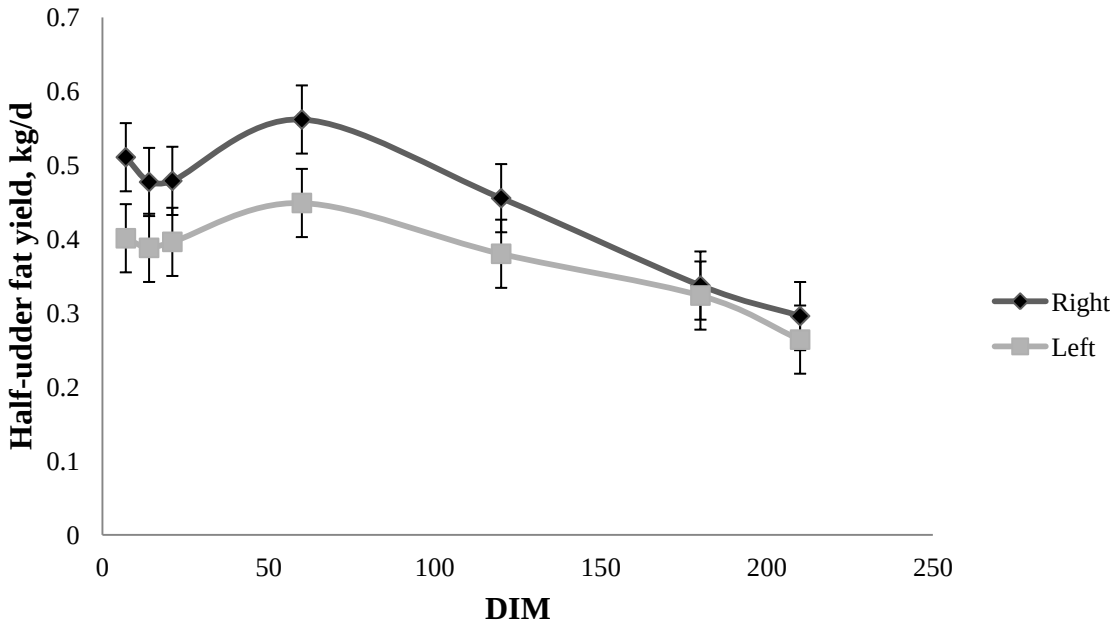

Figure 3.3. Mean fat yield of udder halves for cows unilaterally milked for the first 21 DIM. The right udder was milked 4X (black line) for the first 21 DIM, and the left udder halves were milked 2X (grey line) for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as lsmeans ± SEM; (Treatment $P < 0.0001$, Day $P < 0.03$, Day X Treatment $P < 0.10$).

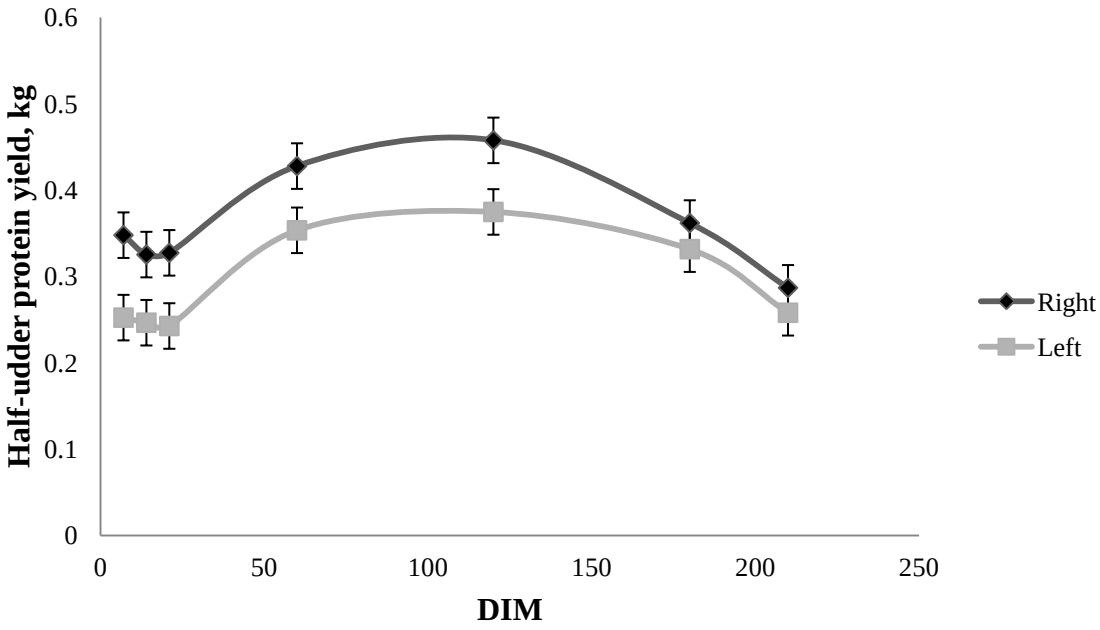

Figure 3.4. Mean protein yield of udder halves for cows unilaterally milked for the first 21 DIM. The right udder was milked 4X (black line) for the first 21 DIM, and the left udder halves were milked 2X (grey line) for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as lsmeans ± SEM; (Treatment $P < 0.0001$, Day $P < 0.002$, Day X Treatment $P \leq 0.05$)

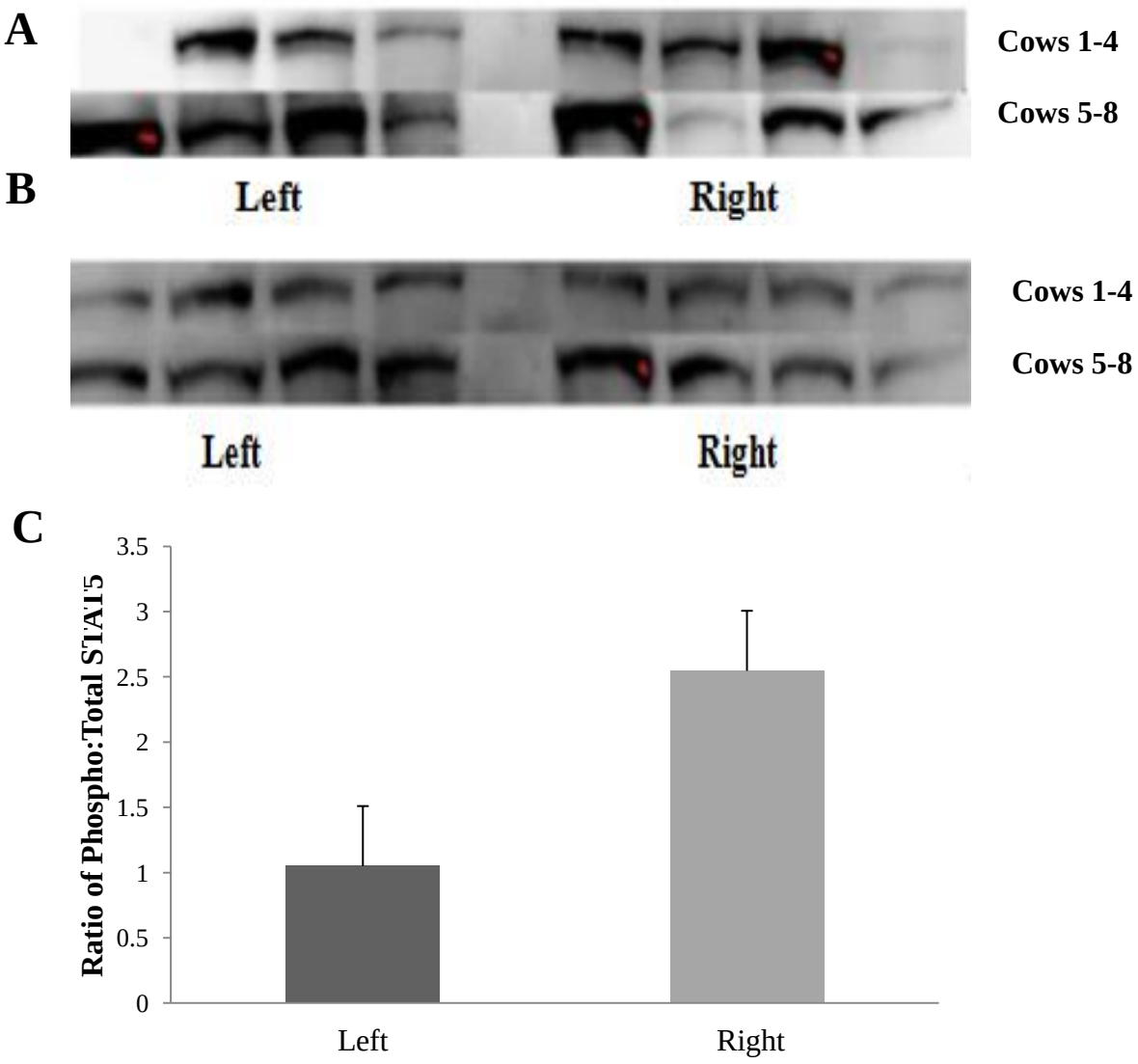

Figure 3.5 STAT5 A) Western blot images for protein expression of phosphorylated STAT5 at 21 DIM. B) Western blot images for protein expression of total STAT5 at 21 DIM. C) Relative abundance of active STAT5 at 21 DIM of mammary gland biopsies for cows unilaterally milked for the first 21 DIM. The right udder was milked 4X for the first 21 DIM, and the left udder halves were milked 2X for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as relative abundance of Phospho/Total STAT5 protein quantified using densitometry software ± SEM; P-value ≤ 0.05.

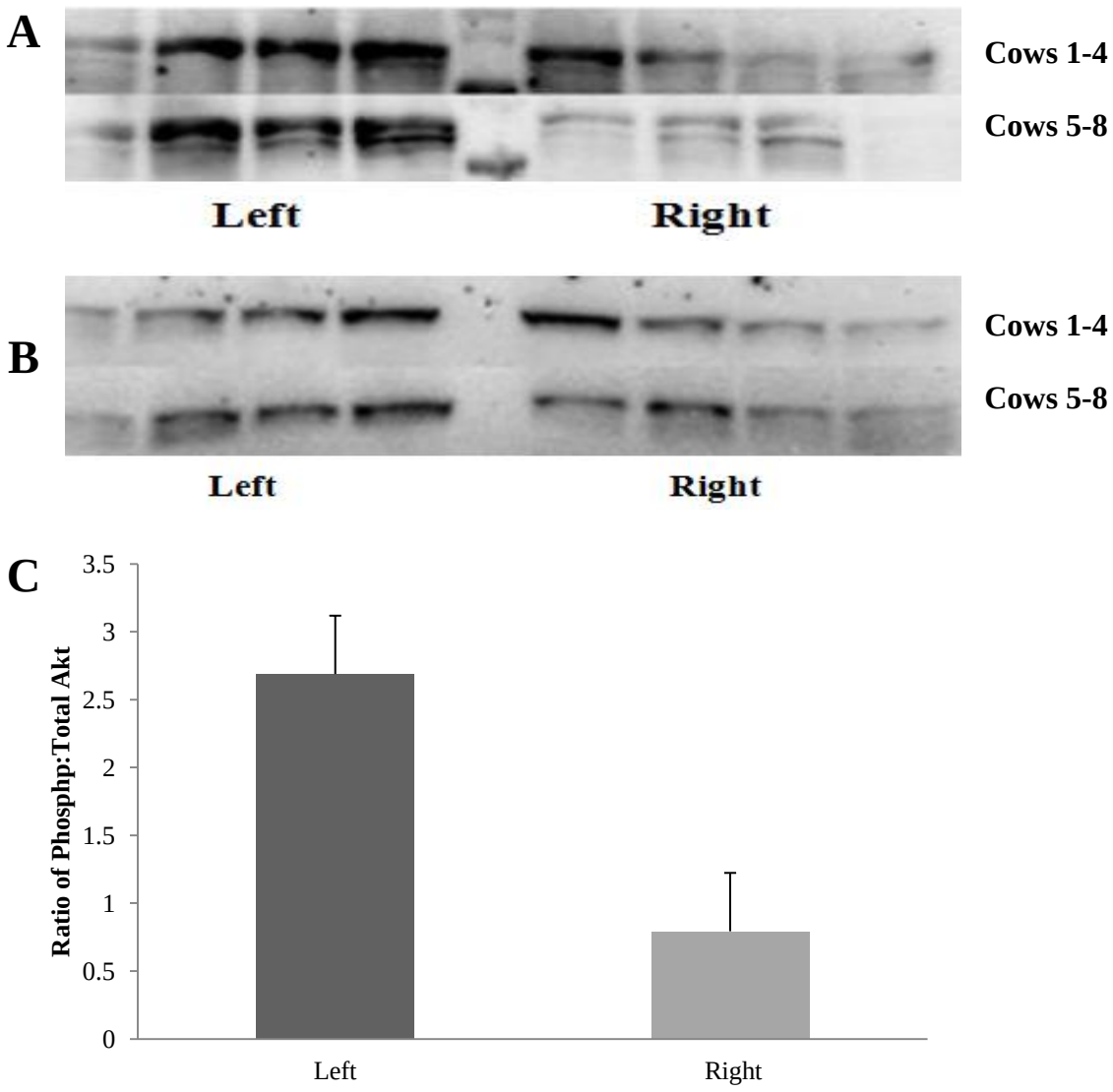

Figure 3.6 Akt A) Western blot images for protein expression of phosphorylated Akt at 21 DIM. B) Western blot images for protein expression of total Akt at 21 DIM. C) Relative abundance of active Akt at 21 DIM of mammary gland biopsies for cows unilaterally milked for the first 21 DIM. The right udder was milked 4X for the first 21 DIM, and the left udder halves were milked 2X for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as relative abundance of Phospho/Total Akt protein quantified using densitometry software ± SEM; P-value ≤ 0.05.

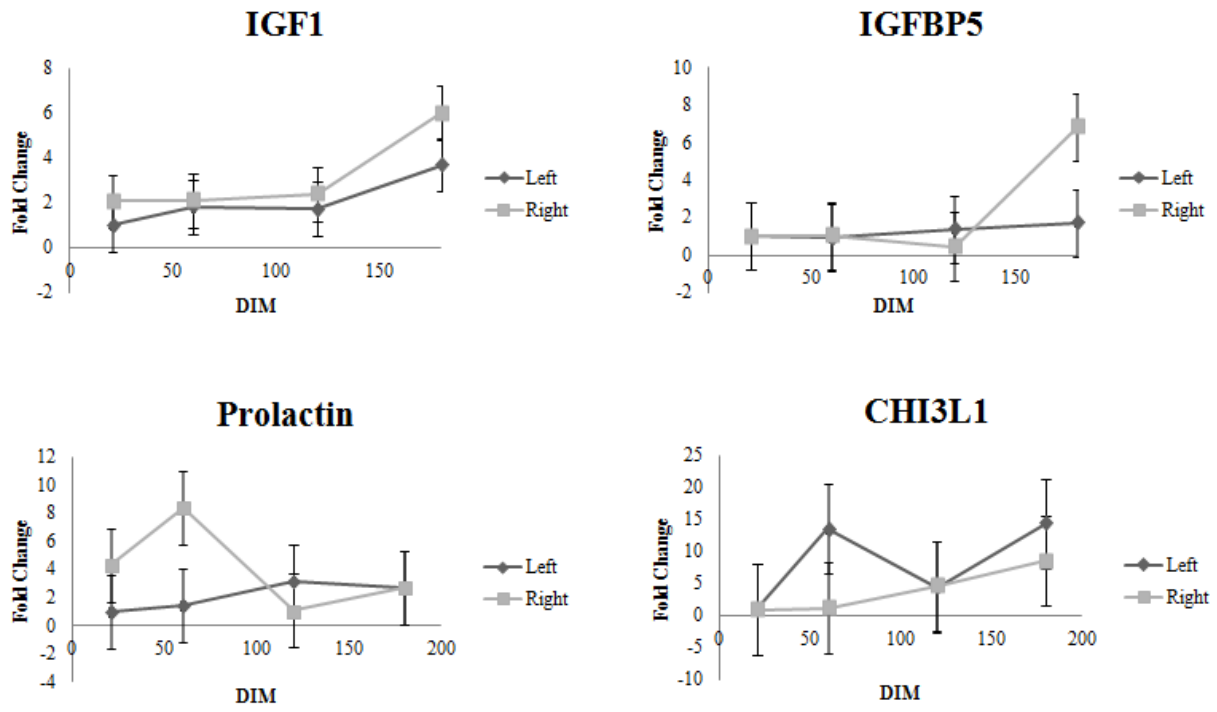

Figure 3.7 Temporal pattern of target gene abundance, relative to three endogenous control genes, in the milk fat of cows unilaterally milked for the first 21 DIM. The right udder was milked 4X for the first 21 DIM, and the left udder halves were milked 2X for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as mean fold change ± SEM; P-value ≥ 0.3.

Table 3.1 List of Antibodies used for Immunoblotting

Antibody	Manufacturer	Catalog Number	Dilution
Stat5	Santa Cruz Biotechnology	SC-835	1:1000
Phospho-Stat5	Cell Signaling	9351	1:1000
Akt	Cell Signaling	9272	1:1000
Phospho-Akt	Cell Signaling	9271	1:1000
Stat3	Santa Cruz Biotechnology	SC-482	1:1000
Phospho-Stat3	Cell Signaling	9145	1:2000
Goat anti-rabbit IgG-HRP	Santa Cruz Biotech	SC-2004	1:2000, 1:4000

Table 3.2 List of Primer Sequences used for Real-Time q-PCR

Transcript	Accession number	Primers (5'-3')	
B2M	BC118352.1	F	TGCTGAAGAATGGGGAGAAG
		R	CCTTGCTGTTGGGAGTGAA
CHI3L1	BT021835.1	F	CGACCAGGAGAGTGTCAAAA
		R	GGCACTTGTGAGAGGAAAGG
EIF3K	BC102250.1	F	GCGATGTTTGAGCAGATGAG
		R	GCATTTTCTTTGGCCTGTGT
IGF1	BC126802.1	F	AGCAATGGGAAAAATCAGCA
		R	AAGAGATGCGAGGAGGATGT
IGFBP5	BC149394.1	F	TCGGAGAAGATGGTGCTCAC
		R	GCACATGGAGAGGGCTTTCT
MRPL39	BC122667.1	F	GGCTTATTGGCGTTCTTGTG
		R	CGCAGGTTCTCTTTTGTTGG
Prolactin	BC148124.1	F	TACCTCCTCCCTTCCTACCC
		R	GCAGCAACCCAAGAATCAA
RPS15	BC102068.1	F	CTCTGTGCATTCGGGTTTTC
		R	GGGCTCTCTGGGTTCCTCT
YWHAZ 554	BC102382.1	F	ACTGGGTCTGGCCCTTAACT
		R	GCTTCAGCTTCGTCTCCTTG

Table 3.3 Half-udder[1] milk yield and milk component data for 1-210 DIM

	Right	Left	Right - Left	SEM	*P*-value
Milk Yield (kg/d)	11.69	9.42	2.27	0.67	< 0.0001
ECM Yield (kg/d)	12.04	9.88	2.16	0.64	< 0.0001
Fat Yield (g/d)	445.6	372.1	73.5	25.91	< 0.0001
Protein Yield (g/d)	358.7	290.7	68	21.92	< 0.0001
Fat Concentration (%)	3.95	4.22	-0.27	0.22	<0.05
Protein Concentration (%)	3.10	3.10	0	0.06	<0.67

[1] Right udder halves were milked 4X for the first 21 DIM and Left udder halves were milked 2X for the first 21 DIM. Following the UFM period, both udder halves were milked 2X for the remainder of lactation. Half-udder milk yields were collected at each milking, and samples for milk composition were sampled once daily on 7, 14, 21, 60, 120, and 210 DIM. Data are presented as treatment lsmeans for each udder half and the difference between the two udder halves and SEM.

Table 3.4 Relative mRNA abundance (expressed as $2^{-\Delta\Delta Ct}$) of select genes in the mammary gland[1] of cows undergoing UFM

Gene	DIM	Udder Half		SEM	P-value
		Left	Right		
		Fold Change			
IGF-1	21	1.49	0.81	0.33	0.36
	60	1.43	1.51	0.66	0.35
IGFBP5	21	1.31	0.98	0.43	0.69
	60	1.42	3.26	0.7	0.24
PRL	21	1.15	1.37	0.34	0.09
	60	4.97	1.5	0.43	0.45
CHI3L1	21	2.60	1.00	0.83	0.29
	60	1.99	2.45	1.2	0.75

[1] Biopsies were taken at 21 and 60 DIM. Fold change is relative to the geometric mean of 3 endogenous control means.

Chapter 4: Conclusion

Early lactation IMF significantly increased milk, milk fat, and milk protein yield, both acutely and persistently, proving it is a viable management practice for producers to consider increasing production. Arguably the most advantageous aspect of early lactation IMF, as described in this experiment, is the low labor requirements associated with 4X milking of cows at the beginning and the end of a milking session. Producers aren't required to incorporate an entire additional milking session into the daily on-farm routine that is required for 3X milking throughout lactation. The only limitation to implementing early lactation IMF on-farm is the necessity for a fresh pen, where cows in early lactation are separated from the remainder of the herd. If a producer is utilizing a fresh pen, then early lactation 4X milking is an advantageous management practice to consider, and potentially more profitable than 3X milking throughout the entire lactation.

Many previous experiments have attempted to identify the mechanism responsible for increased milk yield due to IMF, but expression of proteins involved in cell signaling pathways had not been investigated. Increased STAT5 activation in udder halves milked 4X the first 21 DIM could indicated an increase in PRL signaling; and would explain the increase in milk protein yield associated with early lactation IMF. The decrease in Akt expression in response to IMF cannot be explained, and may warrant further investigation into other potential activators of Akt in the bovine mammary gland. Though there was no difference in STAT3 activation between udder halves, the high level of activated STAT3 in the glands at d 21 was interesting, as it isn't activated in mice until the onset of involution. The lack of significant difference at d 60 may indicate separate mechanisms responsible for

the increase in yield, or may be a result of reduced sample size at d 60, especially for the western blots where only six animals were available for analysis.

In conclusion, early lactation IMF significantly increased milk production, both acutely and persistently; and was accompanied by an increase in STAT5 activation and a decrease in Akt activation. Further research is needed to investigate changes in local PRL signaling in response to early lactation IMF; including, PRL receptor expression, STAT5 target gene expression and changes in DNA methylation of those genes, which would indicate an epigenetic alteration in response to IMF.

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Appendix A

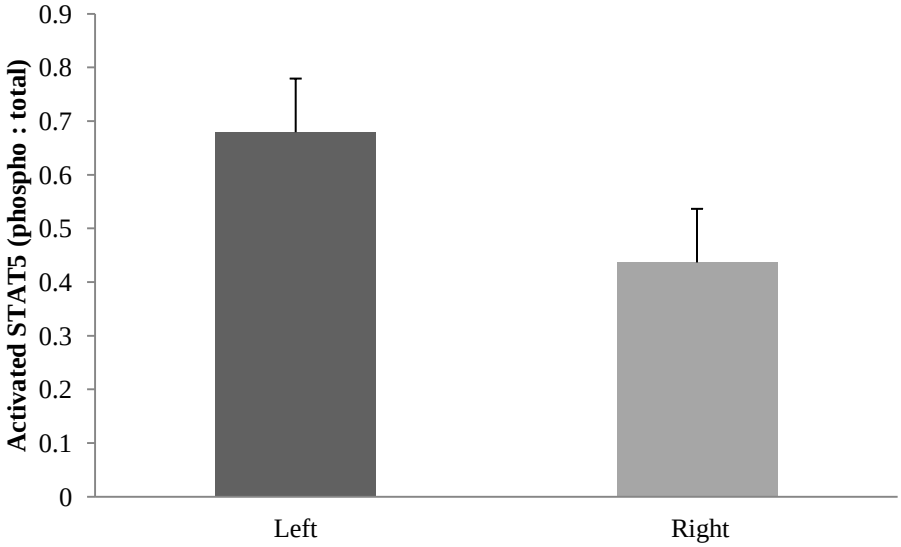

Figure A.1 Relative abundance of active STAT5 at 60 DIM of mammary gland biopsies for cows unilaterally milked for the first 60 d of lactation. The right udder was milked 4X for the first 60 DIM, and the left udder halves were milked 2X for the first 60 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as relative abundance of Phospho/Total STAT5 protein quantified using densitometry software ± SEM; P-value > 0.18

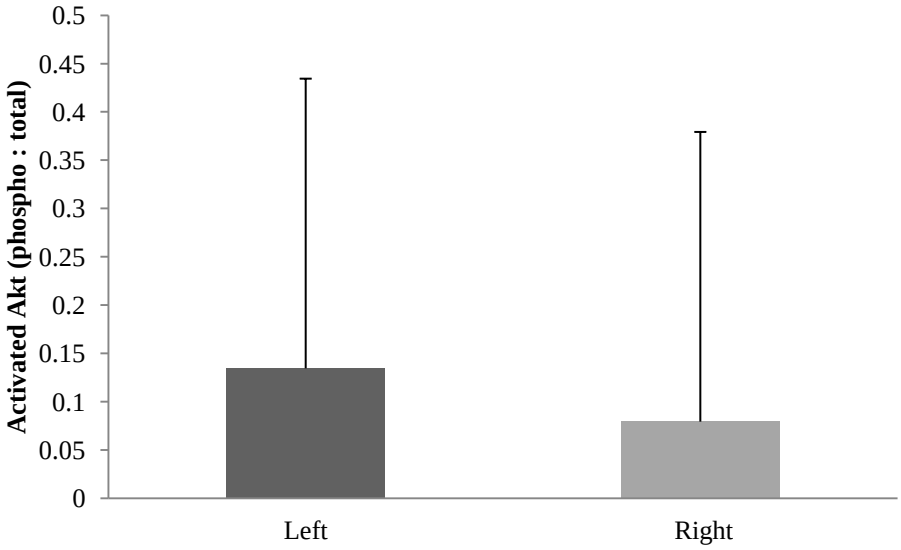

Figure A.2 Relative abundance of active Akt at 60 DIM of mammary gland biopsies for cows unilaterally milked for the first 60 d of lactation. The right udder was milked 4X for the first 60 DIM, and the left udder halves were milked 2X for the first 60 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as relative abundance of Phospho/Total Akt protein quantified using densitometry software ± SEM; P-value > 0.27

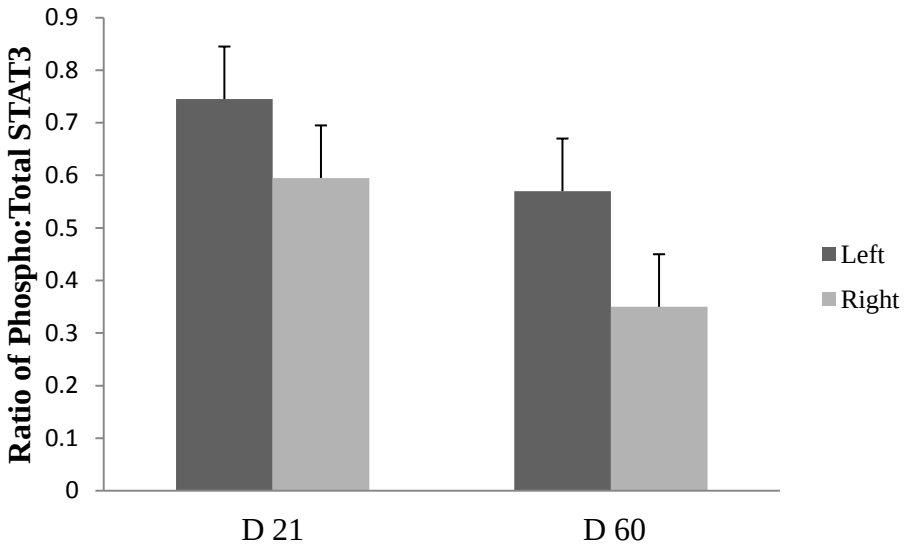

Figure A.3 Relative abundance of active STAT3 at 21 DIM of mammary gland biopsies for cows unilaterally milked for the first 21 d of lactation. The right udder was milked 4X for the first 21 DIM, and the left udder halves were milked 2X for the first 21 DIM. Beginning at 22 DIM, both udder halves were milked 2X for the remainder of lactation. Data are expressed as relative abundance of Phospho/Total STAT3 protein quantified using densitometry software; P-value > 0.69