Determining the Appropriate Duration of Increased Milking Frequency during Early Lactation for Increased Milk Yield and Efficient Production

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ABSTRACT

Increasing the milking frequency of early lactation dairy cows increases their milk yield, both during increased milking and after the cows are returned to a normal twice-daily milking schedule. When milked four times a day (4X) for only the first twenty-one days of lactation, the right udder half produces 3 kg/d more milk than the left half, which is milked only two times a day (2X) over the course of lactation (Hale et al., 2003). Alterations to this increased milking frequency (IMF) approach have been investigated in order to maximize production of the animals and determine the most efficient practice for producers. The aim of this study was to determine the appropriate duration of early lactation IMF treatment by increasing milking frequency of early lactation cows for various lengths of time, and subsequently increasing the use of this management practice on Virginia dairy farms. The right udder half of twenty-three primiparous and multiparous Holstein cows were milked 4X for 10, 20, or 40 days at the beginning of lactation, and the left udder half 2X for the entire lactation. Udder-half milk yields were measured at various time points throughout lactation and used to calculate the difference between right (4X) and left (2X) udder halves. Overall, treatment did not have a significant effect on milk yield difference throughout the entire lactation; the udder half differences for each group were -0.45 kg, 1.92 kg and 4.62 kg for the 10 d, 20 d and 40 d treatments ($P > 0.05$). In addition to the IMF portion of the experiment, two different methodologies were used to investigate the possible mechanism of local regulation of milk yield in response to IMF treatment. Milk fatty acid analysis was performed on milk samples obtained from the above experiment. Three different groups of fatty acids were analyzed to detect potential changes in the right udder half (4X) when compared to the left (2X); the three groups were denovo, C16, and preformed fatty acids. There was no significant effect of treatment on fatty acid composition of right and left udder halves for any of the three groups ($P > 0.05$). The second methodology used to explore a possible mechanism behind increased milk yield following IMF treatment was immunohistochemistry of mammary gland tissue samples obtained after IMF treatment in a previous experiment. The key target investigated was a component of the Hippo signaling pathway, Yes-associated protein (YAP). Intensity of YAP staining in the cytoplasmic area of mammary epithelial cells (MEC) and number of YAP-positive stained nuclei located in the MEC were quantified for each of the images obtained. There was no effect of treatment or day on intensity of staining ($P > 0.05$) with no difference in the intensity of staining between 4X and 2X samples or d 21 and d 60 samples. However, the interaction for treatment × day tended to be significant ($P < 0.06$), with the d 60 samples tending to have higher intensity of staining than d 21 samples. For YAP-positive nuclei, there was a significant effect of day ($P < 0.05$), with d 60 samples having significantly more YAP positive nuclei. There was not a significant effect of treatment or treatment × day interaction ($P > 0.05$) with 2X and 4X samples having the same number of YAP positive nuclei. Results from the first experiment reveal that 40 d of IMF during early lactation is sufficient to produce an increase in milk and component yields throughout lactation. This practice could be implemented on Virginia dairy farms as a way to increase efficiency and milk yield per cow. Results from the second half of this research indicates that further research is needed to investigate the fatty acid content of milk from cows subject to IMF treatment during early lactation. In addition, YAP potentially plays a role in the changes occurring in the mammary gland, with increased intensity of YAP staining and increased number of YAP positive nuclei observed at 60 DIM. Understanding of this protein and its involvement in the mammary gland could lead to identifying a mechanism for which this increase in milk yield and components following IMF is occurring. Further research needs to be done to provide results supporting the current experiment.

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GENERAL AUDIENCE ABSTRACT

The focus of this study is increasing milk yield by increasing milking frequency of early lactation cows. We will use a technique where each half of the cow's udder is milked with different milking frequencies. Previous studies have shown an increase in milk yield due to increased milking frequency. When milked four times a day for 21 days, the right udder half produced 2,032 lb. more milk than the left over the course of a lactation. This project will investigate modifications to the 21-day increase in milking frequency that might increase the use of this management practice on Virginia dairy farms. This experiment will determine the increase in milk yield when cows are milked four times a day for 10, 20, or 40 days. Farmers are concerned about return on investment - including time invested engaging extra milkings. This experiment will determine the effective duration of increased milking frequency needed to gain an increase in milk production with the minimum investment of time necessary to apply increased milking frequency - can the increase achieved with 20 days be achieved in only 10 or does increasing to 40 days provide an even greater benefit than 20-day application?

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List of Abbreviations

4X	Four times a day milking
2X	Twice daily milking
IMF	Increased milking frequency
YAP	Yes-associated protein
MEC	Mammary Epithelial Cells
3X	Three times a day milking
1X	One time a day milking
FM	Frequent milking
DHIA	Dairy herd improvement association
6X	Six-times a day milking
UFM	Unilateral frequent milking
PP	Post-partum
DoT	Days of treatment
FA	Fatty Acid(s)
IHC	Immunohistochemistry

Chapter 1: Introduction

Milk is the primary source of nutrition for neonates but provides many nutritional benefits to adult mammals as well. With a high demand for both milk and milk products, dairy farmers must produce milk more efficiently in order meet the needs of consumers. One factor that can influence the amount of milk produced per cow is milking frequency, the frequency of milk removal from the mammary gland. Milking frequency has a direct influence on milk yield; more frequent milk removal increases both the average secretion rate and total yield for a given period of time. In a study by Stelwagen and Knight (1997), twelve multiparous cows in early or late lactation were milked either once daily or twice daily to examine the difference in milk yield associated with milking frequency. The milk yield from once daily-milked glands was significantly lower during both stages of lactation compared with twice daily-milked glands (Stelwagen and Knight, 1997).

Dairy producers most often milk cows two times (2X) or three times (3X) per day. Milk yield per cow increases 2-3 kg/d when moving from 2X to 3X (Erdman and Varner, 1995, Hart et al., 2013). Along with the benefits to producers, 3X milking can also improve udder health by reducing somatic cell count (SCC) and incidence of mastitis. The lactating mammary gland is particularly sensitive to these changes in milking frequency during early lactation. Increasing milking frequency of early lactation dairy cows increases their milk yield, with this increase persisting into lactation after the cows are returned to a normal schedule of 2X milking. Hale et al. (2003) demonstrated that implementing a frequent milking routine for the first 21 days of lactation is sufficient to increase milk yield immediately after treatment and persists into the remainder of lactation. Wall and McFadden (2007a) also observed effects on milk yield following increased milking frequency (IMF) during early lactation. Only having to milk cows at increased frequency for the first 21 days of lactation versus the normal 305+ day lactation period is important for making this technique applicable in production settings. Efficient milk production is critical to the success of the dairy industry (Wall and McFadden, 2007a) and achieving a 3 kg/d increase in milk yield while only milking 2X for most of lactation would increase income for producers without investing in increased milking equipment capacity.

The increase in milk yield observed with frequent milking is controlled by a number of factors that have not yet been clearly described. It is known that milk yield is regulated at the gland level through both number and activity of secretory MEC; the exact mechanism by which these changes occur is unknown. However, some factors have been identified that could influence number and activity of these secretory MEC, including, genetics, age, lactation stage, udder development, mastitis, nutrient availability, secretion rate and blood flow. While there are many different aspects of production that can have an effect on milk yield, previous research has shown that these effects are locally regulated in the mammary gland.

The first two objectives for this study were to (1) determine the effective duration of early lactation IMF needed to gain an increase in milk production with the minimum investment of time necessary to apply IMF, and (2) identify a second potential mechanism to how the IMF-associated increase in milk yield is occurring, using milk samples obtained from the first experiment, and analyzing them for various fatty acid concentrations. The next objective was to identify a potential mechanism to explain how this increase in milk yield associated with IMF occurs. Tissue samples obtained from a previous experiment were used to determine expression of Yes-associated protein (YAP).

Chapter 2: Literature Review

Increased Milking Frequency

Annual milk production of dairy cows in the U.S. has been steadily increasing over decades due to improved genetic merit and management techniques (Campos et al., 1994). Milking frequency, the number of times per day a cow is milked, is a management technique that can be used to impact milk production. U.S. Dairy producers most often milk cows twice daily (2X) and some are milked three times daily (3X); when increasing from 2X to 3X, an increase of 3.5 kg/day can be observed (Erdman and Varner, 1995). Alternatively, when cows go from 2X milking to being milked once daily (1X), milk yield can decrease by up to 6.2 kg/day (Erdman and Varner, 1995).

Frequent milking is defined as milking cows three or more times daily and is a tool that can be used by producers to increase the milk production efficiency of their cows and utilize facilities more efficiently (Allen et al., 1986, Wall and McFadden, 2007b). In the 1950's and 1960's, only high producing purebred herds were milked 3X, but the increase in labor costs eliminated the use of this practice (Pearson et al., 1979). In 1984, approximately 15% of herds in the Dairy Herd Improvement Association (DHIA) in California were milked 3X. The average production per cow for herds milked 3X was approximately 8534 kg, compared to approximately 7594 kg for herds milked 2X, producing a difference of approximately 940 kg (Allen et al., 1986). While the increasing costs of labor, facilities, feed and utilities are still an issue, an opportunity exists to improve the frequent milking technique. It can be noted that the increase in milk yield associated with increased milking frequency (IMF) appears to be an immediate effect, occurring within hours and can be maintained during and after frequent milking is applied

(Hillerton et al., 1990). This raises the question of when does this frequent milking need to take place; during the entire 305-d lactation or during one specific stage of lactation?

Early Lactation Increased Milking Frequency

Frequent milking is an effective tool for increasing milk yield, but what is the optimal time to apply this practice? In a study by Bar-Peled et al. (1995), milking frequency was increased from 3X to 6 times a day (6X) during the first 6 weeks of lactation. This was the first detailed experiment on frequent milking during early lactation. They reported a 7.3 kg/d (21%) increase in milk production when milking frequency went from 3X to 6X. Other studies researching IMF used groups of cows that were in different stages of lactation and reported an increase in milk production of 11% when milking 4X for 4 weeks compared to 2X milking. Their results demonstrate that although there was a decrease in production when cows moved from 6X back to 3X, there was a clear carryover effect of very frequent milking in early lactation, with cows in the 6X group producing 5.1 kg/d more than cows in the 3X group for the remainder of lactation (Bar-Peled et al., 1995). Another study by Eslamizad et al. (2010) explored the effects of 6X milking during early lactation versus full lactation on milk production. Their results indicated that increasing MF to 6X daily only increases subsequent milk yield when applied during early lactation, regardless of the length of treatment. They found no advantages of 6X milking compared with 3X during the mid and late lactation periods.

While all of the previous studies mentioned up to this point have observed a positive effect on milk yield for IMF treatment, there are other data that support IMF not having an effect on milk yield during early lactation. VanBaale et al. (2005) was the first group to investigate the effects of IMF during early lactation for various lengths of time, less than 21 d. Their objective was to evaluate the time necessary to milk cows 6X during early lactation to have a positive impact on peak yield and lactation persistency. Three groups of cows were milked 6X for either 7, 14, or 21 d postpartum and a fourth group was milked 3X for the entire 305-d lactation. Cows milked 6X were returned to 3X milking at the end of each treatment period (d 8, 15, and 22). They found that cows milked 3X tended to produce more milk (43.2 kg/d vs 41.5 and 41.0 ± 1.1 kg/d) than cows milked 6X for either 7 or 21 d during the first nine weeks of lactation. For weeks 10-43 of lactation, they did not observe a difference between the four groups for milk production. These results were not expected, as they did not agree with most of the previous data in which IMF produced a positive response in milk yield both during and after application. Results from this experiment may have differed due to the distance cows milked 6X were required to walk each day. They estimated the distance to be 87 ± 36 m from the pen where cows were housed to the milking parlor. This large distance caused 6X cows to be away from feed and water longer, as well as decreasing the lying time per day. These factors may have contributed to the lack of effect observed in this study and may indicate that nutrition could potentially play a role in the response, or lack of one, in milk yield following IMF treatment.

One possible reason for the observed increase in milk production following IMF during early lactation is that frequent milking has been shown to increase differentiation and proliferation in bovine mammary cells (Hillerton et al., 1990). Mammary proliferation continues for the first few weeks post-partum (PP), meaning that the gland may be responsive stimuli such as frequent milking during early PP.

Regulation of Milk Yield Response due to Early Lactation IMF

The next question to answer once it was determined that milk yield can be influenced by increasing milking frequency in early lactation was, how is this response regulated? Is it regulated locally in the mammary gland, or by other systemic factors such as lactogenic hormones? One study by Wall and McFadden (2007a) utilized a technique referred to as "unilateral frequent milking" (UFM), in which the left and right udder halves are milked at different frequencies for a length of time in early lactation, to optimize the timing and duration of frequent milking. Multiparous Holstein cows were assigned to have their left udder half milked 2X and right udder half milked 4X for 2 weeks at 2 different times (d 1 to 14, or d 7 to 21) during early lactation. Researchers found that for both treatments, the 4X udder halves produced more milk than the 2X udder halves during UFM, and the full lactation yield of the 4X udder half was greater than the 2X udder half (Wall and McFadden, 2007b). These results indicate that the response to IMF during early lactation is in fact regulated locally in the gland, since only the gland exposed to treatment (4X) responded and not the regularly milked gland (2X). This half-udder model is extremely powerful because it eliminates the variation between animals attributable to environment, nutrition, and genetics. These models also expose both udder halves to the same systemic factors, verifying that the response to IMF treatment is strictly at the level of the mammary gland (Wall and McFadden, 2007a).

In 2003, (Hale et al.) reported that applying FM from d 4-21 of lactation stimulated a similar effect on milk yield to that previously observed for FM for d 1-21 of lactation (Hale et al., 2003). Similarly, Wall and McFadden (2007b) did not find a significant difference between the two different times that they tested (UFM- 1-14 or UFM- 7-21), indicating that there is no clear answer as to when during early lactation should this treatment be applied and further research needs to be done to establish the appropriate duration and timing of application.

Potential Local Regulators of Increased Milk Yield due to Early Lactation IMF

It is known that increased milk yield due to IMF is locally regulated, and treatment is most effective during early lactation; but what exactly is controlling these factors is unknown. Mammary epithelial cells (MEC) are responsible for synthesizing and secreting milk during lactation. These cells, just like any other cell in the body, are capable of undergoing growth, division, and death. This led researchers to the question, "is it an increase in cell number or a change in cell activity that causes the increase in milk yield associated with early lactation increased milking frequency?".

Mammary proliferation continues for the first few weeks post-partum (PP), meaning that the gland may be responsive to stimuli such as frequent milking during early PP. Hillerton et al. (1990) demonstrated that FM increases differentiation and proliferation in bovine mammary cells of glands milked 4X compared to glands milked 2X. They observed a significant increase in milk yield, with the glands milked 4X producing 10.4% more milk than the control glands milked 2X. The activity of several key enzymes involved in the synthesis of milk constituents was measured to identify changes in cellular differentiation following IMF and tended to be higher in 4X milked glands when compared to 2X milked glands (Hillerton et al., 1990). Although the differences only tended to be significant, the consistent trend suggests that cellular differentiation was stimulated in glands milked 4X, and the response seems to be due to differentiation rather than cell hypertrophy.

Hale et al. (2003) performed a similar experiment in which cows were milked either 2X or 4X for the first 21 days of lactation, after which, the 4X cows were returned to 2X milking for the remainder of lactation. They observed an increase in mammary cell proliferation, demonstrated by H-thymidine incorporation, and an increase in epithelial cell apoptosis via TUNEL; however, these results were not consistent throughout all of the 4X milked cows. In addition, another indicator of mammary cell proliferation, Ki-67, was measured and there was no significant increase in expression in 4X glands as opposed to 2X (Hale et al., 2003). These results indicate proliferation is not consistently related to milk yield responses. In agreement, Norgaard et al. (2005) reported an increase in milk yield due to IMF treatment, but no significant effect of treatment on mammary cell proliferation or apoptosis. An increase in proliferation of the MEC following IMF, could indicate more cells are present that are secreting milk, explaining the increase in milk yield observed following IMF. Conversely, an increase in the rate of apoptosis of MEC following IMF, could indicate an increase in the turnover rate of MEC in the mammary gland induced by treatment. This increase in turnover rate could suggest that new MEC are responsible for the increase in milk yield following IMF as opposed to an increase in the milk secreting capacity of old MEC already present in the gland.

Lima et al. (2016) took a different approach in which they assessed the morphological differences between 4X and 2X glands following 21 days of IMF treatment. As a result, they found no changes in mammary morphology when evaluating the appearance of the alveoli and cells of the mammary tissue. Similar results were reported in a study by Wall et al. (2013), in which biopsies were taken at day 21 and 23 PP for cows milked 2X or 4X for the first 21 days of lactation. In summary, all of these experiments lead to the conclusion that it is not cell number that is influenced by IMF during early lactation, but rather some signal that is sent to or is already present the gland to alter the activity of these MEC that are responsible for synthesizing and secreting milk.

Changes in Milk Composition due to Early Lactation IMF

Increasing MF from 2X to 4X increases milk yield, but does it also affect milk components? The increase in milk yield that takes place during and following IMF treatment could be due to an increase in the water component of milk, or an increase in production of milk components, including fat and protein. When milk yield increases, if the gland responds by secreting more fat and protein into the milk, this would be reflected through an increase in component yields rather than percentages. However, a decrease in the percent of components would indicate an increase in the water component of milk. Bar-Peled et al. (1995) reported an increase in fat and protein yields for cows milked 6X compared to cows milked 3X, and no difference in milk fat or protein percentages during or after treatment. In contrast, Hale et al. (2003) found no difference in fat or protein yields throughout the entire lactation of cows milked 2X vs 4X but did report a significant decrease in the fat percentage and no change in protein percentage between the 4X and 2X groups during IMF treatment. A more recent study by Shields et al. (2011) found IMF treatment during the first 21 d of lactation was associated with increased milk fat percent, and milk fat and protein yields.

Milk fat can originate from two different sources; de novo synthesis, fatty acids formed in the mammary gland, or preformed uptake, fatty acids that come directly from the diet or reserves. With the yield of fat increasing following IMF, the source from which this additional fat is originating is not known. Although not much work has been done in cows to investigate the changes in FAs occurring in the mammary gland, Travers and Barber (1993) investigated the effect of milking frequency on the expression of fatty acid synthase genes in the goat mammary gland. They found that increased milking frequency increased the activity of fatty acid synthase, which could possibly increase the de novo synthesis of short- chain fatty acids. Further research is needed to fully investigate the effect of IMF on the content of FAs in milk.

A Question of Cell Activity or Cell Number

As a cow advances through lactation, a gradual decrease in mammary epithelial cell (MEC) population is occurring and results in a decline in milk production. This decline in milk production, is not due to the loss of activity by MEC, but rather the net decline in cell number (~50%) resulting from apoptosis (Capuco et al., 2001). However, cell renewal is also taking place over the course of lactation. By the end of lactation, while ~50% of the original cells have died, most of the cells present in the mammary gland were formed after calving (Capuco et al., 2001). When cows are milked at increased frequency, it is known that they produce more milk. The question of how the gland is accommodating this increased production becomes of interest. With these changes that are taking place in the mammary gland during increased milking frequency (IMF) being locally regulated, the main question researchers have is, is it an increase in the number or activity of MECs?

Ki-67 is an antigen commonly used to indicate cell proliferation was used by Capuco et al. (2001) in mammary gland tissue samples to verify the proliferative status when cows were milked 2X. The number of Ki-67 expressing cells were lower in early lactation and higher during later stages of lactation. These results along with a higher apoptotic index during early lactation suggest that significant mammary growth does not occur during early lactation. A more recent study by Soberon et al. (2010) also used Ki-67 as an indicator of cell proliferation, but cows were milked either 2X or 4X to compare the effects of IMF. The results from this experiment were similar to those of Capuco et al. (2001); there was no effect of IMF treatment on epithelial cell proliferation. The results from both of these studies suggest that there are alternative mechanisms that must be responsible for the changes in lactation performance following IMF treatment. One potential mechanism for regulation of cell activity is a component of the Hippo pathway, Yes-associated protein (YAP). This transcriptional coactivator could possibly be involved in regulating changes occurring in the mammary gland due to IMF in early lactation.

Yes-Associated Protein and the Hippo Pathway

The Hippo pathway has been identified as a key regulator of organ size and tissue homeostasis. This pathway regulates cell proliferation, apoptosis and stemness, by inhibiting YAP and TAZ transcription coactivators, in response to extracellular and intracellular signals such as cell to cell contact, cell polarity, and mechanical cues (Yu et al., 2015). The Hippo pathway was originally discovered in *Drosophilia* as a suppressor of tissue overgrowth (Justice et al., 1995), and is highly conserved in mammals. Upon activation of the Hippo pathway, Ste20-like kinases (MST1 and MST2) phosphorylate and activate large tumor suppressor kinases (LATS1 and LATS2), which in turn phosphorylate Yes-associated protein (YAP) (Kodaka and Hata, 2015). When the hippo pathway is on, YAP is phosphorylated and remains in the cytoplasm. This means that tissue growth is being controlled and no cell proliferation is occurring. When the hippo pathway is off, YAP is not phosphorylated and can translocate to the nucleus, allowing cell proliferation and tissue growth to occur. The functions of this pathway have made it of primary interest in the role of cancer, specifically breast cancer. Evidence suggests that YAP is an oncoprotein that promotes breast cancer cell proliferation and survival, with the *Yap* gene being amplified in breast tumors in mice (Overholtzer et al., 2006). These data suggest implications for Yap in the mammary gland and its most important function, milk production.

With YAP being capable of expression in either the cytoplasm or nucleus of mammary epithelial stem cells, depending on the activity, it's role in the mammary gland is of interest. In a study by Chen et al. (2014), mouse genetics were used to interrogate Hippo signaling in the mammary gland in vivo. The expression of YAP in the mammary gland of virgin mice was examined by immunohistochemistry and was detected in luminal and myoepithelial cells, with the subcellular localization of YAP differing in these cell types. Myoepithelial cells showed nuclear YAP expression, while the luminal cells displayed a more diffuse localization of YAP throughout the cytoplasm and nucleus. During lactation, the amount of YAP staining in the alveoli was significantly decreased. These results indicate that the hippo pathway is unessential for virgin mammary gland development, but is required during pregnancy, when MECs are undergoing rapid growth and differentiation. The dynamic changes of YAP expression at these different stages suggest a potentially critical role for the Hippo pathway in mammary gland growth and development.

The expression and role of YAP has not yet been determined in bovine mammary glands subject to IMF treatment. The role of this protein in growth and development in the mouse mammary gland makes it of interest in the bovine mammary gland as a possible explanation for the changes occurring in the gland during IMF treatment. If YAP activity is altered in mammary glands subject to IMF treatment during early lactation, this could provide some insight to the unknown changes occurring in the mammary gland. An increase in the amount of YAP present in the nuclei of MEC in glands milked 4X, could indicate an increase in MEC proliferation following IMF treatment. No changes in YAP activity and/or location could also mean that YAP does not play an active role in the increased milk yield response to IMF treatment.

CHAPTER 3: Evaluating the duration of increased milking frequency during early lactation for increased yield through lactation

INTRODUCTION

Dairy producers most often milk cows two times (2X) or three times (3X) per day. Milk yield (MY) per cow increases 2-3 kg/d when moving from 2X to 3X (Erdman and Varner, 1995, Hart et al., 2013). The lactating mammary gland is particularly sensitive to changes in milking frequency during early lactation with the increase persisting into lactation after milking is reduced to a lower frequency. In a study by Bar-Peled et al. (1995), milking frequency was increased from 3X to 6 times a day (6X) during the first 6 weeks of lactation. They reported a 7.3 kg/d (21%) increase in milk production during 6X milking and a clear carryover effect with cows in the 6X group producing 5.1 kg/d more than cows in the 3X group for the remainder of lactation.

Unilateral frequent milking (UFM), a method in which each udder half is milked at a different frequency, eliminates environmental, nutritional, and genetic variation that occurs between cows. This technique was first used to examine 4X vs 2X milking by Wall and McFadden (2007a). Cows were assigned to have their left udder half milked 2X and right udder half milked 4X for the first 21 days of lactation. The 4X udder halves produced 3.5 ± 0.2 kg/d more milk than the 2X udder halves during UFM treatment, and 1.8 ± 0.2 kg/d more for the remainder of lactation. It can be inferred that observed treatment differences in this experiment were due local regulation of intramammary factors. However, the duration of IMF treatment during early lactation, which will result in increased milk yield throughout the entire lactation has not yet been determined.

With an increase in MY occurring both during and after IMF treatment, another important aspect to consider is the effect of treatment on milk components. Wall and McFadden (2007a, 2007b) found no effect of UFM on SCC, milk fat percentage, or milk protein percentage. In a review by Erdman and Varner (1995), 22 published reports were used to examine the effect of MF on MY and composition. They reported milk fat and protein percentages tended to decrease as MF increased. Whereas milk fat and protein yields were increased by increased MF due to increased MY. It is known that the responses of the gland to IMF treatment are locally regulated, the changes in component yields, specifically milk fat, can be investigated at the level of the gland.

Mammary epithelial cells (MEC) are responsible for synthesizing and secreting milk during lactation, and are capable of undergoing growth, division, and death. With an increase in both yield and components occurring following IMF treatment, there is either an increase in cell number or a change in cell activity responsible for these increases. Hale et al. (2003) milked cows either 2X or 4X for the first 21 days of lactation, and the 4X cows were returned to 2X milking for the remainder of lactation. This treatment increased mammary cell proliferation and increased epithelial cell apoptosis; however, these results were not consistent among the 4X milked cows. In addition, another indicator of mammary cell proliferation, Ki-67, was used, and there was no significant increase in expression in 4X glands as opposed to 2X (Hale et al., 2003). These results indicate proliferation is not consistently related to milk yield responses. In agreement, Norgaard et al. (2005) reported an increase in milk yield due to IMF treatment, but no significant effect of treatment on mammary cell proliferation or apoptosis.

It appears that an increase in cell number is insufficient to explain the increase in MY following IMF treatment, but cell activity might explain the increase. One measurement that could be used as an indicator of cell activity is milk fatty acid (FA) profile. Fatty acids originate from two different sources; de novo synthesis, meaning synthesis in the mammary gland, and preformed uptake, meaning they come directly from circulation. Because there is an increase in milk fat yield in response to IMF, a change in the activity of MECs may be occurring. Changes in the proportion of FA sources contributing to milk fat might indicate changes in cell activity. Shields et al. (2011) investigated the effect of IMF for the first 21 days of lactation, using UFM, on milk fatty acid (FA) profile, and found that there were no significant changes in the FA profile of 4X glands compared to 2X.

The first objective of this study was to evaluate the duration of increased milking frequency during early lactation for increased MY through lactation, using the UFM model. The second objective of this study was to determine if there is an effect of treatment on the source of fatty acids present in milk by evaluating FA profile.

MATERIALS AND METHODS

Animals and Treatments

The Virginia Tech Institutional Animal Care and Use Committee approved all procedures involving animals. Thirty primiparous and multiparous Holstein cows, that calved between March-April 2017, were provided by the Virginia Tech dairy center and randomly assigned to treatment milked 4X for (1) 10 days, (2) 20 days, or (3) 30 days at the start of lactation (n=10 cows per treatment).

For the first 7-10 days of lactation, cows were housed in a bedded pack. Next, the cows were moved to a lactation pen with free stalls bedded with sand. Around 10 DoT, cows were moved to a lactating pen (free stalls with sand; approximately 70 cows/pen) for the remainder of lactation. Cows were fed a total mixed ration at all times.

Animals were removed from the experiment after being assigned to treatment due to severe mastitis infections (n=4, DoT= 3, 7, 39, 108) and an abscess (n=1, DoT= 180). Cows were not eligible for the experiment because of unbalanced udder halves (>1.5kg, n=3) or dystocia requiring C-section (n=1). Treatment groups were blocked based on parity. Table 3.1 presents the distribution of parities for each of the treatment groups.

Milk Sampling

At the second or third milking post-calving, udder half milk yields were measured from the left and right halves to ensure only cows producing equal amounts of milk in each udder half would be included. Udder half measurements were collected using Surge RX quarter milkers and two milk buckets (Nasco, Fort Atkinson, WI). The milkers have a claw that is divided into four quarters, allowing milk to be collected from each quarter individually, and then combined by udder halves and weighed. Udder half milk samples were weighed in the milking parlor. If udder half milk yields were equal, cows were added to a treatment.

In order to achieve 4X milking of the right udder half, cows were returned to the milking parlor, to milk only the right udder half three hours after the regular milking time. This created, on average, a 9:3:9:3-hour milking interval (4X) for the right udder half, and a 12:12-hour interval (2X) for the left, with the length of treatment application corresponding to treatment group (10 d, 20 d, or 40 d). The two regular milkings were at approximately 3:00 am and 3:00 pm, and the two additional milkings were at approximately 6:00 am and 6:00 pm, where only the right udder half is milked. In order to milk only the right half, inflation plugs (Nasco, Fort Atkinson, WI) were used to plug the left side inflations of the milker.

Milk samples were collected on days 10, 20, 40, 60, 120, 150, 180, 210, 240, and 270 of treatment to measure udder half milk weights. Milk was sampled for analysis of milk fat, true protein, SNF, lactose and somatic cell count. During 4X milking, udder half milk samples were collected at two of the four milkings that day (3:00 pm and 6:00 pm), using the quarter milkers. Following completion of 4X milking and returning to 2X milking, samples were only collected at one (3:00 pm) of the two milkings that day. Milk composition was analyzed by United DHIA (Fairlawn, VA) to measure milk fat, true protein, lactose, SNF and somatic cell count.

Milk Fat Extraction

Milk fat lipids were extracted for methylation according to the method of Hara and Radin (1978) with some modifications. Frozen milk samples were thawed to room temperature and 40 g aliquots were weighed into centrifuge tubes. Milk samples were centrifuged at 17,800 x g for 30 minutes at 8°C. Fat cake that weighed approximately 300-350 mg was transferred to a

16x150 screw top test tube and stored at -20°C. To each sample, 5.4 mL of hexane: isopropanol (HIP) was added, followed by 3.6 mL of sodium sulfate (prepared from 1 g of anhydrous salt and 15 ml of water). Following addition of sodium sulfate, samples were vortexed for 30 s, allowed to sit, and vortexed again for 30 s. Samples were centrifuged at 2000 x g for 5 minutes. Using a glass Pasteur pipette, the organic phase containing the lipids was transferred to a test tube containing 1 g Na_2SO_4, then the test tube atmosphere was purged with N_2 and incubated at room temperature for 30 minutes. The solvent was transferred to a 16x100 screw top test tube and loaded onto a nitrogen evaporator with water bath at 40°C; solvent was evaporated under a stream of N_2. Next 40 mg of lipid was weighed out into a 16x100 screw top test tube, purged with N_2 and stored at -20°C until methylation.

Methylation of Milk Lipid Fractions

Milk samples were prepared for fatty acid analysis by gas chromatography according to the method described in Kelsey et al. (2003) with some modifications. The lipid samples had 2 mL hexane and 40 mL methyl acetate added to them and were vortexed for 30 s. 40 μL of methylation reagent (1.75 mL methanol with 0.4 mL sodium methoxide) was added and the sample was vortexed for 2 minutes and then allowed to stand for 8 minutes. Next 60 μL of termination regent (0.5 g oxalic acid: 15 mL methanol, stored in dark at room temperature) was added and the sample vortexed for 30 s. To reduce deterioration of the GC column, a small scoop (several grains) of calcium chloride, which traps water and methanol, was added and allowed to rest for 1 hour. Samples were centrifuged at 2000 x g for 5 minutes, then transferred to a GC vial.

As described in Stamey et al., 2012, the milk fat samples were analyzed by gas chromatography (Agilent 6890N GC, Agilent Technologies, Palo Alto, CA) using a CP-Sil 88 capillary column (100 m x 0.25 mm i.d. with 0.2 μm thickness; Varian Inc.). The oven temperature, initially 80°C, was increased at 2°C/min to 190°C and maintained for 13 minutes, followed by an increase of 2°C/minute to 210°C and held for 14 minutes. Inlet and detector temperatures were 250°C, and the split ratio was 100:1. The hydrogen carrier gas flow rate was 1 mL/minute. Hydrogen flow to the detector was 25 mL/minute, airflow was 400 mL/minute, and the flow of the nitrogen make-up gas was 40 mL/minute.

Yield Conversions

Calculations were completed to convert milk component data from percentages to kilograms (Table 3.2) and to obtain daily yields and daily percentages (Table 3.3). Daily yields and percentages were calculated for all variables (milk, fat, protein, lactose, SNF) using data collected during the 3 pm and 6 pm milkings and were used as yields from all four milkings on a particular sampling day (3 am, 6 am, 3 pm and 6 pm). These yields and percentages were computed for all variables except somatic cells (1,000 cells/mL).

Table 3.1. Distribution of parities for each treatment group

	Treatment		
Parity	10	20	40
1	1	3	4
2	3	1	1
3	1	2	1
4	2	1	0
5	0	0	1

Table 3.2. Calculations in SAS to convert milk component percentages to kilograms[1]

Parameter	Formula
Fat (kg/milking)	FatkgL=MYkgL*fatpctL/100 FatkgR1=MYkgR1*fatpctR1/100 FatkgR2=MYkgR2*fatpctR2/100
Protein (kg/milking)	ProteinkgL=MYkgL*protpctL/100 ProteinkgR1=MYkgR1*protpctR1/100 ProteinkgR2=MYkgR2*protpctR2/100
SNF (kg/milking)	SNFkgL=MYkgL*SNFpctL/100 SNFkgR1=MYkgR1*SNFpctR1/100 SNFkgR2=MYkgR2*SNFpctR1/100
Lactose (kg/milking)	LactosekgL=MYkgL*lactpctL/100 LactosekgR1=MYkgR1*lactpctR1/100 LactosekgR2=MYkgR2*lactpctR2/100
Denovo (kg/milking)[2]	DenovokgL= FatkgL*denovopctL/100 DenovokgR1= FatkgR1*denovopctR1/100 DenovokgR2= FatkgL*denovopctR2/100
C16 (kg/milking)[3]	C16kgL= FatkgL*C16pctL/100 C16kgR1= FatkgR1*C16pctR1/100 C16kgR2= FatkgR2*C16pctR2/100
Preformed (kg/milking)[4]	PreformedkgL=FatkgL*preformedpctL/100 PreformedkgR1=FatkgR1*preformedpctR1/100 PreformedkgR2=FatkgR2*preformedpctR2/100

[1] MYkgL= milk yield(kg) for left udder half (2X), MYkgR1= milk yield(kg) for right udder half at 3pm, MYkgR2= milk yield(kg) for right udder half at 6pm

[2] Denovo= FA ≤ 15 carbons

[3] C16= C16:0 + C16:1

[4] Preformed= FA ≥ 17 carbons

Table 3.3. Calculations in SAS to calculate daily yields and percentages

Parameter	Formula
Daily milk (kg/d)	MYdaykgL=MYkgL*2 MYdaykgR= (MYkgR1+MYkgR2)*2
Daily fat (kg/d)	FatdaykgL= FatkgL*2 FatdaykgR= (FatkgR1+FatkgR2)*2
Daily protein (kg/d)	ProteindaykgL= ProteinkgL*2 ProteindaykgR= (ProteinkgR1+ProteinkgR2)*2
Daily SNF (kg/d)	SNFdaykgL=SNFkgL*2 SNFdaykgR= (SNFkgR1+SNFkgR2)*2
Daily lactose (kg/d)	LactosedaykgL= LactosekgL*2 LactosedaykgR= (LactosekgR1+LactosekgR2)*2
Daily cells (10^3/mL)	CellsdayL= cellsL CellsdayR= cellsR1*(MYkgR1/MYdaykgR) + cellsR2*(MYkgR2/MYdaykgR)
Daily denovo (kg/d)	DenovodaykgL= denovokgL*2 DenovodaykgR= (denovokgR1+denovokgR2)*2
Daily C16 (kg/d)	C16daykgL= C16kgL*2 C16daykgR= (C16kgR1+C16kgR2)*2
Daily preformed (kg/d)	preformeddaykgL=PreformedkgL*2 preformeddaykgR=(preformedkgR1+preformedkgR2)*2
Daily fat (%)	FatdaypctR= 100*fatdaykgR/MYdaykgR
Daily protein (%)	ProtdaypctR= 100*protdaykgR/MYdaykgR
Daily SNF (%)	SNFdaypctR= 100*SNFdaykgR/MYdaykgR
Daily lactose (%)	LactdaypctR= 100*lactdaykgR/MYdaykgR
Daily denovo (%)	DenovodaypctR= 100*denovodaykgR/MYdaykgR
Daily C16 (%)	C16daypctR= 100*C16daykgR/MYdaykgR
Daily preformed (%)	PreformeddaypctR= 100*preformeddaykgR/MYdaykgR

[1] MYkgL= milk yield (kg) for left udder half (2X), MYkgR1= milk yield (kg) for right udder half at 3pm, MYkgR2= milk yield (kg) for right udder half at 6pm

[2] Denovo= FA $\leq$ 15 carbons

[3] C16= C16:0 + C16:1

[4] Preformed= FA $\geq$ 17 carbons

Statistical Analysis of Yields and Concentrations

Data were analyzed by the GLIMMIX procedure of SAS (SAS 9.4; SAS Institute, Inc., Cary NC), using a statistical model that included treatment (tmt, 10 d, 20 d, 40 d), lactation number (lactno, 1 + 2, 3+) and day as fixed variables, and cow as a random variable (Table 3.4). Tmt, lactno, and tmt × lactno interactions were tested with the random variable cow(tmt ×

lactno) with 15 degrees of freedom (df). Repeated measures of milk yield were collected at days 10, 20, 40, 60, 120, 150, 180, 210, 240, and 270 of treatment. The GLIMMIX procedure was used with a mixed model of both fixed and random effects. The Kenward-Rogers method computed the denominator df (ddfm) for the tests of fixed effects. For the repeated effect of day, AR(1) was used to specify a first-order autoregressive structure, indicating days closer together were more highly correlated. Least squares means were calculated for fixed variables, and the SLICE option was used to test treatments within interactions of treatment × lactno and treatment × day. The random interaction of day × cow(treatment × lactno) was the residual term that tested day and its interactions.

Studentized residuals were generated and plotted to identify non-normality and outliers. Candidates for outliers were designated when residuals were > ± 3 SD from zero. Least squares means were plotted from the tmt × day interaction. Significant values were determined if the difference between udder halves was significantly greater than zero ($P < 0.05$). Figure 3.1 displays time on the x-axis, and MY difference on the y-axis for each of the three treatments. Milk component variables were analyzed with the same model. Figures 3.2-3.6 display least squares means from the tmt × day interaction for milk component variables.

Table 3.4. Statistical model terms for data analysis

Source	Df	Fixed or Random	Denominator df (DDFM)
Tmt[1]	2	Fixed	15
Lactno[2]	1	Fixed	15
Tmt x lactno	2	Fixed	15
Cow(tmt x lactno)	15	Random	
Day	9	Fixed	135
Day x tmt	18	Fixed	135
Lactno x day	9	Fixed	135
Tmt x lactno x day	18	Fixed	135
Residual	135	Random	
Total	209		

[1] Treatment

[2] Lactation number

RESULTS

Udder half milk and component yields and percentages

Milk composition was measured for each udder half at each of the sampling days and used to find the udder half difference. Statistical analysis was performed on the difference between udder halves in order to obtain *P*-values. For 10, 20, and 40 d treatment groups, mean udder half differences (4X-2X) through 270 d of treatment for MY, fat yield and protein yield are presented in Table 3.5. For milk, fat and protein yields, there was no effect of treatment group on the difference between 4X and 2X udder halves. Mean percentages per udder half are reported in Table 3.6. There was no difference between 2X and 4X treatment for fat, protein, lactose or SNF percentages for any of the treatment groups across all sampling days.

The effect of lactation number (lactno) tended to affect milk yield difference between udder halves ($P = 0.06$). Cows with parities 1 and 2 did not have different milk yields for any sampling days, but cows with parity 3 or greater (lactations 3, 4, and 5) had a greater milk yield for the 4X udder half compared to 2X, irrespective of treatment group (Data not shown). Interactions of treatment × DoT and treatment × lactno were not significant for differences between udder halves for any measures. Only cows on the 40 d treatment group with 3 or more lactations had a significant difference in MY between udder halves (Data not shown).

A significant increase in milk yield from IMF was determined if the difference between udder halves (4X-2X) was significantly different from zero ($P < 0.05$). Only the 40 d treatment group had a significant response to IMF and had a difference between udder halves for milk, fat, protein, SNF, lactose, and SCC (1000's/mL). The MY difference between udder halves was different for the 20 d treatment group only during the treatment in early lactation (Figure 3.1). The 40 d treatment had significant differences in MY between udder halves during the treatment period, and then for all sampling days after 180 DoT (Figure 3.1). The 20 d treatment group had a significant effect on fat yield difference on the first sampling day only (Figure 3.2). The 40 d treatment group had a significant effect on fat yield difference between udder halves both during and after IMF treatment, with differences observed until the last sampling day at 270 DoT (Figure 3.2). Differences between udder halves for protein yield were observed for all three treatment groups during treatment and after treatment for the 40 d group only (Figure 3.3). Lactose yield difference between udder halves was significant for the 40 d treatment only, mostly occurring during early lactation (Figure 3.4). Differences in SNF yield were detected for the 20 d treatment group during IMF treatment, and then both during and after treatment, all the way until 270 DoT for the 40 d group (Figure 3.5). Somatic cell difference between udder halves was significant for all three treatment groups at various sampling days, with each of the treatment groups having a difference on different sampling days (Figure 3.6).

Table 3.5. Mean udder half differences (4X-2X) through 270 d of treatment for milk, fat, and protein yields[1]

	Treatment			
	10 d	20 d	40 d	*P*-value[2]
Milk yield (kg/d)	0.87 ± 1.15	1.75 ± 1.11	4.09 ± 1.22	0.18
Fat yield (g/d)	12.6 ± 38.5	61.4 ± 36.9	136.5 ± 40.8	0.09
Protein yield (g/d)	28.5 ± 34.9	52.5 ± 33.6	117.0 ± 37.0	0.23

[1] Data presented are LSMeans ± SEM

[2] $P < 0.05$

Table 3.6. Mean percentages for milk components through 270 d of treatment

	Treatment Group						
	10 d		20 d		40 d		
Component	L	R	L	R	L	R	*P*-value[1]
Fat	3.5 ± 0.87	3.4 ± 0.81	3.8 ± 1.03	3.7 ± 0.92	3.8 ± 0.67	3.8 ± 0.69	0.83
Protein	3.0 ± 0.34	3.0 ± 0.37	3.0 ± 0.26	3.0 ± 0.26	3.0 ± 0.31	3.0 ± 0.31	0.81
Lactose	4.8 ± 0.18	4.8 ± 0.19	4.9 ± 0.23	4.9 ± 0.23	4.9 ± 0.19	4.9 ± 0.17	0.48
SNF	8.7 ± 0.43	8.7 ± 0.42	8.7 ± 0.37	8.7 ± 0.38	8.7 ± 0.40	8.7 ± 0.40	0.17

[1] Values are percentages of total milk yield

[2] *P*-value represents the effect of treatment on the difference between udder halves (4X-2X)

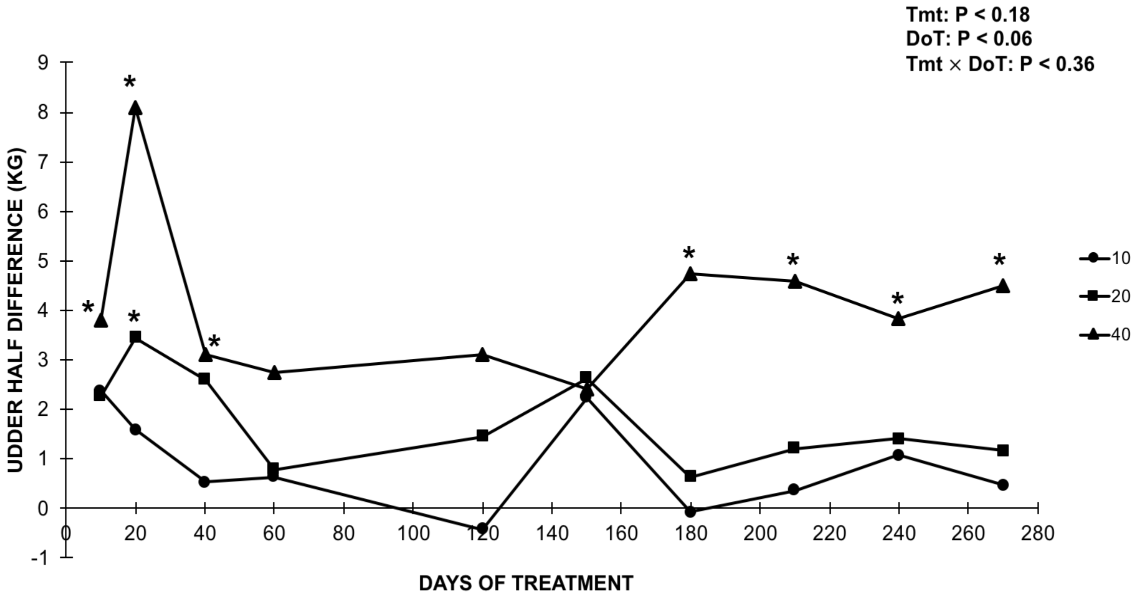

Figure 3.1. Difference in milk yield between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 270 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days, $P < 0.05$

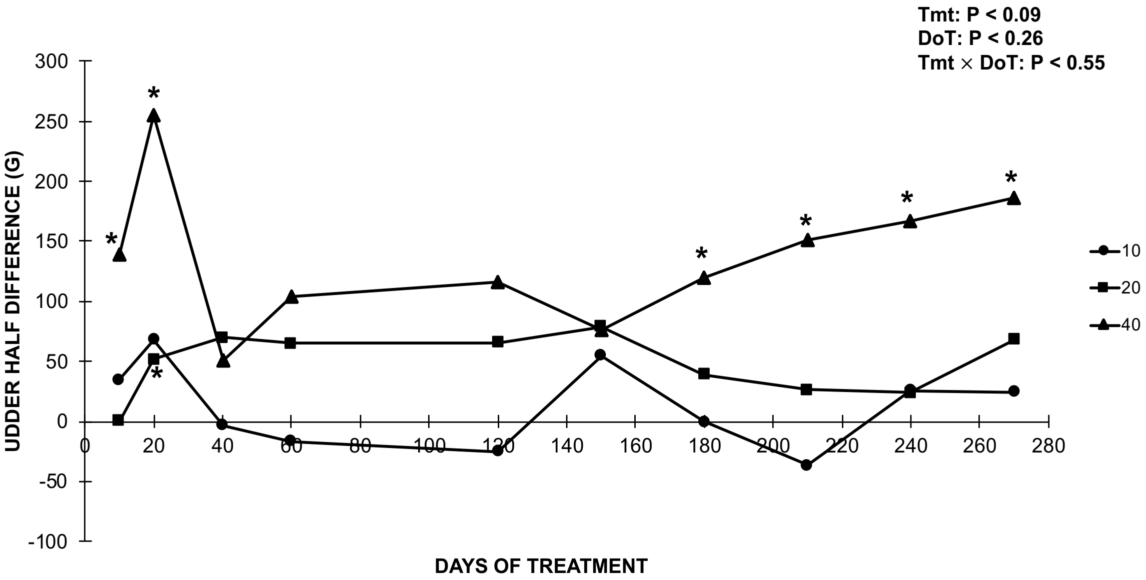

Figure 3.2. Difference in fat yield between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 270 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days, *P* < 0.05

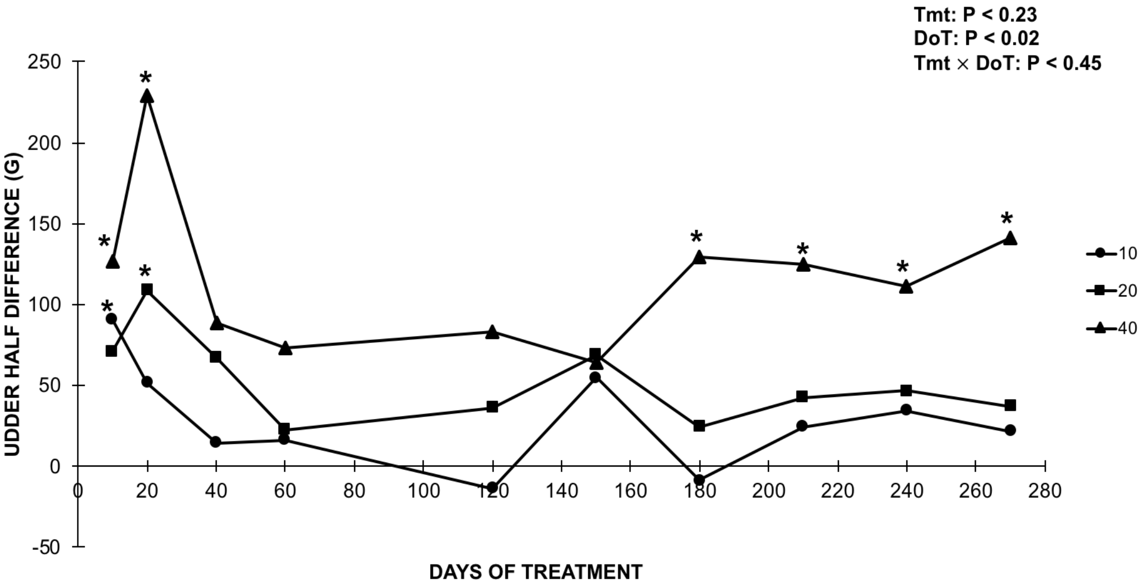

Figure 3.3. Difference in protein yield between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 270 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days, *P* < 0.05

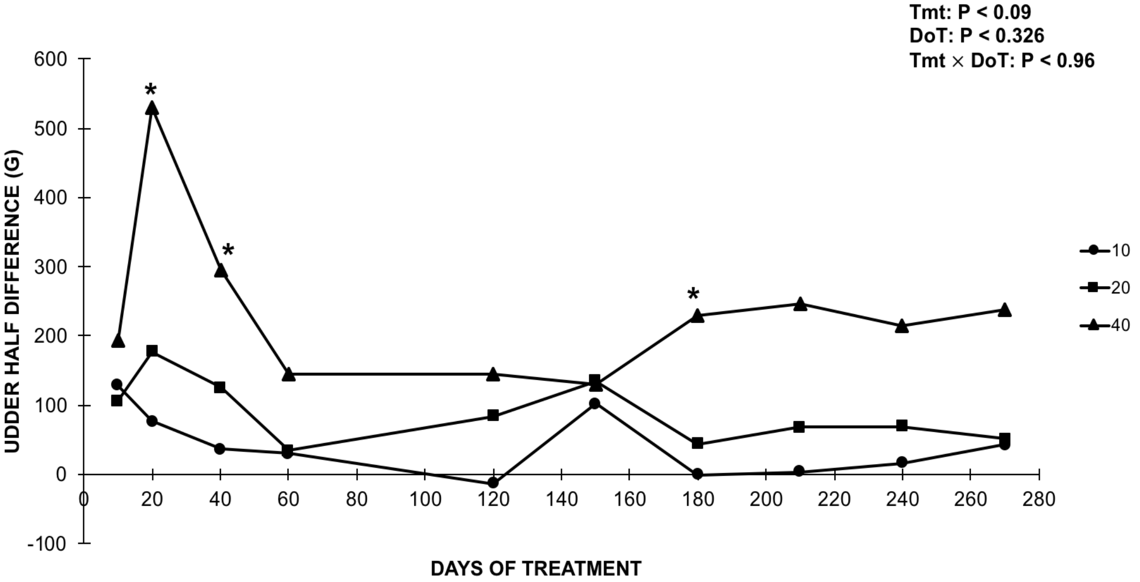

Figure 3.4. Difference in lactose yield between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 270 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days, *P* < 0.05

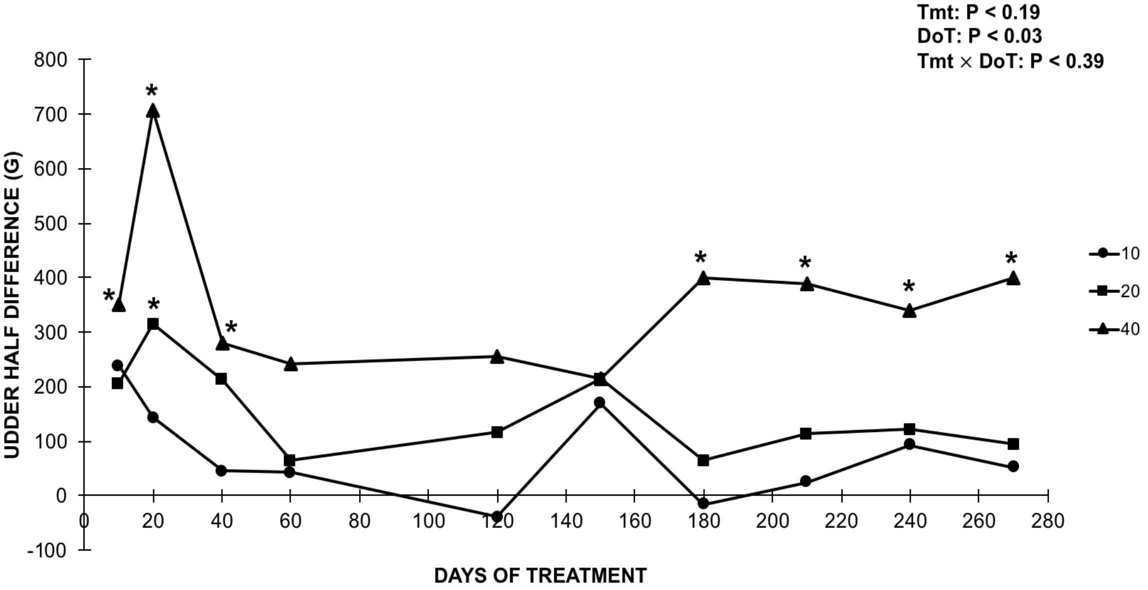

Figure 3.5. Difference in SNF yield between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 270 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days, *P* < 0.05

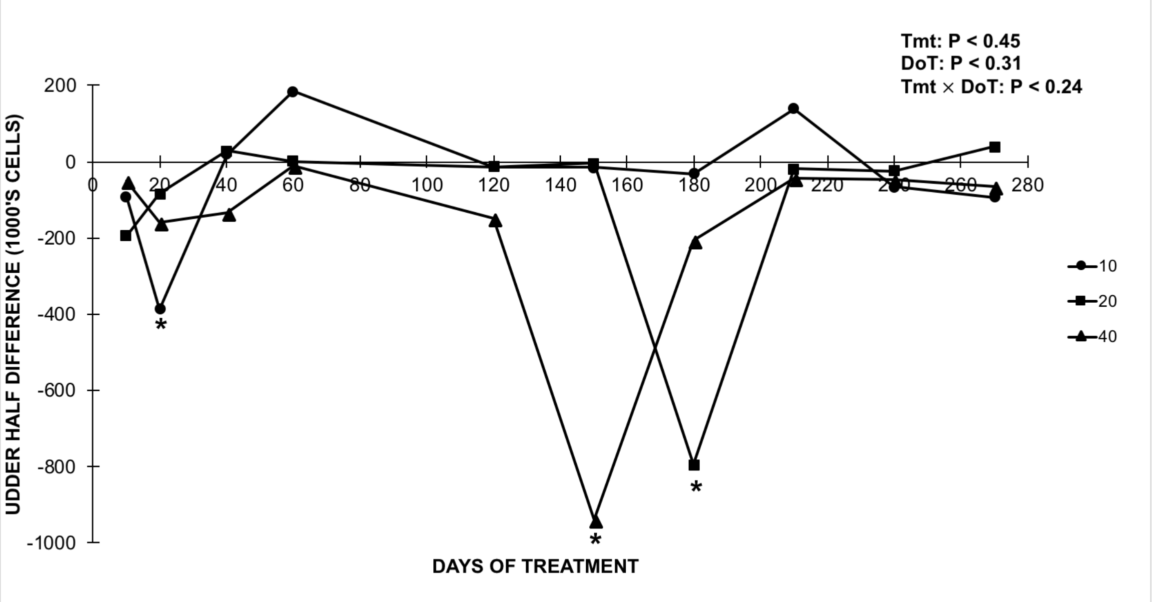

Figure 3.6. Difference in somatic cells between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 270 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days, *P* < 0.05

Fatty acid yields

Fatty acid composition (FA) was characterized to distinguish the sources of milk fat FA and measure concentration relative to IMF treatment. The two sources from which FA originate are de novo synthesis, meaning synthesis in the mammary gland, and preformed uptake, meaning from circulation. Mean udder half differences (4X-2X) were calculated for the 10, 20, and 40 d treatment groups through 60 DoT for denovo, C16, and preformed yields (Table 3.7). There was no effect of treatment for any of the three groups of FAs (Table 3.7). Similar to other analyses, significance was determined if the difference between udder halves (4X-2X) was significantly different from zero *(P* < 0.05; Figures 3.7-3.9). Although there was no significant effect of treatment on denovo FA yield, both the 20 and 40 d treatments were significantly different from zero (data not shown). The yield of denovo FAs differed from zero on d 10, 20, and 40 for the 40 d treatment only. C16 fatty acid yields differed from zero on d 20 and 40 for the 40 d treatment only. Preformed FA yields differed from zero on day 10 and 20 of the 40 d treatment. Interactions of treatment × lactno and treatment × DoT were not significant for any of the three FA groups (data not shown).

Daily percentages of FA were calculated for each udder half at each sampling day, through d 60, and used to find the difference between udder halves (4X-2X). Statistical analysis was performed on the difference between udder halves in order to obtain *P*-values. There was no effect of treatment on percentage of FA for any of the three groups (Table 3.8), meaning the percent of FA produced by the right udder half (4X) was not greater than the percent of FA produced by the left udder half (2X) for any of the three treatment groups. Table 3.8 displays the mean percentages for FA in milk as a percentage of the total milk fat.

Table 3.7. Mean udder half differences (4X-2X) through 60 d of treatment for denovo, C16 and preformed fatty acid yields[1]

	Treatment Group			
	10 d	20 d	40 d	*P*-value
Denovo yield[2]	9.61 ± 8.38	18.39 ± 7.97	35.06 ± 9.15	0.13
C16 yield[2]	-0.41 ± 13.66	24.92 ± 13.09	40.91 ± 14.94	0.12
Preformed yield[2]	17.72 ± 20.28	36.29 ± 19.31	80.54 ± 22.16	0.11

[1] Data presented are LSMeans ± SEM

[2] Measured in g/kg of fat

[3] $P < 0.05$

Table 3.8. Mean percentages for fatty acids in milk through 60 d of treatment

	Treatment Group						
	10 d		20 d		40 d		
	L	R	L	R	L	R	*P*-value[2]
Denovo[1]	21.4 ± 3.1	21.8 ± 3.1	21.9 ± 4.2	21.9 ± 3.6	21.5 ± 3.2	21.6 ± 2.9	0.18
C16[1]	25.8 ± 2.3	25.0 ± 5.3	24.7 ± 4.8	25.6 ± 2.2	24.7 ± 5.0	25.5 ± 2.4	0.09
Preformed[1]	52.9 ± 4.1	53.2 ± 5.2	53.4 ± 6.1	52.5 ± 4.8	53.8 ± 5.7	52.9 ± 4.4	0.23

[1] Values represent percentages of total milk fat yield

[2] *P*-value represents the effect of treatment on the difference between udder halves (4X-2X), $P < 0.05$

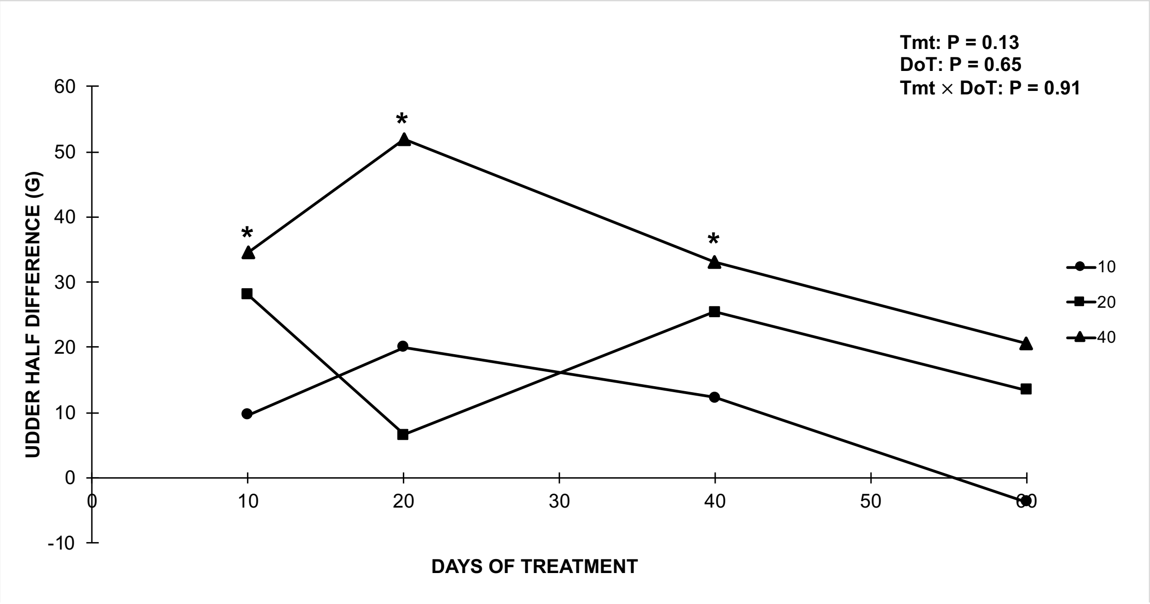

Figure 3.7. Difference in denovo FA yields between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 60 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days $P < 0.05$

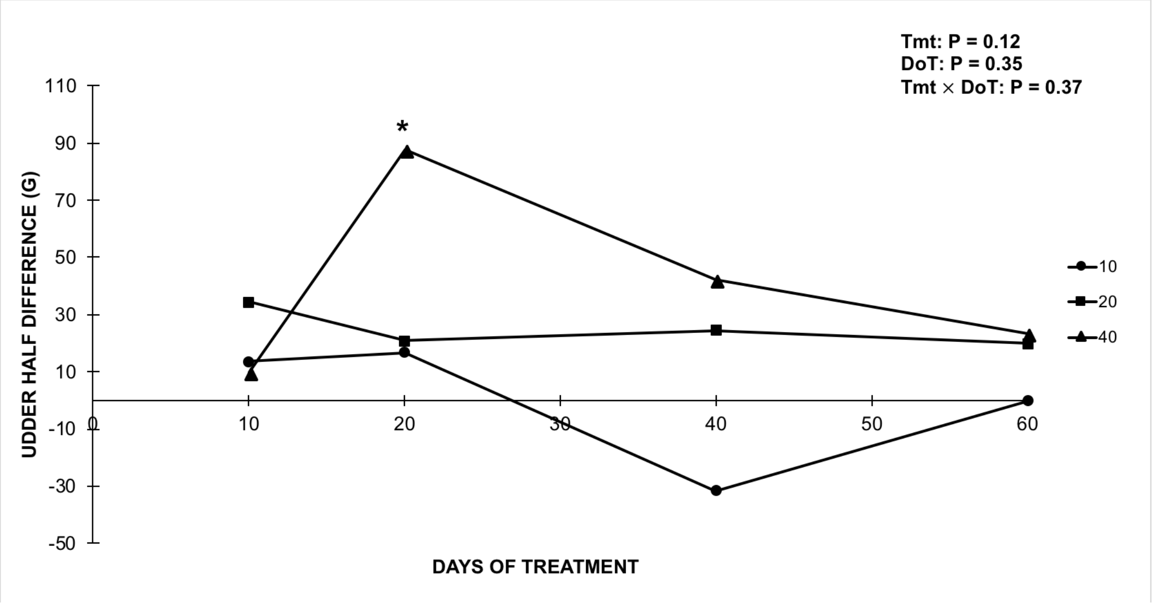

Figure 3.8. Difference in C16 FA yields between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 60 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days *P* < 0.05

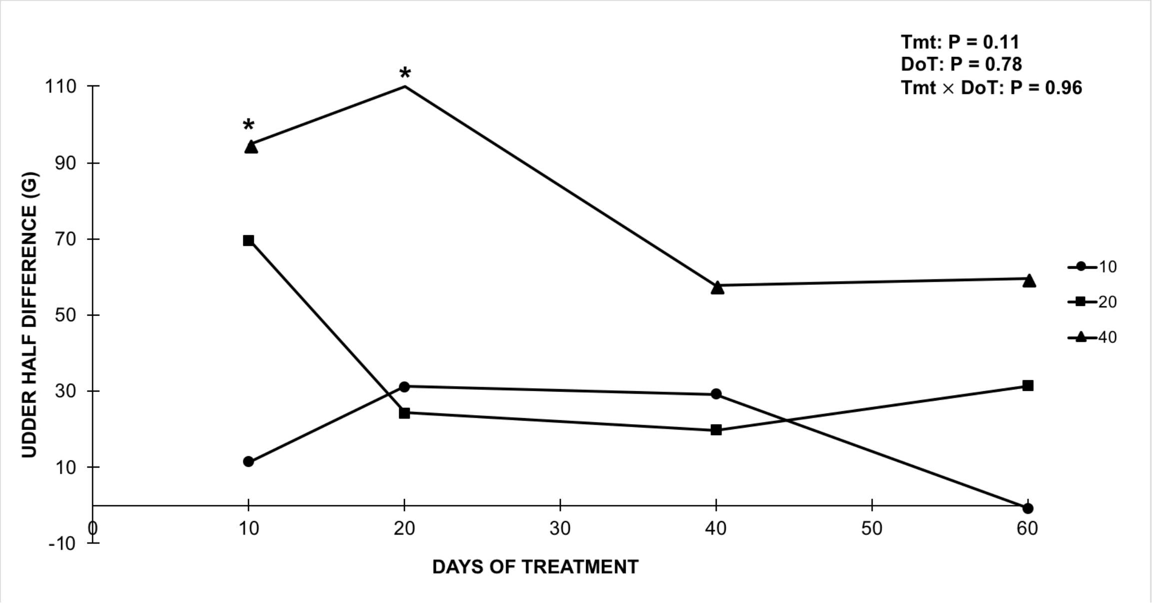

Figure 3.9. Difference in preformed FA yields between udder halves (4X-2X) for 10, 20, and 40 d treatments at various sampling days through 60 d of treatment. Asterisks represent a significant difference between udder halves at individual sampling days *P* < 0.05

DISCUSSION

In this experiment, the duration of IMF treatment during early lactation was investigated, and overall, the 40 d treatment resulted in greater milk and component yields. This was the first study to use the unilateral frequent milking (UFM) method to test these three different lengths of application of treatment during early lactation. The UFM method was first used by Wall and McFadden (2007a), in which one udder half was milked 4X for the first 21 days of lactation and the other half was milked 2X for the entire lactation. Their results revealed that the 4X udder halves produced more milk with a 3.5 ± 0.2 kg/d increase compared to the 2X halves for the remainder of lactation. The results from the current experiment coincide with Wall and McFadden (2007a); there tended to be an increase in milk yield following milking 4X, but no significant difference between the three treatments overall throughout the entire lactation. Another study similar to the current one, which also attempted to assess the timing of IMF treatment during early lactation was done by Wall and McFadden (2007b). The main objective in this study was to determine if IMF during early lactation from d 1-14 or d 7-21 would have a greater effect on milk yield. They found that overall milk yield responses were not significantly different for each of the treatments and were not significantly different than that previously observed for IMF on d 1-21 of lactation. Results from the current study are also in agreement, since the 10 and 20 d treatments did not differ from one another with respect to milk yield, demonstrating that the first 21 days of lactation seem to be responsive to IMF, no matter when or how long treatment is applied.

In the current experiment, the 40 d treatment was created to determine the benefits of an extended period of increased milking frequency, beyond the typical 21 d used in various other studies. Eslamizad et al. (2010) determined the effect of 6X milking vs 3X milking during both early and full lactation on milk production. Researchers found no difference between milk yield for the cows milked 6X for the full lactation and cows milked 6X during the first 90 DoT only. These results suggest that while early lactation is the most opportunistic time during lactation to apply IMF treatment, a treatment length 90 d might exceed the window of opportunity, and a length greater than 21 d but shorter than 90 should be the goal. In the current experiment, the 40 d treatment had the best response to IMF in terms of milk and component yields. This was the first experiment to test IMF treatment application for this length of time.

With an increase in MY occurring both during and after IMF treatment, another important aspect to consider is the effect of treatment on milk components. Wall and McFadden (2007a) found no effect of UFM on SCC, milk fat percentage, or milk protein percentage. The current study reported component yields instead of percentages, but the results were similar to that of Wall and McFadden (2007a). No differences were observed between the three treatments for milk fat, protein, lactose, or SNF yields along with SCC. In a review by Erdman and Varner (1995), 22 published reports were used to examine the effect of milking frequency on MY and composition. They reported milk fat and protein percentages tended to decrease as MF increased. Whereas milk fat and protein yields were increased due to increased MY from IMF. These results do not agree with the current study since we did not observe a significant difference in milk fat and protein yields following IMF during early lactation. While there were numeric differences in the yields, due to increasing milk yields, this difference was not great enough to be considered statistically significant for 2X vs 4X milking.

In attempt to explain how this increase in milk yield following IMF is occurring, milk fatty acid (FA) profile was measured. Fatty acids originate from two different sources; de novo synthesis, meaning synthesis in the mammary gland, and preformed uptake, meaning they come directly from circulation. Because some reports have indicated that there is an increase in milk fat yield in response to IMF, a change in the activity of MECs may be occurring. Shields et al. (2011) investigated the effect of IMF for the first 21 days of lactation, using UFM, on milk fatty acid (FA) profile, and found that there were no significant changes in the FA profile of 4X glands compared to 2X. Results from the current experiment are in agreement with Shields et al. (2011); no difference was observed in FA profile between treatments (4X-2X) for milk samples obtained at d 10, 20, and 40 of treatment. While there was no overall effect of treatment on FA profile, we did observe a significant difference between the 40 d group and the 10 and 20 d treatments, with the 40 d treatment having denovo, C16 and preformed yield differences that were significantly different from zero, while the other two groups did not. These significant differences indicate a difference in the amount of FA generated in both the 4X and 2X udder halves. The 40 d group might have resulted in significant differences between the two treatments because of the greater length of application of treatment, indicating there are some changes occurring in the fat content of milk following IMF treatment. While no other data exists on changes in FA profile in bovine milk following IMF treatment, these results suggest that there may be some changes in the milk fat synthesis program in the mammary gland, occurring due to IMF treatment. Further research needs to be done to investigate this hypothesis.

CONCLUSION

Results from the current study suggest that 40 d of IMF treatment during early lactation is maximal for achieving an increase in milk and components yields throughout lactation compared to 10 or 20 d of IMF. Other research groups utilized 21 d of IMF during early lactation and found an increase in milk yield either for part of lactation (Hale et al., 2003) or for the entire lactation (Wall and McFadden, 2007a). An experiment similar to the current one is needed to validate that 40 d of IMF is superior to 21 d of IMF and results in an increase in milk and component yields for an entire 305-d lactation.

The second portion of this experiment investigated the effect of three durations of IMF on FA concentration in milk in attempt to determine whether the increase in milk fat observed following IMF is coming from local or systemic sources. We observed an increase in both denovo and preformed FA yields glands milked 4X for 40 d compared to glands milked 2X. There were no significant differences between 4X and 2X udder halves for 10 or 20 d of IMF treatment. With an increase in both sources of FA, there was no clear distinction as to the contribution of the two sources to increased milk fat following IMF. Although not statistically tested, the yield of preformed FA was approximately double the denovo yield for 10, 20, and 40 d treatment groups; this trend is worth investigating further. The current experiment measured FA yields only at 10, 20, 40 and 60 DoT, and perhaps measuring FA content at other time points further into lactation would be helpful and provide better insight to the changes in FA that are occurring following IMF.

CHAPTER 4: Immunohistochemical identification of Yes-associated Protein expression in mammary tissue from cows treated with increased milking frequency

INTRODUCTION

The Hippo pathway has been identified as a key regulator of organ size and tissue homeostasis. This pathway regulates cell proliferation, apoptosis and stemness, by inhibiting YAP and TAZ transcription coactivators, in response to extracellular and intracellular signals such as cell to cell contact, cell polarity, and mechanical cues (Yu et al., 2015). The Hippo pathway was originally discovered in *Drosophilia* as a suppressor of tissue overgrowth (Justice et al., 1995), and is highly conserved in mammals. Upon activation of the Hippo pathway, Ste20-like kinases (MST1 and MST2) phosphorylate and activate large tumor suppressor kinases (LATS1 and LATS2), which in turn phosphorylate Yes-associated protein (YAP) (Kodaka and Hata, 2015). When the hippo pathway is on, YAP is phosphorylated and remains in the cytoplasm. This means that tissue growth is being controlled and no cell proliferation is occurring. When the hippo pathway is off, YAP is not phosphorylated and can translocate to the nucleus, allowing cell proliferation and tissue growth to occur. The functions of this pathway have made it of primary interest in the role of cancer, specifically breast cancer. Evidence suggests that YAP is an oncoprotein that promotes breast cancer cell proliferation and survival, with the *Yap* gene being amplified in breast tumors in mice (Overholtzer et al., 2006). These data suggest implications for YAP in the mammary gland and one of its most important functions, production of milk.

It is known that when cows are milked at increased frequency, they produce more milk. The question of how the gland is accommodating this increased production becomes of interest. With these changes that are taking place in the mammary gland during increased milking frequency (IMF) being locally regulated, the main question researchers have is, is it an increase in the number of mammary epithelial cells (MEC), or an increase in MEC activity? With YAP being expressed in either the cytoplasm or nucleus of mammary epithelial stem cells, depending on the activity, this becomes a question of cell activity rather than cell number. In a study by Chen et al. (2014), the expression of YAP in the mammary gland of virgin mice was examined by immunohistochemistry and was detected in luminal and myoepithelial cells. The subcellular localization of YAP differed in these cell types. During pregnancy, myoepithelial cells showed nuclear YAP expression, while the luminal cells displayed a more diffuse localization of YAP throughout the cytoplasm and nucleus. During lactation, the amount of YAP staining in the alveoli was significantly decreased. The dynamic changes of YAP expression at these different stages suggest a potentially critical role for the Hippo pathway in mammary gland growth and development. The main objective of this experiment was to evaluate the relative amount and location of YAP expressed in mammary glands subject to IMF treatment through immunohistochemical analysis.

MATERIALS AND METHODS

Tissue preparation and embedding

Twenty-eight tissue samples obtained from a previous experiment, (Hardin, 2015), were used for immunohistochemistry to investigate YAP expression in bovine tissue. Methods from the experiment are listed briefly; eight cows (seven Holstein and one Jersey) from the Virginia Tech Dairy Center were milked at IMF for 21 days using the unilateral frequent milking method, with the right udder half milked 4X and the left udder half milked 2X. On day 21 and day 60, mammary biopsies were taken, as described in (Hardin, 2015) and fixed in 10% formalin and subsequently stored in 70% ethanol. All of the tissue samples were processed starting with 70% ethanol for one-hour 2X, 80% ethanol for one hour 2X, 95% ethanol for one hour 2X, 100% ethanol for one hour 2X, xylene for one hour 2X, and finally paraffin for one and a half hours 2X. After processing, the cassettes remained in paraffin and transferred one by one into a metal mold. Paraffin was poured into each mold and given time to solidify (approximately 30 minutes).

The blocks were then sliced using a HM 340E Microm GmbH microtome (Walldorf, Germany) in 5µm thick sections and placed on charged microscope slides. (Fisher Scientific Color Frost Plus; Pittsburgh, PA). Three to four tissue sections were placed on each slide, and one slide per tissue sample was made. One tissue section on each slide served as a negative control, to account for background noise. The slides were deparaffinized and rehydrated by submerging in a series of solutions: Xylene substitute (VWR), Ethanol 100%, Ethanol 95%, Ethanol 70% and water, each 2X for 5 minutes. The slides were then placed in a 1x (0.1 M) concentration citrate buffer heated to approximately 95°C for 30 minutes to allow denaturing of the proteins. After being cooled to room temperature, slides were washed with 1x (0.01M) phosphate-buffered saline (PBS) 2X for 5 minutes. A hydrophobic PAP pen for immunostaining (Daido Sangyo Co., Ltd.; Tokyo, Japan) was used to draw separate borders around each tissue section. A universal blocking agent, CAS-block (Invitrogen by Life Technologies; Frederick, MD) was applied and allowed to sit for 30 minutes. A YAP (D8H1X) XP® Rabbit mAb primary antibody (Cell Signaling Technology) (1:200 dilution in CAS-block) was applied to tissue sections and incubated overnight. The following day, the primary antibody was removed and washed 3X with 1x PBS to remove any excess solution. An Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen Molecular Probes; Eugene, OR USA) secondary antibody (1:200 dilution) was applied to each of the tissue sections and allowed to sit for one hour. The slides were then washed again with 1x PBS and the PAP pen sections removed. A Molecular Probes- ProLong Gold antifade reagent with DAPI (Life Technologies; Eugene, OR) was used as a counterstain. Images of the slides were captured using a Nikon Eclipse E600 immunofluorescence microscope using a 40X objective lens.

Slide Evaluation

Images of each slide were evaluated using the program Image-Pro Plus 7 (Media Cybernetics, Inc. Rockville, MD). Areas of epithelial staining were identified and outlined by hand and used to quantify the intensity of staining present in the three replicate images for each udder half for a particular animal. This process was repeated for all day 21 and day 60 images. Intensity areas were reported as intensity per density/area. Averages for each of the three images per slide were obtained, and then udder half averages for each animal were calculated for both day 21 and day 60 samples.

Presence of YAP- positive nuclei were evaluated using the same program, Image-Pro Plus 7. Nuclei that appeared to have staining over top of a clearly blue stained nucleus were identified as YAP-positive nuclei. These were counted by hand and totals for each slide were kept in an excel spreadsheet. Representative images were chosen based on averages for each measurement These data were then analyzed using the SAS program.

Histology Data Modifications

Histology measurements (intensity and nuclei) were recorded for three fields per image, and averages for each image were calculated in Excel. A ratio of YAP-positive nuclei to total number of nuclei per field was calculated and then averaged by treatment to obtain one value for each sampling day per cow.

Statistical Analysis of Histology Data

The averages for intensity and nuclei were analyzed in SAS, using the GLIMMIX procedure described previously and a plot of studentized residuals to identify non-normality and outliers. Studentized residuals are calculated by dividing the residual of an estimate by the standard deviation. This deletes observations one at a time, each time refitting the regression model on the remaining observations. The model included both fixed and random variables (Table 4.1). The fixed variables were treatment (tmt), day, and tmt × day interaction. The random variables were cow and tmt × cow. Least squares means were calculated, and the SLICE option was used to test treatments within interactions of tmt × day. A contrast of the tmt × day interaction was added to test whether the difference between the two treatments (4X-2X) on day 21 was the same as the difference between the two treatments on day 60. The contrast produced the numeric difference between the two treatments and its standard error, as well as the test of significance which gave the same *P*-value as obtained from the tmt × day interaction. This was due to testing only two treatments and two days. The contrast coefficients are listed in Table 4.2.

Table 4.1. Statistical model for histology experiment

Source	df	Fixed or Random	Denominator df (DDFM)
Tmt	1	Fixed	6
Cow	6	Random	6
Tmt × cow	6	Random	
Day	1	Fixed	6
Day × tmt	1	Fixed	6
Residual	12	Random	12
Total	27		

Table 4.2. Contrast coefficients for treatment × day interaction for histology experiment

	2X-d21	2X-d60	4X-d21	4X-d60
D60(4X-2X) – D21(4X-2X)	1	-1	-1	1

RESULTS

Intensity of YAP staining was reduced by 50% in 2X compared to 4X milking at d 60 *(*$P < 0.04$; Figure 4.1). There was no difference between the treatments at d 21 *(*$P< 0.56$; Figure 4.1). The contrast for the treatment × d interaction, which compared the difference between the two treatments at d 21 and subtracted that value from the difference between the two treatments at d 60, was not significant *(*P=0.0602), in agreement with the results from the type III test of fixed effects on the interaction. There was no significant effect of sampling day on intensity of staining, meaning that the amount of staining at d 21 was not significantly different from the amount of staining at d 60, irrespective of treatment (Figure 4.1). Figure 4.2 shows representative images of intensity of Yap staining for each of the two treatments at both sampling days.

The effect of treatment on the average number of YAP-positive nuclei tended to be significant *(*P=0.0557), with the 4X samples tending to have more positively stained nuclei than the 2X. The effect of day was significant *(*P= 0.0332), indicating there was a 6% increase in the number of YAP-positive nuclei at d 60 compared to d 21, irrespective of treatment (2X or 4X; Figure 4.3). The treatment × d interaction was not significant *(*$P < 0.18$), but when sliced by d, an increase of 10% was observed when going from 2X to 4X milking at d 60*(*$P < 0.0204$; Figure 4.3). No difference was found between treatments at d 21 *(*$P < 0.5107$; Figure 4.3). The contrast for the treatment × d interaction, which took the difference between the two treatments at d 21 and subtracted it from the difference between the two treatments at d 60, was not significant *(*P=0.1842), in agreement with the results from the type III test of fixed effects on the interaction. Figure 4.4 shows representative images containing nuclei stained positively for YAP at both sampling days for each of the two treatments.

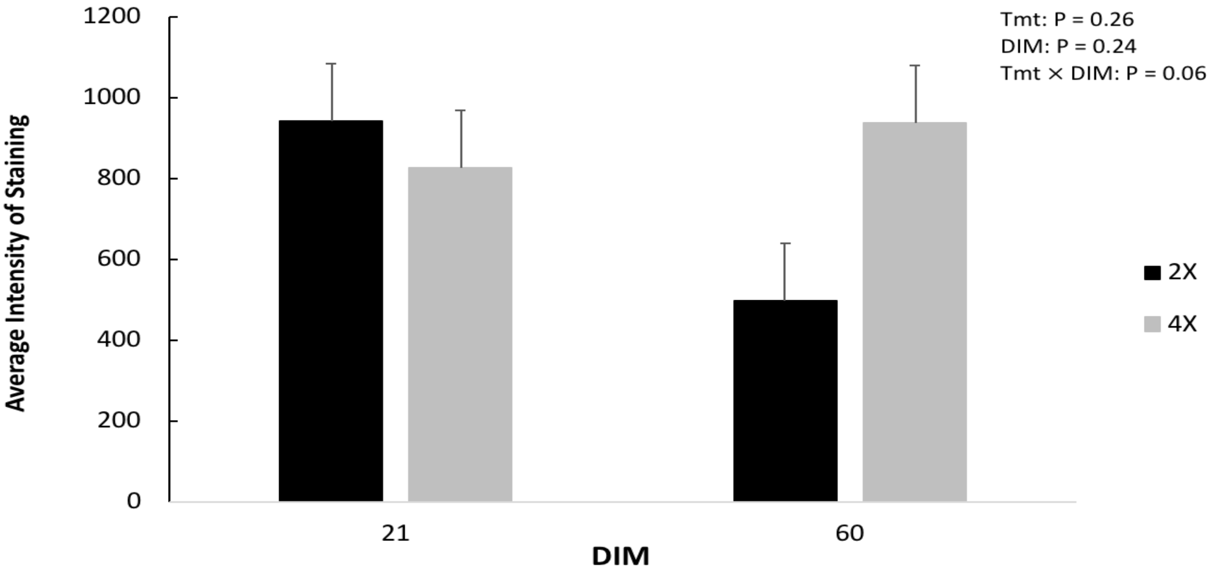

Figure 4.1. Average intensity of YAP staining for tissue samples from mammary glands milked either 2X or 4X at DIM 21 and 60. Asterisks represent significant differences between treatments at d 60.

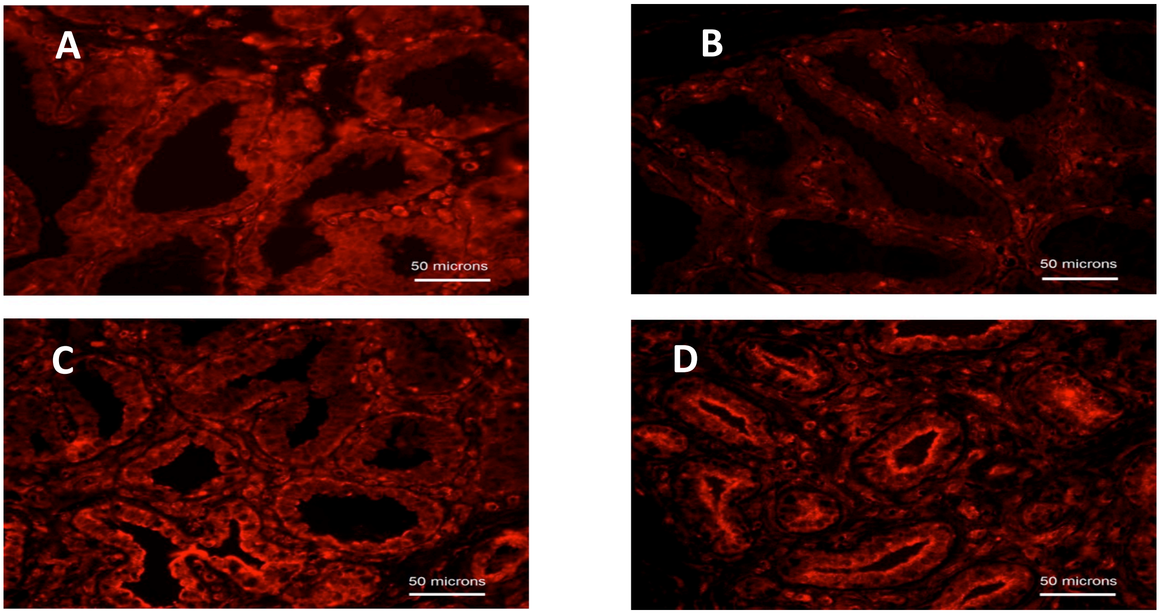

Figure 4.2. Representative images for intensity of Yap staining for both treatments and each sampling day. (A) day 21 sample for 2X treatment, (B) day 60 sample for 2X treatment, (C) day 21 sample for 4X treatment, (D) day 60 sample for 4X treatment. Magnification for images was 40×.

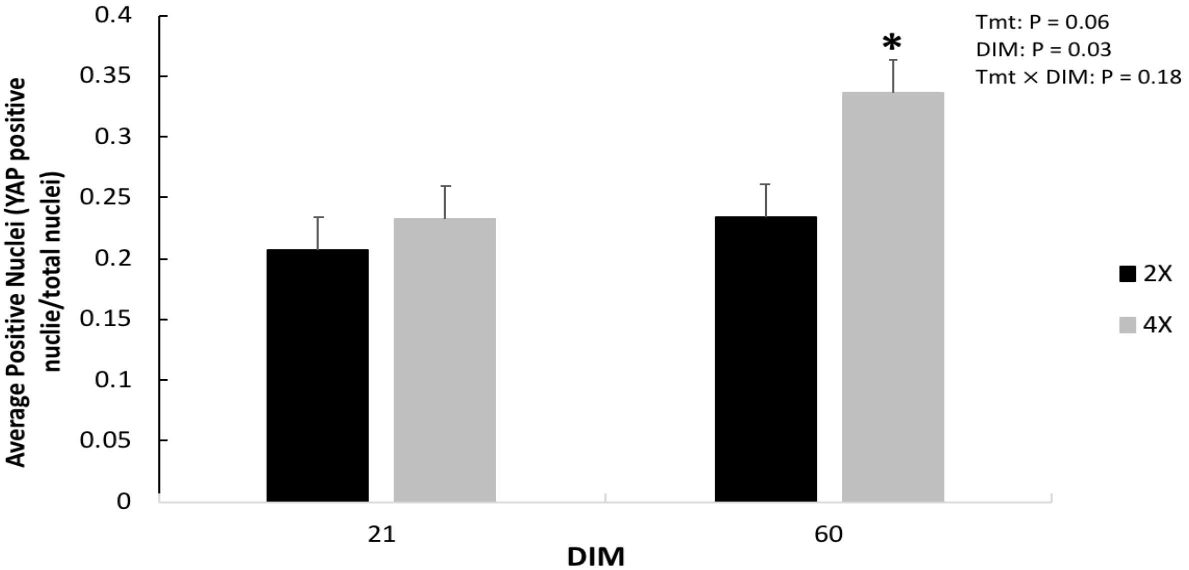

Figure 4.3. Average number of positively stained Yap nuclei (nuclei/image) for tissue samples from mammary glands milked either 2X or 4X at DIM 21 and 60. Asterisks represent a difference between the two treatments at d 60.

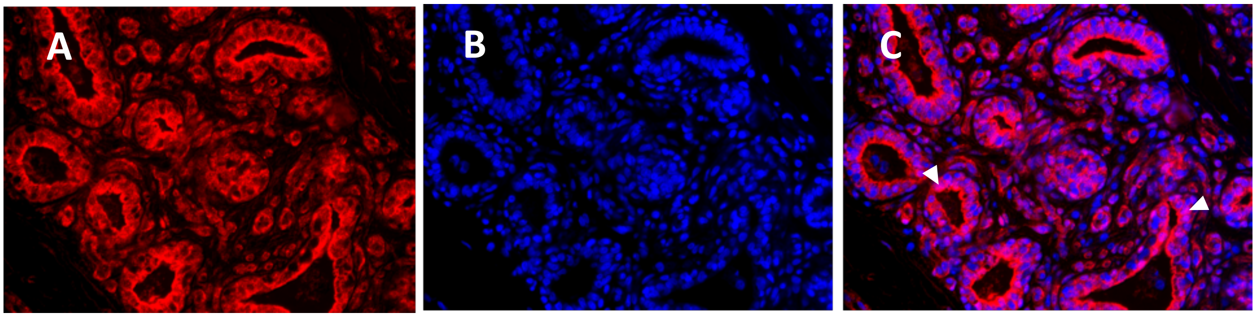

Figure 4.4. Representative images from 4X udder half sample at 60 DIM for (A) intensity of YAP staining, (B) nuclei stained with DAPI, (C) composite image with both YAP staining and DAPI staining. Arrowheads denote nuclei positively stained for YAP. Magnification for images was 40×.

DISCUSSION

The main objective of the current experiment was to evaluate the relative amount and location of YAP expressed in mammary glands subject to IMF treatment through immunohistochemical analysis. The two methods of measuring YAP activity were intensity of staining and number of positively stained nuclei. With no prior research on YAP expression in lactating mammary glands subject to IMF treatment, information on the relative amount and location of YAP obtained from this experiment were novel. The amount of YAP staining in each of the tissue samples appeared to be more than expected. Representative images were presented in the results and demonstrate that the average amount of YAP staining that was observed. The majority of the YAP staining was located in the mammary epithelial cells (MECs), in the alveoli for each sample. Overall, there were no statistical differences in the intensity of YAP staining between the two treatments (2X vs 4X). This means that increased milking frequency did not have an effect on the amount of YAP that was present in mammary gland tissue samples. There did tend to be significance of the tmt × d interaction, with the difference between the two treatments being significant at d 60, but not at d 21. At d 60, the intensity of YAP for the 4X treatment was significantly higher than the 2X treatment. These results indicate that there may be a difference in the activity of YAP, measured by difference in treatments, in the mammary gland during IMF treatment (d 21) versus the activity after IMF treatment is complete (d 60). The changes occurring in the mammary gland during IMF treatment that result in increased milk yield, are thought to have a carryover effect, since the increase in milk yield is observed after treatment has ceased. If YAP activity is related to this increase in milk production, a carryover effect in the expression of YAP would also be expected. Results from this experiment are in agreement with this hypothesis since a change in intensity of YAP staining was observed at d 60.

A future study in which biopsy samples are taken earlier in the IMF treatment, as opposed to the last day of treatment (i.e. the current study), would better compare the expression of YAP during and after treatment, and examine any changes that could be taking place in the mammary gland.

Once it was determined that YAP was present in these tissues samples for both treatments, in the MECs, the next step was to determine where it is present in these MECs. When YAP is expressed in the nucleus of cells, it is unphosphorylated, and allows cell proliferation and tissue growth to occur. When YAP is expressed in the in the cytoplasm, it is phosphorylated and is inactive. Chen et al. (2014), examined the expression of YAP in the mammary gland of virgin mice using immunohistochemistry and detected it in luminal and myoepithelial cells. The subcellular localization of YAP differed in these cell types with myoepithelial cells showing nuclear YAP expression, and luminal cells displaying more diffuse localization of YAP throughout both the cytoplasm and nucleus. These researchers also found that during lactation, the amount of YAP staining in the alveoli was significantly decreased. In the current experiment, the location of YAP was mostly in the cytoplasm and more concentrated on the luminal side of the cells. A marker for myoepithelial cells was not used here, so different cell types could not be distinguished as they were in Chen et al. (2014). There was nuclear staining present, with treatment tending to have an effect on the number of positively stained nuclei. Day did have a significant effect of day on number of positive nuclei, and these results indicate that at d 60 there were significantly more positive nuclei than there were at d 21. These results are not in agreement with the ones from Chen et al. (2014), where they found the amount of YAP staining was decreased during lactation, but in the current study, an increase was seen in the number of positively stained nuclei as lactation progressed.

CONCLUSION

Overall, there was a change in the activity of YAP, demonstrated through an increase in the intensity of YAP staining and the number of YAP positive nuclei for 4X tissues compared to 2X tissues at 60 DIM, but not at 21 DIM. More YAP in the nuclei of mammary cells of glands milked at increased frequency, compared to the glands milked 2X could indicated an increase in cell proliferation associated with IMF. Other research groups have investigated cell proliferation for glands milked at increased frequency using markers such as Ki-67 and found no difference between control and treatment glands (Hale et al., 2003, Norgaard et al., 2005). Further research should investigate whether there is an increase in proliferation associated with increased YAP expression in mammary glands following IMF.

CHAPTER 5: Conclusion

Increasing milking frequency during early lactation increases milk yield during treatment and once treatment has ceased (Hillerton et al., 1990, Bar-Peled et al., 1995, Erdman and Varner, 1995). This practice can be used by dairy producers as a tool to increase milk production efficiency of their cows and utilize facilities more efficiently (Allen et al., 1986, Wall and McFadden, 2007b). Bar-Peled et al. (1995) increased the milking frequency of cows from 3X to 6 times a day (6X) during the first 6 weeks of lactation and reported a 7.3 kg/d (21%) increase in milk production demonstrating that increased milking frequency (IMF) during early lactation was successful in increasing milk production for those cows. This was the first detailed study looking at very frequent milking during early lactation and demonstrated that applying treatment only during early lactation is sufficient to increase production throughout the entire lactation. A second study by Wall and McFadden (2007b), investigated duration of IMF during early lactation using the unilateral frequent milking (UFM) technique in which both udder halves are milked at different frequencies. One udder half was milked 2X while the other was milked 4X for either d 1-14 of d 7-21 of lactation. They found no significant differences between the two treatment groups for milk production, but udder halves milked 4X still produced more milk than udder halves milked 2X. These results reveal that there is no clear answer as to when during early lactation this treatment should be applied, and further research was needed to investigate the appropriate duration of frequent milking application.

The main objective of this study was to determine the appropriate duration of early lactation IMF treatment by increasing milking frequency of early lactation cows for various lengths of time to refine the approach and promote the use of this management practice on Virginia dairy farms. Results revealed that 40 d of IMF would maximize the efficient use of this practice in comparison to 10 or 20 days, demonstrated through an increase in milk, fat, and protein yields. Although the 40 d group had the best response to treatment, the 10 and 20 d groups did not respond to treatment as expected, and therefore, a similar study would need to be repeated to confirm the 40 d treatment's superiority to the other two durations.

The response in milk yield to early lactation IMF could be mediated by an increase in mammary epithelial cell number, increased mammary epithelial cell activity, or both. Along with milk components, concentration of various fatty acids (FA) were determined as an approach to examine a possible mechanism involving cellular activity to cause the increase in milk fat yield following IMF treatment. Overall, there was no effect of IMF on levels of denovo, C16 or preformed FAs in milk samples across the three treatment groups. Just as was seen with milk, fat and protein yields, the 40 d treatment group had significantly more denovo, C16 and preformed FAs in the 4X udder half than in the 2X udder half. A significant increase in only one of the groups of FAs would have indicated a change in a specific component of the fat synthesis pathway, but an increase in all three groups of FA denotes an increase in the overall milk fat synthesis program. Further research is needed to investigate the changes mammary epithelial cell activity.

In addition to investigating concentration of various FA, a second experiment was used to explore a possible change in proliferation related to the increase in milk yield observed following IMF. Tissue samples from a previous experiment were analyzed via immunohistochemistry to determine YAP expression following IMF treatment in early lactation. Changes in intensity of YAP staining were observed between 2X and 4X treatments, with 4X udder halves expressing 50% more YAP than the 2X udder halves. This pattern was also observed when looking at the number of YAP positive nuclei in these tissue samples; the 4X tissue samples at d 60 had more positively stained nuclei than the 2X udder halves at that day. The increase in intensity of YAP staining could indicate an increase in the number of cells in the mammary gland expressing YAP for udder halves milked 4X compared to 2X while an increase in the number of YAP positive nuclei could signify that cell proliferation and tissue growth are occurring in the mammy gland following IMF treatment. These results reveal a potential role for the Hippo pathway and YAP in regulating the increase in milk yield that occurs after IMF treatment is applied. Additional studies would be needed to determine if these results are repeatable, and possibly investigate changes in YAP expression and activity at other time points during lactation.

This experiment demonstrated that duration of IMF during early lactation does have an effect on milk yield. Although activity is up in udder halves milked 4X, indicated by an increase in milk yield, milk composition was largely unaffected with all components of milk fat synthesis being elevated, but not individual parts. We also demonstrated some effects of YAP following IMF, demonstrated by an increase in intensity of staining and number of YAP-positive nuclei, which could imply cell number or cell proliferation effects.

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