Effects of transition cow management strategies on regulation of metabolism, immune signaling, and mammary function

by

Caroline M. Ylioja

B.S., Michigan State University, 2013

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

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Abstract

The weeks around parturition and the adaptation to lactation continue to present a challenge for the dairy cow and for the producers responsible for dairy cow management. Physiological adaptations to lactation can easily become dysregulated by factors such as insufficient nutrient availability to meet energy demands, oxidative stress, reduced immune competence, and excessive systemic inflammation, which can have lasting negative effects on the subsequent lactation. Recent literature shows that treatment strategies to reduce inflammation in early lactation have promoted sustained increases in milk production. Evaluation of transcriptional differences in mammary tissue due to anti-inflammatory treatment revealed no obvious differences to explain long-term effects, but differences in methylation of mammary tissue DNA warrant further investigation into potential epigenetic effects of inflammation mitigation.

Management strategies to prevent excessive adiposity in transition cows are also often employed in commercial herds, as cows with excess body condition and lipid mobilization often have altered immune function and poor metabolic health in early lactation. MicroRNA, a type of non-coding RNA, facilitate communication and signaling between immune cells. We evaluated associations between maternal adiposity and microRNA in colostrum. Although no large-scale differences were detected in the colostrum profile of microRNA, further investigation of microRNA found in the mammary gland may help to determine the role of microRNA in the regulation of metabolism and immune signaling in the mammary gland, and to identify strategies to optimize cow health and production at the post-transcriptional level.

Mitigation of the disparity between nutrients required by the dairy cow at the onset of lactation and the nutrients consumed through feed can also improve metabolic and immune function and promote the successful adaptation to lactation. We evaluated the effects of altered nutrient demand by reducing frequency of milking in early lactation. Although we did not observe large differences in glucose metabolism, reduced milking frequency did alter circulating glucose concentrations as well as some inflammatory markers in tissue and in circulation. Further development of strategies to optimize the resources available to the cow will help facilitate a successful navigation through this transition period and optimize both cow health and milk production.

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Chapter 1 - Review of the literature: Inflammatory signaling in the mammary gland

Physiological challenges that face the dairy cow during the transition period often include immune suppression, subclinical hypocalcemia, systemic inflammation, and negative energy balance caused by a combination of reduced feed intake and rapidly increasing nutrient demand by the mammary gland (Trevisi and Minuti, 2018). Often overlooked is the mammary gland itself, which undergoes a change in state from quiescence to a state of secretion of large volumes of milk and components. These components include not only fatty acids and proteins, but also components with roles in immune function, such as acute phase proteins, oxylipids, cytokines, and immune cells (Alnakip et al., 2014).

It is hypothesized that the mammary gland existed first as a gland with an immunological role before development of its nutritional function (Vorbach et al., 2006). Investigation of immune signaling in the mammary gland may shed light on mechanisms employed by the mammary gland itself during development, during other phases of mammary tissue remodeling (i.e., involution), and during the adaptation to the lactating state.

Conventionally, immune signaling in the mammary gland is described in the context of response to disease or infection. This review will first briefly cover what is known about inflammation in the mammary gland during mastitis and characteristics of this immune response. This will lead to examination of inflammatory signaling components in the context of the healthy mammary gland, during organ development as well as around the remodeling required for the onset and

cessation of lactation. Lastly, recent literature describing non-coding RNA in the mammary gland and its involvement in immune signaling will be summarized.

Inflammatory signaling during mastitis

Overview of mastitis

The immune response of the mammary gland to pathogen challenge has been well described (Sordillo, 2018; Butler et al., 2015; Alnakip et al., 2014; Thompson-Crispi et al., 2014). As is typical of pathogen challenges in other tissues, mastitis begins with recognition of the pathogen or pathogen components, followed by initial response by the innate immune system and adaptive immune cell activation and recruitment. Pathogen recognition is accomplished through pathogen recognition receptors (PRRs) that are expressed not only by immune cells but also epithelial, endothelial, and fibroblast cells in the mammary gland (Sordillo, 2018). Receptor binding activates intracellular signaling that results in the release of inflammatory mediators, including cytokines and oxylipids (Oviedo-Boyso et al., 2007). These mediators recruit neutrophils to the infected tissue, which is facilitated by the expression of adhesion molecules to signal the entry point for migrating neutrophils. Neutrophils then phagocytose the invading bacteria, and cells of the adaptive immune response, including T cells, B cells, and NK cells, are recruited to eliminate any surviving pathogens.

Immune cells present in bovine mammary glands during infection

A variety of immune cells are present in the mature mammary gland that aid in activation of the inflammatory response to pathogenic challenge. Soluble factors associated with innate immune defense, such as Complement, cytokines, lactoferrin, lysozyme, reactive oxygen species, and

acute phase proteins, are present even prior to bacterial invasion. Innate immune cells include

macrophages, neutrophils, and NK cells; they are present at the early stages of infection (Sordillo

et al., 2002). Adaptive immune cells are activated by antigen-presenting cells, and are recruited

to the mammary gland if the innate immune cells are not able to neutralize the pathogen threat.

Adaptive immune cells during mastitis include CD4+ T cells, which can instigate either a T

helper type 1 (Th1) or Th2 immune response, depending on the type of immune challenge and

the cytokines that are then secreted. Cytokines associated with the Th1 response include IL-2

(interleukin 2) and IFNγ (interferon-gamma), while the Th2 response is characterized by IL-4,

IL-5, and IL-10 (Sordillo et al., 2002). Ruminants also produce γδ T cells, which are elevated in

milk and in blood during mastitis, though their specific role is unknown (Bröker et al., 2016).

Although the obvious etiology of mastitis involves signaling and recruitment of immune cells to

eliminate invading pathogens, inflammatory signaling and the ensuing immune response is a

concerted effort involving many cell types in the mammary gland, including those not

conventionally thought of as part of the immune system, such as mammary epithelial cells

(MEC). Luminal epithelial cells serve as one of the first cell types exposed to bacteria in the

lumen of the mammary gland, and feature a host of defense mechanisms, including phagocytic

ability (Günther and Seyfert, 2018). Stromal cell types have also demonstrated inflammatory

activity. Mammary stromal fibroblasts treated with conditioned culture media from MEC that

were exposed to either lipopolysaccharide or lipotechoic acid (components of *Escherichia coli*

and *Staphylococcus aureus*, respectively) showed increased expression of inflammatory genes,

reduced proliferation, and increased migratory activity (Zhang et al., 2016). Reciprocally, an *in

vitro* co-culture experiment using stromal fibroblasts harvested from the mammary glands of

cows infected with *E. coli* demonstrated that signaling from fibroblasts promoted TNFα (tumor necrosis factor-α) and IL-8 production by co-cultured healthy MEC (Chen et al., 2016). Additionally, MEC proliferation was reduced along with production of β-casein, suggesting that immunomodulatory signals from fibroblasts may play a role in the inhibitory effects on mammary cell function that often follow intramammary infections (Chen et al., 2016).

Interactions with other cells in the mammary environment also impact the immune response. Transcriptional responses of primary bovine MEC stimulated with heat-inactivated *E. coli* were compared to the *in vivo* response of the mammary gland infected with *E. coli* (Günther et al., 2009). Inflammatory response genes were upregulated in both, but to a greater extent in mammary tissue than in MEC alone. For example, gene transcripts for the anti-inflammatory cytokine IL-10 were upregulated in mammary tissue, but not detected in primary MEC. Common to both were chemokines involved in leukocyte recruitment. Expression of Complement factors were increased in MEC but downregulated in mammary tissue (Günther et al., 2009). Although some variation in response may be due to heat inactivation of bacteria used for treating primary cells, this highlights the point that the immune response within the mammary gland is shaped by crosstalk between multiple cell types. *In vitro* studies that report activity of a single cell type provide only a limited view of cellular activity and may not be physiologically accurate.

Soluble factors: acute phase proteins, cytokines

Acute phase proteins are those which are produced by the liver in response to inflammatory stimuli. Those elevated during mastitis include serum amyloid A and haptoglobin, in both serum and milk (Eckersall et al., 2001). Cytokines are proteins released by cells and are involved in

cell signaling, including recruitment and activation of immune cells. A variety of cytokines are elevated in the mammary gland. Treatment with lipopolysaccharide (LPS) from *E. coli* resulted in elevations in gene transcripts for IL-8, TNFα, and IL-1β in the bovine mammary gland (Vernay et al., 2012), which are likely involved in the chemoattraction of innate immune cells. In the murine mammary gland, *E. coli* infection induced elevation of IL-22, IL-6, TNFα, and IL-10 (Porcherie et al., 2016). Following neutrophil invasion, regulatory T cells producing IL-17 decreased bacterial numbers and strengthened the neutrophil response to bacteria, demonstrating that IL-17 is likely an important cytokine for combatting *E. coli* infections, at least in rodents (Porcherie et al., 2016). Comparisons between *E. coli* and *S. aureus* infections reveal similar induction of IL-6 expression, but the timing of the response is delayed with *S. aureus* challenge (Günther et al., 2011); additionally, *S. aureus* failed to induce IL-1 and TNFα production. These studies demonstrate a typical cytokine profile in response to mastitis is difficult to describe, as responses vary depending on the type of invading pathogen, the host species, and likely other factors as well.

Effect of mastitis on the mammary gland

Following resolution of inflammation, the mammary gland ideally returns to a non-infected state. This transition, beginning with the resolution of inflammation, features a rise in anti-inflammatory molecules such as IL-10, which decrease inflammatory cell numbers and activity and downregulate the production of inflammatory mediators (Alnakip et al., 2014). Damaged cells are removed and tissue is repaired through transforming growth factor (TGF)-α and TGFβ signaling. Timely reduction in local pro-inflammatory mediators is necessary to prevent uncontrolled tissue damage. In lactating cows, mammary function and milk production are both

negatively affected by mammary infection due to mechanisms related to impaired nutrient transfer into milk (Kobayashi et al., 2013), increased MEC apoptosis, and increased membrane permeability (Akers and Nickerson, 2011). Even after infection is resolved, structural effects on mammary tissue may persist through the remainder of that lactation, resulting in long-term reductions in milk production (Akers and Nickerson, 2011). Infection during the non-lactating state also has negative impacts on mammary structure. *E. coli* infection introduced during heifer mammary growth resulted in decreased epithelial growth compared with non-infected glands, showing altered tissue development due to direct pathogen exposure, the host immune response, or both (Enger et al., 2018).

Inflammatory signaling during non-disease states

In contrast to the inflammatory response of the mammary gland in response to exogenous pathogenic influence, immune signaling pathways activated during other times of mammary remodeling are under the influence of hormones of pregnancy. Reed and Schwertfeger (2010) suggested the initiating signals for immune cell infiltration during the tissue wound healing response are connected to platelet aggregation, while in contrast, immune cells involved in mammary development may instead be responsive to hormonal signals. This likely helps to distinguish between immune signaling cascades intended to mount an inflammatory response to pathogen challenge from the immune signaling that is tied to mammary remodeling and alterations in tissue structure and function.

Tissue development: puberty, gestation, onset of lactation

Immune cells present during mammary development

Contrary to mastitis, where the ultimate effect on the mammary gland often results in a reduction in milk-producing ability, the effect of tissue remodeling during puberty or gestation is a maturation of tissue or a transition from quiescent state to active secretion. Studies evaluating mammary development have often focused on the tissue components directly involved in milk secretion, but the stromal cells, including adipocytes, fibroblasts, immune cells, and vascular endothelial cells, also heavily influence mammary development and function (Akers, 2017).

Immune cells present during mammary development vary by stage of development and by location within the gland. During puberty, terminal end buds (for rodents) infiltrate the mammary fat pad to form the branching alveolar structure of the mammary ducts (Paine and Lewis, 2017). Immune cells localized to the terminal end bud region include mast cells, macrophages and eosinophils. Macrophages are likely involved in collagen remodeling during ductal growth, clearance of apoptotic bodies within ducts, and production of epidermal growth factor (EGF), which is needed for ductal elongation (Paine and Lewis, 2017). The presence of mast cells and eosinophils are needed for ductal growth and branching, respectively (Paine and Lewis, 2017; Reed and Schwertfeger, 2010), and the inhibition of mast cell degranulation in developing mouse mammary tissue inhibited terminal end bud formation (Reed and Schwertfeger, 2010). An overabundance of mammary eosinophils induced by IL-5 overexpression also resulted in impaired ductal development (Sferruzzi-Perri et al., 2003). In contrast to other species, forward growth of the ductal system in ruminants does not only occur at the terminal end bud region as in rodents and other species, but rather occurs along the developing ducts (Capuco and Ellis, 2013). However, the presence of macrophages, eosinophils,

and mast cells during early mammary development appears to be similar for the bovine as it is in other species (Beaudry et al., 2016).

At least in rodents, eosinophils and macrophages are also important for mammary development and function during pregnancy and lactation. Similar to during puberty, macrophage absence due to colony-stimulating-factor knockout resulted in impaired lactation (Pollard and Hennighausen, 1994), likely at least partially due to abnormal murine mammary ductal development. Additionally, both B and T lymphocytes are present in the healthy murine lactating mammary gland (Reed and Schwertfeger, 2010). Adipocytes, though not conventionally considered immune cells, produce adipokines such as leptin, adiponectin, and chemerin during mammogenesis (Dzięgelewska and Gajewska, 2019), and are thought to play a role in mammary development in humans.

In addition to immune cells, the rest of the stromal tissue environment plays an important role in supporting development of the mammary parenchymal tissue. Sheffield briefly described studies that transplanted mammary tissue of male or fetal origin into mammary fat pads of female mice and observed normal mammary growth and differentiation, (Sheffield, 1988). Additionally, testicular cells or embryonic stem cells transplanted into mammary extracellular matrix were able to acquire the branching morphology exhibited by mammary epithelium (Bruno et al., 2017).

Cytokine/chemokine signaling during mammary development

Cytokines and other proteins act as chemokines during mammary development to induce migration of immune cells. For example, CCL28 (C-C motif chemokine ligand 28) in the lactating mammary gland promotes B cell recruitment and the secretion of immunoglobulin A into milk (Reed and Schwertfeger, 2010). During puberty, MEC produce colony stimulating factor 1 (CSF1) and eotaxin to recruit macrophages and eosinophils, respectively, to the terminal end bud region during mammary ductal development (Paine and Lewis, 2017). Mice deficient in CSF1 exhibited impaired ductal growth, tied to the absence of mammary macrophages (Gouon-Evans et al., 2000). IL-5 also promotes eosinophil recruitment and activation; knockout of either IL-5 or eotaxin in mice reduced eosinophil presence and ductal development during puberty, and increased pup mortality during lactation (Reed and Schwertfeger, 2010; Gouon-Evans et al., 2000). Eosinophils also recruit macrophages through secretion of CCL6 (Gouon-Evans et al., 2002). TGFβ in the mammary gland acts as an inhibitor of ductal proliferation but promotes stromal proliferation and development of the extracellular matrix (Paine and Lewis, 2017). The reduction in ductal development observed with an overabundance of eosinophils in the terminal end bud region may be tied to production of TGFβ by eosinophils (Reed and Schwertfeger, 2010).

Cytokine presence in the mammary gland differs by developmental stage. In an immortalized murine MEC cell line, undifferentiated cells produced Th1 cytokines such as IL-1β, TNFα, IL-12a, and IFNγ, while exposure to prolactin and dexamethasone to promote differentiation induced the production of Th2-associated cytokines, including IL-4, IL-13, and IL-5 (Khaled et al., 2007). Knockout mouse studies showed that the absence of IL-4 and IL-13, as well as their downstream target STAT6, impaired MEC differentiation and alveolar development during

pregnancy (Reed and Schwertfeger, 2010; Khaled et al., 2007), but whether they influence immune cells or strictly developing MEC is unknown. Observations of human mammary tissue revealed the expression of Complement components C3, C4 and factor B in tissue during gestation but not lactation or normal resting tissue (Laufer et al., 1999). These patterns were similar to those observed in inflamed tissue, but the cause of inflammation was not well-characterized.

Immune cells present during involution

Mammary involution follows the cessation of milk removal and is marked by the switch from actively secreting tissue back to a quiescent state. This involves extensive remodeling and homeorhetic adjustments to mammary morphology and function. The onset of involution (up to 48h of milking cessation) is marked by the brief presence of neutrophils and is reversible. As the cessation of lactation persists, there is a temporary elevation in the proportion of macrophages present, as well a slower but more persistent increase in T cell numbers, at least in mice (Hojilla et al., 2011). A rise in plasma cells capable of producing immunoglobulins has also been observed in the mammary tissue within days after involution begins (Stein et al., 2004).

Mast cells are also present in the involuting mammary gland. In addition to their ability to release inflammatory mediators, they bind to and assist in the localization of plasma kallikrein to the mammary gland. Kallikrein is a protease which facilitates the conversion of plasminogen to plasmin, and lack of either kallikrein or plasminogen has resulted in impaired progression of involution (Reed and Schwertfeger, 2010).

Macrophages present during involution likely participate in phagocytosis of apoptotic cells, and have a similar phenotype to the M2 macrophages present during the tissue repair phase of activated inflammation (Reed and Schwertfeger, 2010). Studies have shown that phagocytosis of apoptotic cells and milk fat globules is also carried out by neighboring MEC (Monks et al., 2008; Hughes and Watson, 2018).

Studies with mice have found immunosuppressive CD4+ T cells and dendritic cells in the involuting murine mammary gland (Betts et al., 2018). Dendritic cells during lactation and involution both exhibited reduced antigen binding and uptake compared with cells present in the pre-lactating mammary gland, as well as reduced ability to activate T cells (Betts et al., 2018).

Cytokines, acute phase proteins, and other immune factors present during involution

Expression of cytokine genes during murine involution suggested that CXCL1 initially acts to recruit neutrophils, before macrophages invade and initiate cleanup of apoptotic MEC (Reed and Schwertfeger, 2010; Stein et al., 2004). The macrophage cell surface marker CD68 was detected in mouse mammary tissue beginning on d 2 of involution, but not during lactation, indicating an increase in macrophage infiltration during this time (Atabai et al., 2007; Hanayama and Nagata, 2005). It is probable that tissue resident macrophages also play a role in early recruitment of other immune cells through cytokine signaling, but may feature altered surface markers. For example, human mammary tissue-resident macrophages exhibit CD68 but murine mammary tissue-resident macrophages do not (Roszer, 2018). This may have resulted in failure to detect their presence in some studies.

Regarding specific cytokines, as during mammary development, TGFβ is present during involution and serves to promote apoptosis of MEC; tissue-specific mouse knockout models showed that loss of either TGFB3 or TGFBR2 genes delayed apoptosis and prolonged lactation, and overexpression of *Tgfb3* increased MEC apoptosis (Guo et al., 2017). *In vitro* studies suggest that phagocytosis by MEC may be activated by TGFβ signaling (Fornetti et al., 2016). Additionally, TGFβ promotes further secretion of TGFβ and IL-10, which act to inhibit pro-inflammatory signaling and promote tissue remodeling (Guo et al., 2017).

Other inflammatory mediators are also present during involution. Clustering of immune related genes expressed in the involuting murine mammary gland showed associations with inflammation, acute phase response, and humoral immunity (Clarkson et al., 2004). Acute phase mediators expressed included leukemia inhibitory factor [LIF], IL-11, CXCL1, and CAAT-enhancer binding protein [C/ebp]), some of which are regulated through STAT3. The deletion of either Stat3 or IKKB (a regulator of NFKB) genes resulted in delayed involution, demonstrating that signaling through these factors is necessary for involution to proceed (Watson et al., 2011).

Contrast of immune signaling in in healthy vs. infected mammary cells

Soluble factors found in the healthy mammary gland during involution (such as transferrin, Complement, LBP, CD14) overlap with those found during mastitis, and are likely involved with both mitigation of bacterial infection and the proinflammatory signaling required for involution (Clarkson et al., 2004). However, while the tissue repair stage may be common to both involution and pathogenic responses, immune signaling during involution differs markedly from the inflammatory signaling induced in response to pathogenic challenge. Evaluation of gene

expression changes occurring during involution of the mouse mammary gland suggested that despite the upregulation of acute phase proteins LIF and C/ebp, the upregulation of uterocalin, clusterin, and oncostatin suggest suppression of neutrophil numbers and potential tissue-damaging activity (Clarkson et al., 2004). The presence of TGFβ during involution, in addition to promoting apoptosis and phagocytosis, also suppresses inflammation (Guo et al., 2017), as does the presence of tolerogenic dendritic cells and regulatory T cells. This suggests that the inflammatory signaling occurring during tissue remodeling is unique from the response observed during pathogenic challenge.

MicroRNA in the mammary gland

A discussion of immune regulation in the mammary gland would not be complete without mentioning noncoding RNAs and their role in post-transcriptional regulation. Noncoding RNAs are derived from genomic regions that do not code for proteins, or from intergenic regions that are spliced out during pre-mRNA processing, and include microRNAs, piwi-associated RNAs, circular RNAs, and long noncoding RNAs, among others (Fu, 2014). Probably the most widely studied type of noncoding RNAs are microRNA (miRNA), which are short sequences of RNA averaging 22 nucleotides in length. Many recent reviews describe our related knowledge to date, including miRNA biogenesis, regulatory activity, trafficking, and mechanisms of action (Krol et al., 2010; Ha and Kim, 2014; O'Brien et al., 2018), and the number of papers mentioning miRNA is steadily increasing even in livestock journals, such as Journal of Dairy Science, as their potential involvement in production animal performance is realized.

The canonical process of microRNA synthesis begins in the nucleus with pri-miRNA, which folds into a hairpin structure for processing by RNase enzymes. One of these, Drosha, cleaves the hairpin structure to allow for transport to the cytoplasm; whereas the second, Dicer, cleaves the loop of the hairpin to leave an RNA duplex (Krol et al., 2010). One strand of the duplex is then assembled into a miRNA-induced silencing complex (RISC), which contains the RNA strand bound to an Argonaute protein. In this form, the miRNA can recognize mRNA targets with full or partial complementary sequences and prevent translation. In some cases, depending on other proteins associated with the RISC complex, miRNA can also upregulate gene expression through interactions with ribosomal proteins or with promotor regions of the DNA (O'Brien et al., 2018).

In addition to regulation within the cell, miRNA can be exported from the cell to circulate in extracellular fluids. MiRNA in circulation may be enclosed in extracellular vesicles, associated with HDL particles (Allen et al., 2018), or associated with proteins such as Argonaute; in these forms, the RNA is less susceptible to degradation by patrolling RNase enzymes. MiRNA can act in an endocrine manner on target cells through receptor-mediated signaling or through uptake by target cells.

miRNA associated with mammary infection

MicroRNAs may play a regulatory role in immune function during infection or disease. In dairy cows, infection of the mammary gland has resulted in differential expression of miRNA that may be involved with the innate or adaptive response to pathogen presence. *In vitro*, primary bovine mammary epithelial cells challenged with *S. uberis* showed altered expression of 22 miRNA within 6 h of treatment, and analysis of the predicted targets of down-regulated miRNA

suggested likely involvement in innate immune function (Lawless et al., 2013). These data highlight potential roles of miRNA in immune responses to mastitis, as well as the contribution of MEC to innate immunity. The same group performed a similar experiment *in vivo* and looked at miRNA profiles in monocytes isolated from both the blood and milk of cows infected with *S. uberis*. They found a greater number of DE miRNA in monocytes from milk, demonstrating tissue-specific miRNA production. In addition, the DE miRNA in these monocytes were enriched for innate immune function (Lawless et al., 2014).

The miRNA response to infection is also pathogen-specific. *In vitro*, miRNA expression induced by LPS was different from that induced by *S. uberis* (Lawless et al., 2013). Bovine monocyte-derived macrophages challenged with 2 different strains of *S. agalactiae* both induced inflammatory cytokine production promoting macrophage activation (Lewandowska-Sabat et al., 2018). The miRNA profile of treated macrophages differed by bacterial strain, however, with predicted differences involving macrophage activation, apoptosis, cell migration and inflammatory signaling. These differences may shed light on mechanisms behind variation in virulence between even closely related strains of bacteria. In immortalized mammary epithelial cells, responses to infection with *E. coli* differed from responses to *S. aureus* in both the timing of changes in miRNA and in the specific miRNA differentially expressed (Jin et al., 2014), which were related to cell growth, metabolism, and immune function. It is apparent that the differing inflammatory responses by the immune system to Gram negative and Gram positive cells is also reflected in the specific miRNA expressed during times of pathogen challenge.

Studies described thus far examined miRNA isolated from either immune cells or epithelial cells. Also of interest are the extracellular miRNA, or those found in milk, because of their ability to traffic between cells. Examination of miRNA isolated from milk exosomes of cows infected with *S. aureus* found 14 DE miRNA for which predicted targets were enriched for immune processes (Sun et al., 2015). Additionally, miRNA in peripheral blood in response to mammary *E. coli* infection were potentially involved in multiple pathways including metabolism, cellular processes, and immunity (such as T-cell signaling, cell migration, and cytokine signaling) (Luoreng et al., 2018), which is supportive of a role for miRNA trafficking in recruitment of immune cells to target organs in times of infection.

miRNA in the healthy mammary gland

The presence of miRNA in the mammary gland has also been of great interest in the context of lactation in general. Different sources of miRNA within the mammary gland have been evaluated for their similarity to mammary tissue, to provide alternative collection methods to conducting invasive mammary biopsies. Li et al. (2016) examined milk fat, whey, and milk cells isolated from milk and found that the miRNA profile from milk fat was most closely related to that of mammary tissue. The difference between milk fractions may be reflective of the origin of the miRNA or their form in circulation (i.e. enclosed in exosomes versus protein-bound).

Profiles of mammary miRNA associated with stage of lactation have been evaluated in multiple species, with results relatively consistent across species. In sows, evaluation of miRNA profiles of milk exosomes revealed that the most abundant miRNA were consistently expressed over the course of lactation, and have been linked to immune responses in the literature (Gu et al., 2012).

In the wallaby, the top miRNA expressed in milk varied somewhat from early to late lactation, likely related to the nutritional or development needs of the neonate over the roughly 250-day lactation. The upregulation of immune-related miRNA in colostrum compared to mature milk has been described in multiple species, including the cow (Samuel et al., 2017; Izumi et al., 2012; Sun et al., 2013; Chen et al., 2010), panda (Ma et al., 2017), and pig (Gu et al., 2012). As a side note, almost all of these studies pooled samples within a lactation day before analysis, and therefore are lacking appropriate replication, but results across studies are nevertheless fairly consistent. Alsaweed et al. (2015) summarized in tabular form the milk miRNA that are involved in immune cell activation.

Plasma miRNA profiles have also been compared with that of milk. It is likely that at least some miRNA traffic from peripheral circulation to mammary glands, but clear differences suggest that a large proportion of those found in milk are of local origin (Modepalli et al., 2014; Alsaweed et al., 2016; Chen et al., 2010). Maternal and neonatal factors may also influence the miRNA profile found in milk; miRNA related to adipose metabolism were found to be decreased in the milk of mothers with greater body mass index, and the same miRNA were also greater in the milk of those mothers with female babies (Xi et al., 2016).

The roles of miRNA in regulating gene function, combined with the abundance of miRNA in milk, raises questions regarding effects on the neonate consuming colostrum. Piglets consuming colostrum and those consuming mature milk were compared in terms of the miRNA circulating in their plasma (Gu et al., 2012), and the abundance of almost all analyzed miRNA in piglet plasma were significantly elevated for those consuming colostrum. This does not necessarily

show absorption of miRNA, but rather the effects of colostrum consumption on circulating miRNA of the piglets.

Considering the wide consumption of dairy products by humans, the miRNA profile in milk must also be considered in the context of potential impacts on human health. A similar study to that evaluating plasma miRNA in piglets examined whether milk consumption by humans would alter their plasma miRNA profiles (Baier et al., 2014). Two miRNA that are abundant in milk were elevated in human plasma following consumption, in a dose-dependent and time-dependent manner. Additionally, these miRNA were elevated in circulating PBMC (peripheral blood mononuclear cells), and at least one target gene was differentially expressed. Whether the miRNA were absorbed from the gastrointestinal tract or whether endogenous production was stimulated by milk consumption, it is clear that consumption of bovine milk can alter miRNA circulating in human plasma.

The immune modulatory effects of milk exosomes has been studied in mouse models for arthritis, showing that oral consumption of these exosomes delayed the onset of disease and attenuated arthritic symptoms (Arntz et al., 2015). Although the effects of bovine milk exosomes were not distinguished from effects of their contents, further research may better characterize effects of specific milk-derived miRNA on bone health.

The involvement of miRNA in mammary gland function was reviewed by Gigli and Maizon (2013). *In vitro* studies with mammary epithelial cells have highlighted miRNA involved in cell proliferation (Jiao et al., 2019) or milk component synthesis (Wang et al., 2017; Tang et al.,

2017; Lian et al., 2016). Bovine MEC treated with lactogenic hormones showed differential expression of several miRNA related to angiogenesis and transcript processing (Muroya et al., 2016), indicating a potential role in mammary tissue development. Murine MEC during gestation compared with early lactation showed clear groupings of miRNA associated with each phase of mammary development/function (Heinz et al., 2016), and miRNA in luminal epithelial cells of sheep were also differentially expressed between early and late gestation (Galio et al., 2013). These provide at least associative evidence for differential expression of miRNA during specific phases of mammary development and function.

Target prediction programs can be utilized to predict which genes might be affected by specific miRNA based on RNA sequence, but a single miRNA might give hundreds of potential target genes. Pathway or function enrichment is often used to estimate functional changes, but without conducting experiments using miRNA-specific silencing or treatment with miRNA mimics, it is difficult to confirm effects on specific targets. A mammary epithelial cell line containing a permanent population of self-renewing progenitor cells was studied for its miRNA profile and their potential role in self-renewal (Ibarra et al., 2007). These authors showed that forced expression of let-7 miRNA eliminated this self-renewal ability.

Regulation by miRNA on a global scale has also been tied to immune function through the enzymes involved in miRNA processing. Knockdown of Dicer1, resulting in a global reduction in all miRNA expressed, has been tied to a shift in macrophage phenotype (Lewandowska-Sabat et al., 2018). In gastrointestinal tissue, Dicer knockdown also altered the microbiome and promoted colitis (Liu et al., 2016). It is likely that regulatory activities of miRNA in the

mammary gland as well are vital for tissue function and homeostasis as well as immune response to pathogens.

Applications

Elucidation of the role for immune signaling in normal mammary function leads to practical applications for dairy production and cow management. Blockade or reduction of inflammation through bioactive feed components or pharmaceuticals during times of mammary remodeling may inhibit crucial processes in tissue function. Additionally, immune cells and their activity are often assigned to either the innate or adaptive immune response. The extensive involvement of immune cells and associated signaling pathways in a non-pathogenic tissue remodeling environment suggests that these labels are somewhat limiting and should be updated or expanded to reflect the role of immune cells in non-pathogenic functions.

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Chapter 2 - Effects of anti-inflammatory treatment in early lactation on mammary tissue proliferation, gene transcript profile, and DNA methylation

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ABSTRACT

Previous studies have demonstrated non-steroidal anti-inflammatory drug (NSAID) treatment in early lactation had a positive impact on whole-lactation milk production in older cows. The objective of this study was to evaluate transcriptional and epigenetic changes in the mammary gland that could explain increased production responses due to NSAID treatment. Sodium salicylate (SAL; 125 g/d) or water (CON) were administered via oral drench to multiparous cows ($n = 8$/treatment) once daily for 3 d beginning approximately 24 h after parturition, and mammary tissue was collected on d 1, 4, and 45 postpartum. Day 1 tissue was collected immediately preceding the initial drench, and d 4 tissue was collected 24 h following the final drench. Blood was collected twice weekly and analyzed for plasma glucose, insulin, beta-hydroxybutyrate, free fatty acids, and prolactin. Cows were milked twice daily until d 7 of lactation, and thrice daily for the remainder of the study. Total RNA extracted from tissue was deep-sequenced and analyzed for differential gene expression using DESeq2. We detected no treatment effect on milk yield or plasma metabolites through 45 d of lactation. Comparison of SAL vs. CON revealed that only 16 of 18,286 genes were differentially expressed (false discovery rate < 0.1) in mammary tissue collected on d 45, while no differentially expressed

genes due to treatment were detected on d 1 or d 4. Analysis of transcriptional differences over time showed downregulation of pathways related to immune cell recruitment and differentiation, and extensive overlap with pathways related to cholesterol synthesis and liver X receptor signaling. Global DNA methylation of mammary tissue was decreased on d 4 for CON compared with SAL. Transcriptome analysis emphasized extensive involvement of immune-related signaling pathways in the switch from lactogenesis to galactopoiesis, and changes in methylation with SAL treatment merit future investigation into epigenetic effects on milk production.

INTRODUCTION

Inflammatory markers such as acute phase proteins are elevated in even healthy cows during the transition period (Qu et al., 2014; Akbar et al., 2015). Excessive systemic inflammation in early lactation can have short-term implications for risk of metabolic disease as well as long-term implications on milk production. Anti-inflammatory treatment in early lactation has shown positive short-term effects on inflammatory markers (Trevisi and Bertoni, 2008) as well as long-term effects on milk yield, especially in older cows (Carpenter et al., 2016; Farney et al., 2013b). Decreased somatic cell count in early lactation has also been observed (Carpenter et al., 2016; Shock et al., 2018). Effects of inflammatory signaling in the mammary gland can suppress the transcription of milk protein genes (Beaton et al., 2003), reduce cell proliferation, and alter chromatin accessibility near milk protein promotor regions (Vanselow et al., 2006). The objective of this study was to examine the mechanistic effects of anti-inflammatory treatment on transcriptional and epigenetic changes of the mammary gland in early lactation, with the hypothesis that anti-inflammatory treatment would increase proliferation or transcriptional changes related to milk component synthesis.

METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University.

Treatments and experimental design

Sixteen Holstein cows in third or greater lactation were enrolled between January and September 2014 and alternately assigned to treatment groups at the time of parturition. Beginning 12-36 h after parturition, cows were treated once daily at 0700 h for 3 d with an oral drench containing either 375 mL of water (CON; $n = 8$) or 125 g of sodium salicylate dissolved in water (SAL, Wintersun Chemical, Ontario CA; final volume 375 mL; $n = 8$).

Animal management

Cows were housed in tie stalls and offered feed at 110% of *ad libitum* intake and had ad libitum access to water. Total mixed ration was mixed once daily and cows were fed twice per day at 0630 and 1730 h. Primary dietary ingredients were corn silage, corn gluten feed, alfalfa hay, and corn grain, and total mixed ration contained 18% CP, 33% NDF, and 1.67 Mcal NE_L/kg DM (Carpenter et al., 2018). Individual feed intake was recorded daily by subtracting feed refused from feed offered, and daily water intake was recorded via water meters located on each cow's water supply line. Cows were milked twice per day (0700 and 1500 h) until d 7, and then 3 times per day (0700, 1500, and 2300 h) for the duration of the study.

Sample collection

Daily milk weights were recorded, and milk samples representative of a full milking were collected twice per week. Milk samples were analyzed for fat, protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments Inc., Chaska, MN), milk urea nitrogen (MUN spectrophotometer; Bentley Instruments Inc.), and somatic cell count (SCC; SCC 500, Bentley Instruments Inc.) at Heart of America DHIA (Manhattan, KS). SCC was converted to a linear score (SCLS = $\log_2(SCC/100)+3$; Shook, 1993). Energy corrected milk yield was calculated as ECM = [(0.327 × milk yield) + (12.95 × fat yield) + (7.65 × protein yield)], and 3.5% fat corrected milk yield was calculated as FCM = [(0.432 × milk yield) + (16.216 × fat yield)].

Blood samples were collected from coccygeal vessels on d 1 (prior to first treatment), 4, 7, 11, 15, 18, 21, 25, 28, 31, 35, 39, 42, and 45. Samples were collected before morning feeding into Vacutainer tubes containing potassium oxalate with sodium fluoride or potassium EDTA (Becton, Dickinson and Co., Franklin Lakes, NJ). Blood was centrifuged at 2800 × g for 15 min and isolated plasma was frozen at -20°C until analysis. Using a multi-spectral plate reader (PowerWave XS spectrophotometer, Biotek Instruments, Inc., Winooski, VT), plasma was analyzed for glucose (catalog no. 439-90901; Wako Chemicals USA Inc., Richmond, VA), insulin (catalog no. 10-1201-01, Mercodia AB, Uppsala, Sweden), β-hydroxybutyrate (BHBA; catalog no. H7587, Pointe Scientific, Inc., Canton, MI), and free fatty acids (FFA; HR Series kit, Wako Chemicals USA Inc., Richmond, VA). Each assay was carried out in duplicate and averaged for analysis. Samples from d 1, 4, and weeks 2 (d 7 and 11 pooled), 4 (d 21 and 25), and 6 (d 35 and 39) were analyzed in duplicate for prolactin (Bovine Prolactin ELISA; catalog no. MBS2022462, MyBioSource.com, San Diego, CA. Detection range 31.2 - 2000 pg/mL; spike recovery 80-92%; intra-assay CV < 10%, inter-assay CV < 12%).

Mammary biopsies were performed on d 1, 4, and 45 after parturition. One hour prior to biopsy, the target gland was treated with 50 mg intramammary prophylactic antibiotic pirlimycin hydrochloride (Pirsue; Zoetis, Parsippany, NJ). Biopsies were conducted at 0900 h, approximately 2 h after morning milking. Cows were administered xylazine hydrochloride (Akorn, Inc., Decatur, IL; 0.025 mg/kg BW) intravenously via the coccygeal vessel for partial sedation. Surgical area was scrubbed alternately with Betadine surgical scrub (Purdue Products LP, Stamford, CT) and ethyl alcohol (70 % v/v) three times. Local anesthetic (10 to 20 mL of 2% lidocaine-HCl) was administered subcutaneously in a horizontal line just dorsal to the incision site; a 1-cm incision was made with a #22 scalpel blade (Integra York PA, Inc., York, PA) and mammary tissue was collected using a Bard Magnum biopsy instrument (catalog no. MG1522; Bard Peripheral Vascular, Inc., Tempe, AZ) with a 12 G × 10 cm tissue biopsy needle (catalog no. MN1210; Bard Peripheral Vascular, Inc., Tempe, AZ). One core of tissue (~100 mg) was placed in 10% formalin for 24 h and stored in 70% (v/v) ethyl alcohol at 4°C for later immunohistochemistry. Tissue for RNA extraction (~100 mg) was immersed in Trizol Reagent (Life Technologies Corporation, Carlsbad, CA) before storage at -80°C; tissue samples for global DNA methylation analysis (~100 mg) were snap frozen in liquid nitrogen before storage at -80°C. Biopsies on d 1 and 4 were collected from alternate rear mammary glands; d 45 biopsy was conducted on the same gland as d 1 but ~3 cm below the site of first incision.

Immunohistochemistry

Formalin-fixed biopsy cores were embedded in paraffin. For this, biopsy cores were removed from storage containers, placed into individually labeled fine-gauge plastic baskets, processed,

and embedded into paraffin blocks. Microscope slides were prepared by slicing 5-μm-thick sections from the paraffin-embedded tissue blocks with a microtome. Four to 5 serial tissue sections from each sample were mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Approximately 5 microscope slides were prepared from each tissue block. General immunohistochemistry protocol methods are described elsewhere (Daniels et al., 2009). Secondary antibodies for Ki67 (Alexa 532 Goat Anti-rabbit IgG [A1109]; 1:200 dilution; Invitrogen [Carlsbad, CA, USA]), counterstaining, and imaging and quantification methods are described by Tucker et al. (2016).

RNA isolation and sequencing

Total RNA was isolated from tissue using the RNeasy mini kit (Qiagen, Valencia, CA) and the concentration was determined using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA), whereas quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Samples with RIN less than 4 were excluded from further analysis, resulting in a total of 43 samples submitted for sequencing. Libraries were created using a TruSeq RNA Library Prep Kit v2 (Illumina, Hayward, CA) and sequenced on a HiSeq 2500 at University of Kansas Medical Center's Genome Sequencing Facility (Lawrence, KS). Average library size was 280.7 bp; samples were run as single read with 100 bp read length.

Global DNA methylation

DNA was extracted from snap-frozen mammary tissue using the Quick-DNA Miniprep Plus kit (Zymo Research Corp., Irvine, CA). Samples were first crushed with mortar and pestle in liquid nitrogen before incubation with Proteinase K and solid tissue buffer (supplied by kit) for 1 h at

55°C. RNase A (2 *u*g/*u*L; Zymo Research Corp., Irvine, CA) was added and incubated for 2 min, after which samples were centrifuged for 1 min at 10,000 × *g*, and the supernatant was used for the remainder of the kit protocol. DNA concentrations were determined with a Take3 microvolume plate (Biotek Instruments, Inc.). Global DNA methylation was determined via colorimetric assay (5-methylcytosine Enzyme-Linked Immunosorbent Assay Kit; Zymo Research Corp., Irvine, CA) using 100 ng of DNA from each sample in duplicate, and plotted against a standard curve created from *E.coli* methylase-treated gDNA, according to kit protocol. After horseradish peroxidase developer substrate was added, the plate was incubated in the dark for 10 min before reading at 450 nm wavelength on a PowerWave XS spectrophotometer (Biotek Instruments).

Data Analysis

Feed and water intake, plasma analytes, milk yield and components, and DNA methylation were analyzed with SAS (version 9.3, SAS Inst., Inc., Cary, NC) using repeated measures with treatment, time, and their interaction as fixed effects and cow as a random effect. BHBA and insulin were natural log-transformed prior to analysis. Contrasts between SAL and CON on d 4 as well as d 45 were used to assess treatment effects on DNA methylation. Immunohistochemistry data were analyzed with JMP (version 11, SAS Inst., Inc., Cary, NC), with fixed effects of treatment, day, and their interaction, and cow as a random effect. Significance was declared at $P \leq 0.05$; P-values > 0.05 and < 0.10 were considered tendencies.

Raw sequencing data were processed using CLC Genomics Workbench 8.0.2 (www.qiagenbioinformatics.com). Counts were analyzed in R (version 3.3.2) using the

Bioconductor package DESeq2 (Love et al., 2014). Genes with less than 3 total reads across all samples were filtered out, reducing the number of genes included in the analysis from 24,616 to 18,286. Principal component analysis (PCA) was used to visualize data and identify outliers; 4 samples were removed from analysis, leaving 39 samples to be included in analysis. Effects of treatment as well as treatment by time interaction were analyzed. Genes with FDR (P value adjusted for false discovery rate) < 0.10 were considered differentially expressed.

Fold change and adjusted P-values of DE genes were uploaded into Ingenuity Pathway Analysis (IPA) software (Qiagen, Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) for functional pathway analysis. Genes with fold changes > 1.5 and adjusted P-values < 0.05 were used for the following contrasts: d 1 vs. d 4; d 4 vs. d 45; d 1 vs. d 45. The contrast of CON d 45 vs. SAL d 45 only resulted in 16 DE genes, of which only 4 met cutoff conditions for inclusion in IPA, rendering pathway enrichment analysis infeasible. Evaluation of differences over time utilized both CON and SAL samples.

RESULTS

Milk production and feed intake

Results of production parameters are reported in Table 2.1. Weekly means of milk production parameters revealed no detectable differences for fat content or yield, milk urea nitrogen, somatic cell score, energy-corrected milk yield, or fat-corrected milk yield. Treatment interacted with week for average protein yield ($P = 0.02$), with SAL cows increasing more (1.07 to 1.49 ± 0.09 kg/d) over the 7 weeks of the study than CON (1.19 to 1.37 ± 0.09 kg/d). Lactose content tended to be greater for SAL (4.89%) compared with CON (4.74 ± 0.05%; $P = 0.06$). Milk yield

tended to increase more for SAL (from 26.6 to 55.8 ± 2.6 kg/d) than CON (from 30.7 to 52.3 ± 2.3 kg/d) over time (interaction $P = 0.10$).

No differences in water intake were detected that could be attributed to anti-inflammatory treatment. Effects of NSAID treatment on feed intake was not detected, but treatment interacted with week ($P=0.03$; Table 2.1, Figure 2.1), such that those treated with SAL exhibited a more rapid increase in intake following parturition, mirroring the pattern of milk production over time.

Plasma analytes

Results of plasma metabolites are presented in Table 2.2 and in Figure 2.2; no differences were detected between treatments for plasma glucose, insulin, BHBA, FFA, or prolactin. Circulating glucose and insulin both reached a nadir around day 11 after parturition (Figure 2.2 A and B), corresponding with the peak in circulating BHBA (Figure 2.2 D) and prolactin (Figure 2.2 E). Circulating FFA levels were greatest at the sampling point closest to parturition and decreased steadily over time until the end of the study (Figure 2.2 C).

Mammary epithelial cell (MEC) proliferation

Results of Ki67 labeling are shown in Figure 2.3. No treatment difference was detected at any of the 3 sampling time points, but the proportion of Ki67-positive cells was greatest on d 1 of lactation and decreased over time ($P < 0.001$).

Transcriptional analysis

A total of 24,616 RNA species were detected in mammary biopsy samples through high-throughput sequencing. After removing those with 3 or less reads across all samples, 18,286 remained for differential expression analysis. Analyzing for the main effect of sodium salicylate treatment identified no differentially expressed (DE) genes. Comparing day 1 to day 4, day 1 to day 45, and day 4 to day 45 samples identified 901, 6623, and 3770 DE genes, respectively. Samples clearly grouped within day when plotted by PCA (Figure 2.4). Additionally, day by treatment interactions were explored such that treatment effects were analyzed within day of lactation; on d 45, 16 genes were DE between SAL and CON, but no DE genes were detected on d 1 or d 4 of lactation. The few genes that were DE on d 45 due to anti-inflammatory treatment are found in Table 2.3.

Most abundant transcripts

The gene transcripts most abundantly detected are shown in heatmap form (Figure 2.5). Transcripts involved in milk protein synthesis (*CSN1S1, CSN2, CSN1S2, LGB, CSN3, LALBA*) collectively accounted for 62% of all transcripts detected. Averaging across all samples, casein alpha S1 was the most abundant mRNA transcript detected, accounting for 20% of all gene transcripts identified. After milk protein genes, functions of the other most abundant gene transcripts related to mitochondrial activity (*COX1, COX3, MT-CYB, ATP6, ND1, CO2, MT-ND4*), lipid synthesis and oxidation (*FASN, FABP3, GPAM*), translation and protein transport (*EEFA1_3*), purine degradation (*XDH*), and cell adhesion (*GLYCAM1*).

Transcriptional changes over time

Data loaded into IPA were analyzed for canonical pathways and upstream regulators that differed over time. Using a comparison analysis, which allows for viewing time-course type data, those outcomes that were affected similarly over multiple timepoint comparisons were identified. Most of the identified pathways were upregulated in early lactation relative to d 45, including dendritic cell maturation, TREM1 signaling, inflammasome pathway, role of NFAT in regulation of the immune response, and CDK5 Signaling. Those pathways that were downregulated in early lactation included IL-1 signaling, STAT3 pathway, PPAR signaling, and GnRH signaling. Pathways elevated especially on d 4 of lactation included role of PRRs in recognition of bacteria and viruses, IL-8 signaling, and acute phase response signaling. A more complete list can be found in Table 2.4.

Examination of individual contrasts between d 1 and d 45 revealed enrichment of molecules involved in cholesterol synthesis and LPS/IL-1 mediated inhibition of RXR function. Prediction of directional changes revealed that pathways of acute phase response signaling, EIF2 signaling, dendritic cell maturation, and HMGB1 signaling were upregulated on d 1, while LXR/RXR activation was downregulated. Contrasting d 4 with d 45, significantly enriched canonical pathways included immunodeficiency signaling, T cell differentiation, retinol biosynthesis, and TR/RXR activation. Complement system was upregulated, while growth hormone signaling was downregulated on d 4 relative to d 45. Between d 1 and d 4, top pathways included cholesterol biosynthesis, FXR/RXR activation, phospholipases, and hematopoiesis. Acute phase response signaling, STAT3 pathway, and cell cycle regulation were downregulated, and ATM signaling, eNOS signaling, and CDK5 signaling were upregulated on d 1 relative to d 4 of lactation. More complete lists can be found in Tables 2.5-2.7.

Global DNA methylation

SAL treatment tended to increase global DNA methylation in mammary tissue ($P = 0.06$; Figure 2.6). Analysis using contrasts on d 4 and d 45 showed that this was largely attributed to the decrease in methylation in the CON cows on d 4 of lactation, but not for SAL ($P = 0.04$). Methylation on d 45 followed a similar numeric trend but was no longer significantly different between treatments ($P = 0.21$).

DISCUSSION

Milk production

Biopsy timepoints were designed to assess the mammary transcriptome immediately prior to and following the SAL treatment period, as well as closer to peak lactation. We hypothesized that short-term transcriptional responses would occur during the time of treatment administration, as well as sustained or later-occurring transcriptional differences that could help to explain long-term increases in milk production observed in previous studies with early lactation anti-inflammatory treatments (Carpenter et al., 2016; Farney et al., 2013b). No overall differences were observed in milk yield in this study over 7 wk of lactation; perhaps more transcriptional changes in mammary tissue would have been evident in studies where milk yield was significantly increased by anti-inflammatory treatment.

Plasma analytes

Lemini et al. (2015) observed that increasing concentrations of TNFα administration *in vitro* increased both mRNA and protein levels of prolactin in a rodent GH4C1 pituitary cell line, and

also mice fed a high fat diet displayed elevated circulating TNFα but reduced circulating prolactin. This led us to wonder if systemic inflammation and elevated inflammatory cytokines around calving could interfere with prolactin release by the pituitary gland, and that NSAID treatment may affect milk production through increased prolactin levels. However, we did not observe any effects on plasma prolactin to support this. Similarly, no differences in other metabolites were detected. Previous research in our lab observed decreased plasma glucose concentrations and increased insulin sensitivity indices for NSAID-treated cows at d 7 of lactation (Farney et al., 2013a; Montgomery et al., 2019), but the reduced duration of treatment used in this study likely prevented a similar outcome, as was also observed in other studies with 3-d NSAID treatment protocols (Carpenter et al., 2016, 2018).

Transcriptional analysis

Samples of mammary tissue analyzed likely included not only mammary epithelial cells (MEC) but also other cell types, including immune cells, fibroblasts, endothelial cells, myoepithelial cells and adipocytes (Kass et al., 2007). Gene transcripts present may therefore originate from any of these cell types, but histological analysis as well as the overwhelming abundance of milk protein transcripts suggest that MEC account for a large proportion of cells present. Other than milk protein genes, the most abundant gene transcripts detected were all related to either protein synthesis, nutrient metabolism, or mitochondrial function, with the exception of *GLYCAM1*. The prevalence of transcripts related to mitochondrial function reflects the high level of metabolic activity in the early lactation mammary gland, as was demonstrated by increased ATP synthesis, mitochondrial number, and electron transport chain activity in postpartum murine mammary tissue (Hadsell et al., 2010).

GlyCAM1 is a mucin-like glycoprotein found on endothelial cells that is involved in cell adhesion via interactions with L-selectin; high expression levels were also detected in other studies of bovine mammary tissue, including one comparing mammary tissue, somatic cells, milk fat globules, and laser-dissected MEC (Cánovas et al., 2014). This gene was not detected in the mammary tissue of sows (Palombo et al., 2018) or women (Maningat et al., 2009) during lactation, but other studies have been described that did find *GLYCAM1* expressed in non-ruminant species (humans, mice) (Le Provost et al., 2003). Those authors showed sheep *GLYCAM1* expression is activated by stimulation by lactogenic hormones, and its expression follows that of milk protein genes, which may be linked to the location of STAT5 regulatory elements near the *GLYCAM1* promotor region. At least in mice, the form of GlyCAM detected in MEC and in milk was a modified form from that found on endothelial cells, lacking the ability to interact with L-selectin, which suggests an alternate function in the mammary gland (Dowbenko et al., 1993; Nishimura, 2003). Evaluation of protein activity of GlyCAM1 is needed to further examine functional differences between endothelial and mammary forms.

Treatment effects

The low number of DE genes detected between CON and SAL agrees with the histological lack of treatment differences on mammary epithelial cell proliferation. Additionally, many of the genes that were differentially expressed between CON and SAL on d 45 of lactation have not been previously characterized and functions are currently unknown. Dicer is an endonuclease enzyme responsible for microRNA processing (Ha and Kim, 2014). Decreased Dicer abundance with SAL treatment may have a global reduction on microRNA-mediated gene regulation, but it

is difficult to know what effects this might have on mammary function around peak lactation. Another endonuclease, Nuclear RNase P, was upregulated by NSAID treatment. This ribozyme is involved with transfer RNA processing (Chamberlain et al., 1998); altered activity may have broader impacts on amino acid trafficking for ribosomal protein synthesis. Histone 1 D is a member of the Histone 1 family, which is involved in formation of chromatin structure, and may regulate epigenetic modifications including DNA methylation (Yang et al., 2013). Its upregulation with SAL treatment could be linked to the increased global methylation observed in SAL-treated cows, though differences in *HIST1H1D* expression were not observed on d 4 of lactation when the greatest difference in DNA methylation occurred. Histones have also been linked to inflammation and immune suppression, especially related to autoimmunity (Kusano et al., 2015). Additionally there is at least an indirect link between salicylate and decreased histone phosphorylation via cyclin-dependent kinase activity (Dachineni et al., 2016), which may have impacts on cell cycle regulation. *C4BPA* is a glycoprotein that inhibits Complement by binding to C4 or C3; a downregulation of complement binding protein transcripts is consistent with the activation of Complement observed with aspirin treatment in the literature (Lee et al., 2006). Lastly, it is also possible that the low number of DE genes detected (16 out of 18,286 analyzed) were merely false positives, and overinterpretation of these results without further supporting data should be avoided.

Transcriptional changes in mammary tissue over time

The canonical pathways that appeared to be most enriched in differentially expressed genes across timepoints include pathways involved in cytokine signaling and immune cell development, as well as cholesterol synthesis and intracellular receptor signaling (ie. LXR/RXR

activation). The downregulation of GnRH Signaling in early lactation agrees with data showing the response to GnRH is decreased post-calving and increases over time (Fernandes et al., 1978; Kadokawa et al., 1998), likely as the cow resumes normal estrous activity. Additionally, although the IL-1 Signaling Pathway was predicted to be downregulated, IL-1 itself was upregulated in our data, along with other pro-inflammatory cytokines, which can also downregulate GnRH secretion and signaling (Sheldon et al., 2008). The immune pathways enriched in early lactation are likely involved in mammary tissue remodeling and differentiation that occurs during pregnancy and during the onset of lactation. IL-1 and CDK signaling were shown to mediate TGFβ induction of MEC proliferation (Karakas et al., 2006), which may lend insight to the role of elevated mammary IL-1 independent from typical downstream inflammatory signaling. IL-1 and IL-18 are also involved in inflammasome-mediated activation of inflammation; however, a recent review on the role of the inflammasome in the GI tract shows its importance in the maintenance of epithelial homeostasis, and that IL-18 can also act to mitigate inflammatory signaling through promotion of regulatory T cells and tolerogenic activity (Rathinam and Chan, 2018).

CDK5 enrichment in early lactation may play a role in tissue organization and membrane trafficking, as has been observed in neural development (Kawauchi, 2014), or differentiation of multiple cell types (Dhavan and Tsai, 2001), though activity of CDK5 differs from other members of the cyclin-dependent kinase family and its functions are not as well understood (Dhavan and Tsai, 2001).

The downregulation of STAT3 Signaling in early lactation reflects the role of this transcription factor in the mammary gland, which unlike STAT5, is predominant during involution and promotes apoptosis of MEC (Clarkson et al., 2006). Both STAT and PPAR signaling pathways are crucial components of mammary function and additionally can each be regulated by the other (Shipley and Waxman, 2004); crosstalk between these pathways is likely part of the orchestrated onset of milk production and component secretion.

Pathways related to pattern recognition receptor signaling, the acute phase response, and IL-8 were especially enriched on d 4 of lactation. Murine mammary IL-8 mRNA expression fluctuates by lactation stage (Li et al., 2012a); in addition to inflammatory signaling, it regulates angiogenesis and stromal remodeling (Rabot et al., 2010). Our data shows that expression of acute phase protein transcripts downstream of NF-IL6, including haptoglobin, alpha-1-antitrypsin, and inter-alpha-trypsin inhibitor, are upregulated on d 1 relative to d 4 or d 45, while a different group of acute phase mediators, including anti-chymotrypsin, fibrinogen, C3, LPS-binding protein, fibronectin, serum amyloid A, and C-reactive protein, is predominantly expressed on d 4. Alterations in PRR signaling and acute phase response pathways between d 1 and d 4 likely reflect the nuanced progression of immune signaling in homeorhetic mammary tissue.

In many enriched canonical pathways, IPA did not determine whether the pathway was up- or down-regulated, because although the molecules involved in the pathway were differentially expressed, the direction of changes were often contradictory within the pathway. For example, NFKB signaling on d 1 relative to d 45 has a positive z-score, indicating predicted upregulation,

but a closer look at the pathway (see Figure 2.7) shows that although the molecules that would activate NFKB are upregulated (*IL-1, TNFa, LTA, CD40, TNFR*) the intermediate signaling molecules are downregulated (*PI3K, AKT*), resulting in a predicted downregulation of NFKB activation. Conversely, NFKB also mediates activation of the inflammasome pathway; in this case it is predicted to be upregulated according to downstream molecule expression (Figure 2.8). This disparity in predicted NFKB activity could be at least partially explained by differences in pathway regulation (i.e., transcriptional vs. post-translational), as PI3K and AKT activity is regulated more via protein phosphorylation than through transcript abundance. However, signaling pathways involved in acute phase response predicts simultaneous up- and down-regulation of *NFKB*, and both up- and down-regulation of the downstream inflammatory molecules (Figure 2.9). This supports the idea that the activation of specific immune signaling pathways may be regulated in multiple ways (i.e. not only through NFKB) and that signaling in the developing mammary gland may differ from conventional activation of inflammation.

The canonical pathways most enriched on d 1 relative to both d 4 and d 45 related to cholesterol synthesis, followed by LXR/RXR activation. Directional changes in transcript abundance of those molecules in the cholesterol synthesis pathways collectively indicate downregulation on d 1, although the magnitude of fold changes for these molecules likely prevented IPA from specifying the direction of regulation. It is tempting to attribute changes in cholesterol to milk output by the mammary gland, but studies with other epithelial cell types have observed similar results. Transcriptional analysis of rumen epithelium of beef cattle exposed to acidosis challenge (Steele et al., 2011) or transitioning to a higher grain diet (Zhao et al., 2017) also reported differences in cholesterol synthesis, suggesting that epithelial tissue charged with adaptation to

environment or physiological state may feature changes in cholesterol as an adaptation mechanism. In addition to being an integral part of cellular membranes, changes in cholesterol metabolism can mediate inflammatory response, cellular proliferation, and oxidative stress (Steele et al., 2011). Additionally, the LXR acts as both a mediator of cholesterol signaling in the cell as well as a regulator of the inflammatory response. Studies with T cells have reported that activation and proliferation required for an adaptive immune response require the LXR for regulation of cholesterol synthesis (Bensinger et al., 2008). Similarly, alterations in cholesterol synthesis are linked to TLR4 signaling in activated macrophages (Carroll et al., 2018). Conversely, in primary bovine mammary epithelial cells, pro-inflammatory cytokine production in response to LPS was inhibited by LXR activation, and this included the inhibition of TLR4 translocation to the plasma membrane (Wang et al., 2018). The similarities in pathway enrichment related to cholesterol synthesis, LXR activity, and immune signaling activity in mammary tissue suggest that increased proliferation of mammary epithelial cells leading up to the onset of lactation may involve similar mechanisms as those observed during immune cell activation, and that signaling pathways attributed to immune cell activation are a feature of proliferating cells or tissue remodeling rather than inflammation per se. This concept is supported by a review of the signaling pathways involved in organism development, which includes those acting through NFKB and JAK/STAT signaling (Perrimon et al., 2012). Those authors also described the diverse biological processes that can be carried out through surprisingly few regulatory pathways, and stressed the influence of other contributing factors on ultimate cell or tissue response.

Epigenetic effects of anti-inflammatory treatment

Global DNA methylation in the mammary gland appeared to decrease for the control cows over our study relative to levels on d 1, and then at least partially recover by d 45, while SAL treatment prevented these changes over time. The onset of milk production is marked by chromatin remodeling to allow for the rapid increase in milk protein production (Rijnkels et al., 2013); however, simplistically, the increased methylation observed in SAL cows should indicate less accessible chromatin and decreased transcription of milk component genes, which is not consistent with production responses to salicylate treatment. However, we only measured global methylation and did not examine specific regions of the DNA, and it's possible that hypomethylation in specific regions could support enhanced milk production, as has been observed for casein gene promotor regions (Singh et al., 2012). Yiannakopoulou (2014) reviewed studies that looked at effects of aspirin, salicylates and other NSAIDs on DNA methylation and found that NSAIDs were able to promote both hypo- and hyper-methylation. Suggested mechanisms included direct effects on DNA methyltransferases or induction of epigenetic changes through changes in reactive oxygen species (Yiannakopoulou, 2014), but these have yet to be confirmed. From the perspective of cow performance, because the changes in methylation were no longer significant by d45 of lactation, it is unclear whether or not epigenetic mechanisms could be responsible for whole-lactation milk production responses observed in previous studies. A more detailed assessment of site-specific epigenetic mechanisms as well as histone modifications seems warranted in light of the global methylation response on d 4.

CONCLUSIONS

Sodium salicylate treatment in early lactation did not affect milk production or circulating metabolites over the first 45 d of lactation. Similarly, there were only a few gene transcripts differentially expressed in mammary tissue on d 45, with potential ties to RNA processing, Complement regulation, and histone activity. Histological analysis showed decreased mammary epithelial cell proliferation as lactation progressed. Transcriptional changes over time highlighted the involvement of cholesterol synthesis and LXR signaling, as well as the upregulation of immune-related signaling pathways in early lactation. Global DNA methylation was increased in mammary tissue by anti-inflammatory treatment; further research may help clarify links between epigenetic alterations and milk production.

ACKNOWLEDGEMENTS

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TABLES

Table 2.1 Feed intake and milk production responses to anti-inflammatory treatment

Item	Treatment[5]		SEM	P-value		
	CON	SAL		Trt	Day	Trt*Day
	n = 8	n = 8				
Milk yield, kg/d	45.4	47.8	2.91	0.57	<0.0001	**0.10**
Fat, %	4.32	4.31	0.15	0.97	<0.0001	0.71
Fat yield, kg/d	1.93	1.99	0.15	0.76	<0.01	0.36
Protein, %	2.90	3.02	0.078	0.28	<0.0001	0.30
Protein yield, kg/d	1.29	1.39	0.070	0.33	<0.0001	**0.02**
Lactose, %	4.74	4.89	0.051	**0.06**	<0.0001	0.40
Lactose yield, kg/d	2.18	2.36	0.15	0.42	<0.0001	0.21
MUN[1], mg/dL	13.05	12.78	0.71	0.79	0.10	0.50
SCLS[2]	3.10	2.34	0.83	0.53	<0.0001	0.79
ECM[3], kg/d	49.7	52.0	3.26	0.62	<0.0001	0.23
3.5% FCM[4], kg/d	50.8	52.9	3.13	0.64	<0.0001	0.19
Dry matter intake, kg/d	25.7	26.8	1.4	0.60	<0.0001	**0.03**
Water intake, L/d	107.9	113.2	6.1	0.55	<0.0001	0.92

[1]MUN = milk urea nitrogen

[2]SCLS = somatic cell linear score; log2(SCC/100)+3.

[3]ECM = energy corrected milk; calculated as (0.327 × milk yield) + (12.95 × fat yield) + (7.65 × protein yield).

[4]FCM = fat-corrected milk; calculated as (0.432 × milk yield) + (16.216 × fat yield)

[5]Multiparous cows (parity 3+, n = 8 per treatment) were treated once daily with an oral drench of sodium salicylate (SAL; 125 g/d) or water (CON) for the first 3 days of lactation. Milk samples representative of a full milking were collected and analyzed twice weekly until d 45 of lactation. Data presented are the overall mean values of the first 45 d of lactation.

Table 2.2 Plasma metabolite response to anti-inflammatory treatment[1]

Item	Treatment			P-value		
	CON	SAL	SEM	Trt	Day	Trt*Day
Glucose, mg/dL	52.0	52.2	3.9	0.97	<0.0001	0.88
Insulin, ng/mL	0.22	0.22	0.03	0.95	<0.001	0.73
Free fatty acids, uM	518	491	35.5	0.60	<0.0001	0.49
BHBA, uM	716	600	69	0.25	<0.001	0.25
Prolactin, pg/mL	23.6	20.1	4.2	0.58	<0.01	0.43

[1] Multiparous Holstein cows (n = 8 per treatment) were administered sodium salicylate (SAL; 125 g/d) or water drench (CON) for the first 3 days in lactation. Blood samples were collected at 0600 h twice weekly until d 45 of lactation. Values reported are overall means.

Table 2.3 Gene transcripts differentially expressed in mammary tissue on d 45 of lactation[1]

Gene	Mean count[2]	log₂ fold change	FDR adjusted *P*-value	Gene name / description
ADAMTSL1	33838.7	-1.62	<0.001	ADAMTS-like-1 (extracellular matrix protein)
ANLN	5872.6	-1.70	<0.001	anillin actin binding protein (regulates cytoskeleton and promotes cell cycle)
CEP120	28317.5	-1.73	<0.001	centrosomal protein 120 (promotes centriole elongation during cell division)
Metazoa_SRP_5	99.3	-1.66	<0.001	uncharacterized
VAV3	6779.6	-1.53	<0.001	vav guanine nucleotide exchange factor 3
ENSBTAG000000 47264	33068.5	-1.62	<0.001	uncharacterized
Metazoa_SRP_4	431.3	-1.44	0.004	uncharacterized
7SK_51	75.9	-1.46	0.004	uncharacterized
RNaseP_nuc_2	23.3	-1.27	0.037	Nuclear RNase P (processes pre-tRNA)
C1orf146	13.8	1.24	0.069	chromosome 1 ORF146; uncharacterized
C4BPA	99.7	1.11	0.069	Complement component 4 binding protein alpha (inhibits Complement)
DICER1	269.5	0.44	0.069	ribonuclease3 (processes microRNA)
HIST1H1D	335.2	-0.97	0.074	histone cluster 1 h1 memberD; (involved in chromatin structure)
Metazoa_SRP_20	27.5	-1.17	0.077	uncharacterized
Metazoa_SRP_6	28.1	-1.17	0.077	uncharacterized
BOLA1	107.3	-0.41	0.094	bola family member 1 (mitochondrial REDOX function)

[1]Multiparous Holstein cows (n = 8 per treatment) were administered sodium salicylate (SAL; 125 g/d) or water drench (CON) for the first 3 days in lactation. Mammary tissue samples were collected on d 1, 4, and 45 of lactation; RNA was sequenced and counts were analyzed for differential expression using DESeq2.

[2]Mean count column displays normalized average of all d 45 samples. Normalized counts are calculated by DESeq2 by first dividing raw counts for each gene by the geometric mean of that gene across all samples. Of the resulting ratios, the median value within each sample becomes the normalization factor (for that sample) by which raw counts are divided to produce the normalized counts (Love et al., 2014). Fold change values are for CON cows relative to those treated with SAL. No gene transcripts were DE on d 1 or d 4 of lactation.

Table 2.4 Top 25 enriched canonical pathways using timepoint comparison analysis

Canonical Pathway[1]	d 1 vs. d 4	d 1 vs. d 45	d 4 vs. d 45
Dendritic Cell Maturation	0.71	3.57*	3.41*
TREM1 Signaling	N/A	2.60*	3.05*
Inflammasome pathway	0.00	2.83*	2.24*
Role of NFAT in Regulation of the Immune Response	N/A	2.79	2.24*
CDK5 Signaling	1.67*	1.40*	1.90
IL-1 Signaling	-2.00	-1.61*	-0.71
Colorectal Cancer Metastasis Signaling	0.00	1.02*	3.27*
STAT3 Pathway	-1.41*	-1.00*	-1.73*
Acute Myeloid Leukemia Signaling	2.00	1.80*	0.33
Gαi Signaling	1.00	2.40*	0.71
PPAR Signaling	N/A	-2.40*	-1.67
GNRH Signaling	-2.24	-1.18*	0.63
HMGB1 Signaling	0.45	1.95*	1.50*
Chemokine Signaling	0.00	1.34*	2.53*
Glioma Invasiveness Signaling	N/A	2.29*	1.51*
Gαs Signaling	-1.13*	-1.22*	1.41
Glioblastoma Multiforme Signaling	-0.45	1.48*	1.81
EIF2 Signaling	N/A	3.67*	0.00
Gαq Signaling	0.45	1.26*	1.94
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	-0.82	1.10*	1.73*
Leukocyte Extravasation Signaling	0.00	1.85*	1.61*
IL-8 Signaling	-0.38	1.07*	2.00
Acute Phase Response Signaling	-0.58*	1.48*	1.39*
NF-κB Signaling	1.34	1.85*	0.23*

[1]Canonical pathways with predicted direction of regulation across multiple timepoints are displayed. Z-score values indicate direction of regulation for the first day listed relative to the second (ie. for day 1 relative to day 4); *$P < 0.05$ for differential pathway enrichment between the 2 days.

Table 2.5 Top 25 canonical pathways enriched by genes differentially expressed between days 1 and 4 of lactation

Ingenuity Canonical Pathways	-log(*P*-value)	Ratio	z-score[1]
Superpathway of Cholesterol Biosynthesis	16.30	0.57	NaN
Cholesterol Biosynthesis I	14.30	0.85	NaN
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	14.30	0.85	NaN
Cholesterol Biosynthesis III (via Desmosterol)	14.30	0.85	NaN
Zymosterol Biosynthesis	4.73	0.67	NaN
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	3.71	0.29	NaN
Acute Phase Response Signaling	3.68	0.09	-0.577
LXR/RXR Activation	3.63	0.11	0
ATM Signaling	3.44	0.13	0.333
Eicosanoid Signaling	3.39	0.13	0
Mevalonate Pathway I	3.14	0.31	NaN
eNOS Signaling	3.09	0.09	1
Epoxysqualene Biosynthesis	2.94	1.00	NaN
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	2.91	0.14	-0.816
Endothelin-1 Signaling	2.74	0.08	-0.775
STAT3 Pathway	2.49	0.11	-1.414
FXR/RXR Activation	2.43	0.09	NaN
Phospholipases	2.31	0.11	NaN
TR/RXR Activation	2.22	0.09	NaN
CDK5 Signaling	2.19	0.09	1.667
Hematopoiesis from Multipotent Stem Cells	2.17	0.25	NaN
Antioxidant Action of Vitamin C	2.08	0.09	1
Bupropion Degradation	2.03	0.16	NaN
Unfolded protein response	2.02	0.11	NaN

[1]Z-score indicates direction of regulation for d 1 relative to d 4; NaN indicates that expression of genes in pathway are not sufficiently consistent to determine direction of regulation. Ratio indicates proportion of genes in pathway that are differentially expressed. *P*-value of 0.05 corresponds to -log(*P*-value) of 1.301.

Table 2.6 Top 25 canonical pathways enriched by genes differentially expressed between days 1 and 45 of lactation

Ingenuity Canonical Pathways	-log(*P*-value)	Ratio	z-score[1]
Superpathway of Cholesterol Biosynthesis	13.80	0.79	NaN
Hepatic Fibrosis / Hepatic Stellate Cell Activation	10.00	0.32	NaN
Cholesterol Biosynthesis I	9.31	0.92	NaN
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	9.31	0.92	NaN
Cholesterol Biosynthesis III (via Desmosterol)	9.31	0.92	NaN
LXR/RXR Activation	7.88	0.34	-0.87
LPS/IL-1 Mediated Inhibition of RXR Function	7.43	0.28	0
Acute Phase Response Signaling	7.24	0.30	1.48
EIF2 Signaling	7.19	0.28	3.667
Colorectal Cancer Metastasis Signaling	7.01	0.26	1.016
Atherosclerosis Signaling	6.72	0.32	NaN
Dendritic Cell Maturation	6.31	0.27	3.571
Axonal Guidance Signaling	6.16	0.22	NaN
HMGB1 Signaling	6.13	0.30	1.947
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	5.81	0.24	NaN
IL-6 Signaling	5.80	0.30	1
Fc Epsilon RI Signaling	5.66	0.30	-0.169
Molecular Mechanisms of Cancer	5.62	0.23	NaN
FXR/RXR Activation	5.45	0.29	NaN
TR/RXR Activation	5.41	0.32	NaN
STAT3 Pathway	5.16	0.34	-1
Mevalonate Pathway I	5.15	0.69	NaN
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4.99	0.25	0.729
Complement System	4.96	0.43	0.302

[1]Z-score indicates direction of regulation for d 1 relative to d 45; NaN indicates that expression of genes in pathway are not sufficiently consistent to determine direction of regulation. Ratio indicates proportion of genes in pathway that are differentially expressed. *P*-value of 0.05 corresponds to -log(*P*-value) of 1.301.

Table 2.7 Top 25 canonical pathways enriched by genes differentially expressed between days 4 and 45 of lactation

Ingenuity Canonical Pathways	-log(*P*-value)	Ratio	z-score[1]
Primary Immunodeficiency Signaling	3.89	0.23	NaN
T Helper Cell Differentiation	3.89	0.19	NaN
Atherosclerosis Signaling	3.54	0.15	NaN
Complement System	3.49	0.24	1.89
Axonal Guidance Signaling	3.30	0.10	NaN
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	3.27	0.12	NaN
Leukocyte Extravasation Signaling	3.25	0.12	1.606
Retinol Biosynthesis	3.22	0.23	NaN
TREM1 Signaling	3.21	0.17	3.051
Colorectal Cancer Metastasis Signaling	3.17	0.12	3.272
Dendritic Cell Maturation	3.17	0.13	3.411
VDR/RXR Activation	3.04	0.17	0
Hepatic Fibrosis / Hepatic Stellate Cell Activation	3.03	0.13	NaN
TR/RXR Activation	3.02	0.15	NaN
T Cell Receptor Signaling	2.98	0.15	NaN
Communication between Innate and Adaptive Immune Cells	2.97	0.16	NaN
Crosstalk between Dendritic Cells and Natural Killer Cells	2.97	0.16	NaN
Endothelin-1 Signaling	2.91	0.12	0.209
Growth Hormone Signaling	2.88	0.16	-0.302
Granulocyte Adhesion and Diapedesis	2.86	0.12	NaN
Pathogenesis of Multiple Sclerosis	2.85	0.44	NaN
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.81	0.11	NaN
Antigen Presentation Pathway	2.80	0.22	NaN
STAT3 Pathway	2.79	0.16	-1.732

[1]Z-score indicates direction of regulation for d 4 relative to d 45; NaN indicates that expression of genes in pathway are not sufficiently consistent to determine direction of regulation. Ratio indicates proportion of genes in pathway that are differentially expressed. *P*-value of 0.05 corresponds to -log(*P*-value) of 1.301.

Figure 2.1 Milk yield and feed intake over the first 7 weeks of lactation.

Holstein cows (parity 3+, $n = 8$ per treatment) were administered an oral drench of sodium salicylate (SAL; 125 g/d) or water (CON) for the first 3 d of lactation. Milk yield (**A**) was recorded at each milking. Amounts of feed offered and refused were recorded daily to calculate dry matter intake (DMI; panel **B**). Despite significant interaction of treatment and week for overall DMI, no differences were detected on any individual week.
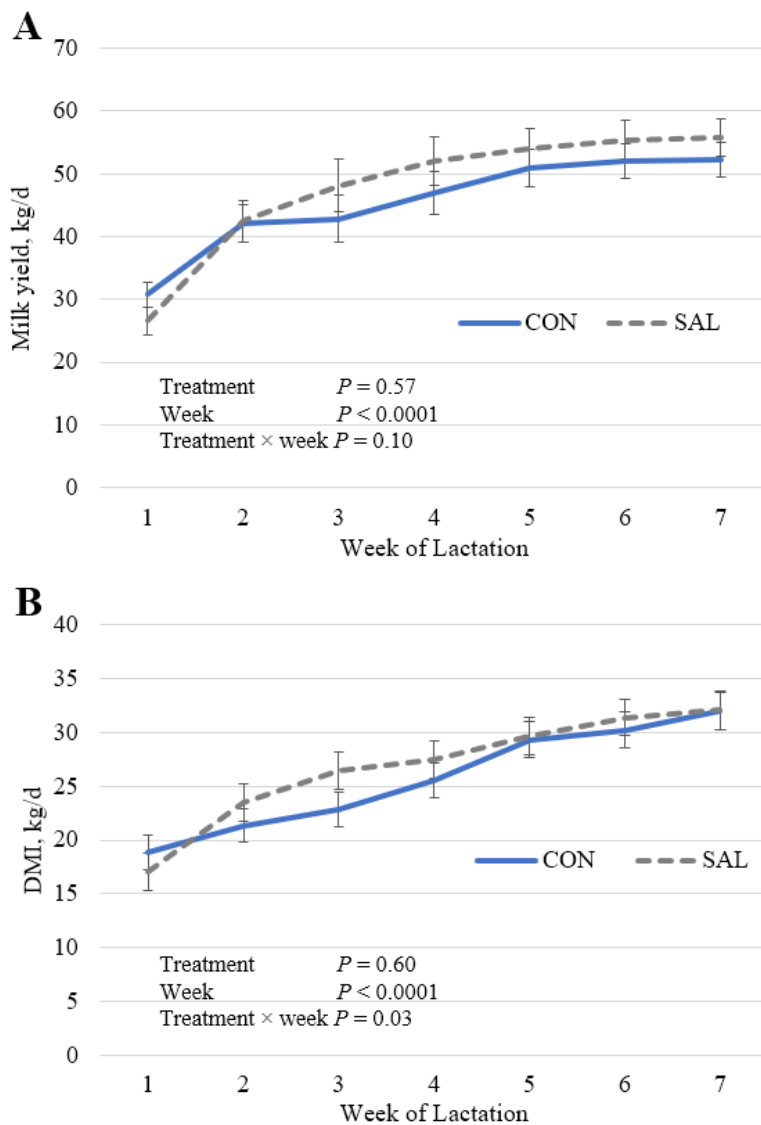
Figure 2.2 Plasma analytes of multiparous dairy cows for 45 d postpartum

Multiparous Holstein cows (n = 8 per treatment; parity 3+) were administered sodium salicylate (SAL; 125 g/d) or water drench (CON) for the first 3 d in lactation. Blood samples were collected at 0600 h twice weekly until d 45 of lactation, and analyzed for plasma glucose (A), insulin (B), free fatty acids (C), β-hydroxybutyrate (βHBA; panel D), and prolactin (E). Values for insulin and βHBA were natural-log transformed for statistical analysis and reverse-transformed for figures.
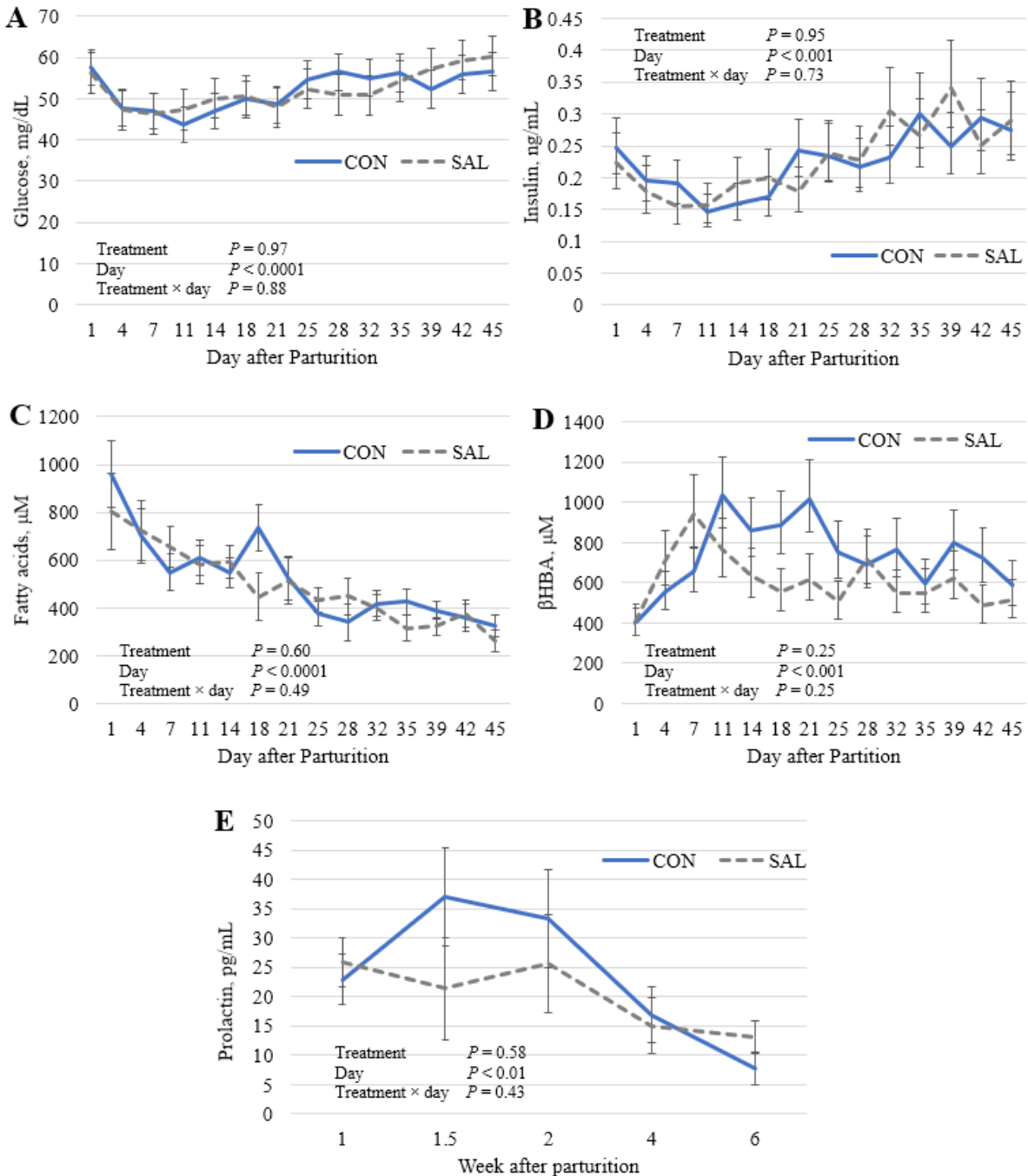
Figure 2.3 Proportion of mammary epithelial cells positive for Ki67 in early lactation

Cows were administered sodium salicylate (SAL; 125 g/d) or water (CON) once daily on d 1 (after first biopsy), 2, and 3. Mammary biopsies were conducted on d 1, 4, and 45 of lactation. Proliferation of mammary epithelial cells was assessed with immunohistochemistry, using antibodies for Ki67, and expressed as Ki67-positive cells (as a % of total epithelial cells).
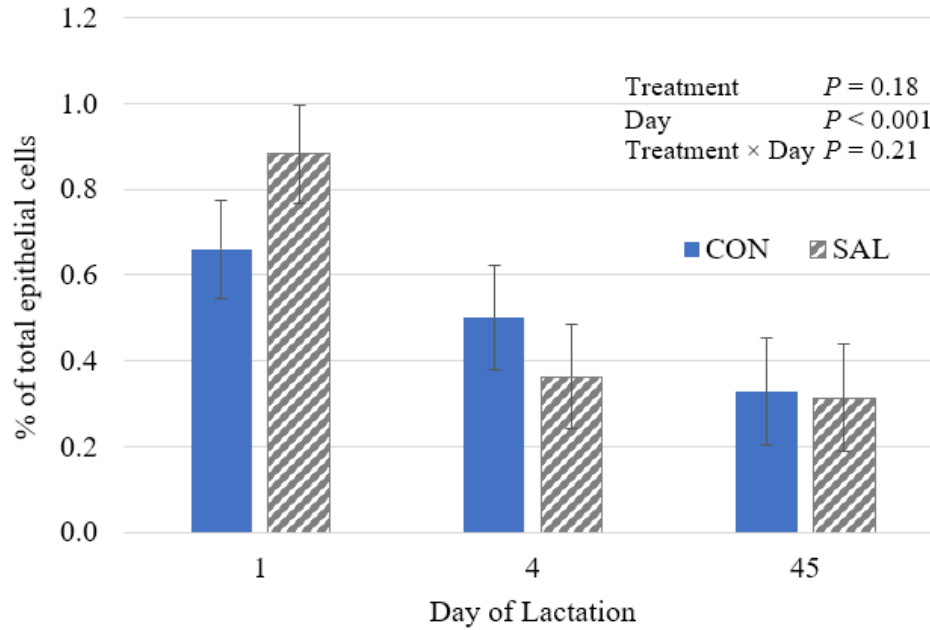
Figure 2.4 Principal Component Analysis plot

Principal component analysis plot of the $\log_2$-transformed normalized data of RNA sequenced from mammary tissue of dairy cows, obtained on d 1, 4, and 45 of lactation. Cows were enrolled at parturition and treated with (SAL; 125 g/d sodium salicylate) or without (CON) anti-inflammatory over the first 3 days of lactation.

Figure 2.5 Heatmap displaying the 20 most abundant genes in mammary tissue

Heatmap showing the top 20 mRNAs detected, which were grouped horizontally by similarity between samples (treatment and day of sampling) and vertically by similar expression patterns between genes. Color scale values indicate $\log_2$ transformed normalized mean counts, averaged across all samples. Gene expression was analyzed from mammary tissues sampled on d 1, 4, and 45 of lactation from cows treated with (SAL; 125 g/d sodium salicylate) or without (CON) anti-inflammatory treatment over the first 3 days of lactation.
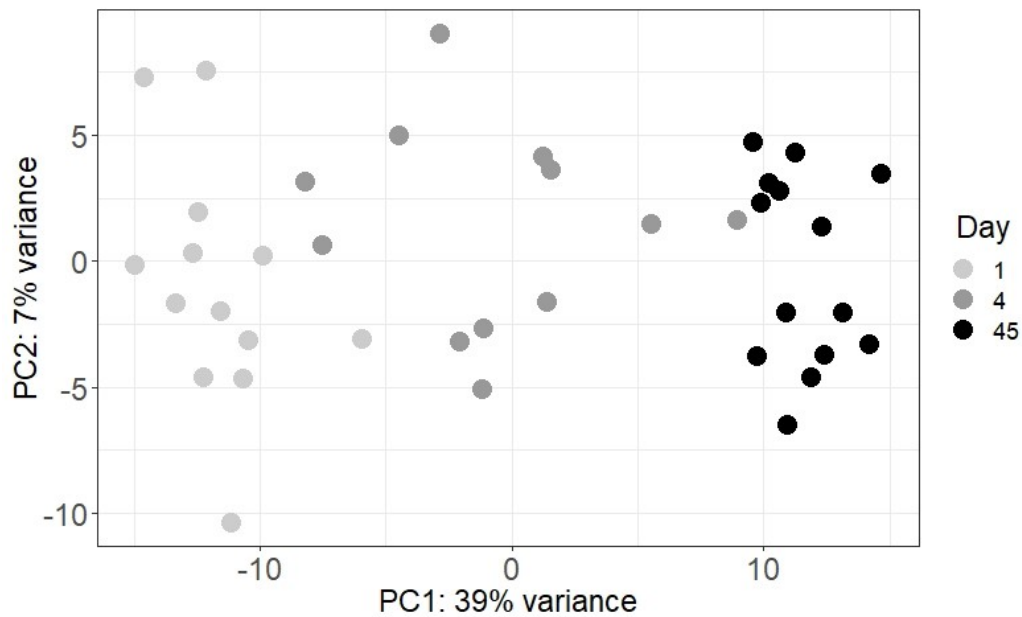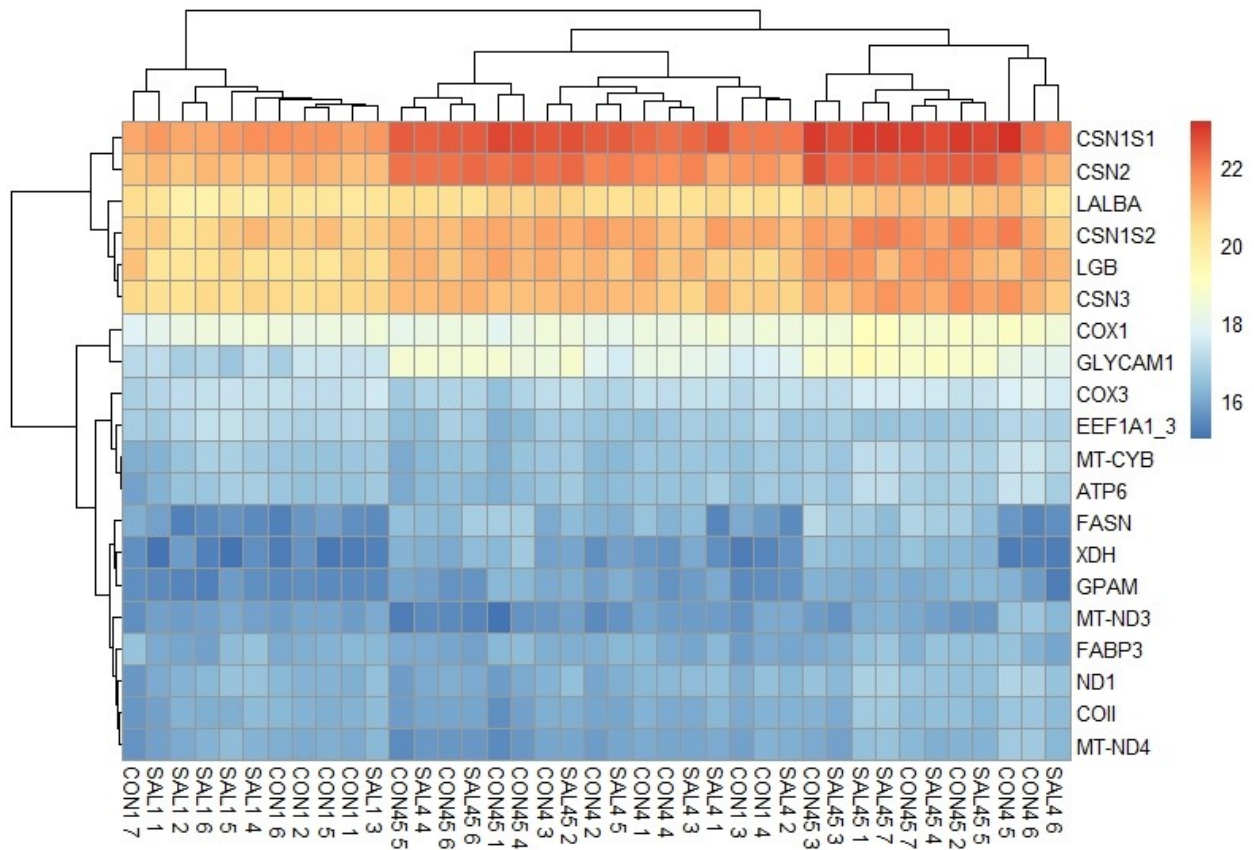
Figure 2.6 Global mammary DNA methylation

Mammary DNA was tested for global methylation using a 5-methylcytosine calorimetric assay.
Tissue samples were obtained on d 1, 4, and 45 of lactation; cows were administered with (SAL)
or without (CON) anti-inflammatory treatment tissue for the first 3 days of lactation. D 1
samples were obtained prior to the beginning of treatment. Analysis of treatment effects on d 4
and on d 45 were performed using contrasts; treatment effect was detected on d 4 ($P = 0.04$) but
not on d 45 ($P = 0.21$). Main effect P-values were obtained after removal of interaction term
from the model (interaction $P = 0.47$).
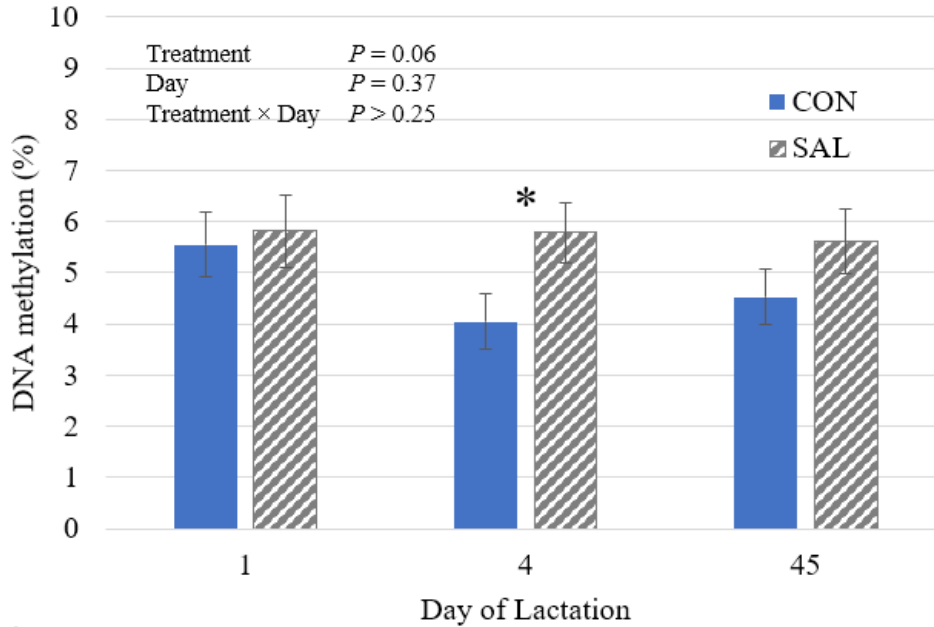
Figure 2.7 NFKB signaling in mammary tissue on d 1 relative to d 45 of lactation
Molecule color key: Green = down-regulated (according to gene expression data), blue = predicted inhibition; pink = up-regulated; orange = predicted activation. Cows in 3rd or greater lactation (*n* = 8 per treatment) were orally administered anti-inflammatory (SAL; 125 g/d sodium salicylate) or control (CON) from days 1 to 3 following parturition. Mammary biopsies were obtained on d 1 (prior to treatment initiation), d4, and d 45, and analyzed for gene expression. Canonical pathway enrichment for NFKB Signaling was obtained through Ingenuity Pathway Analysis of data analyzed by DESeq2.
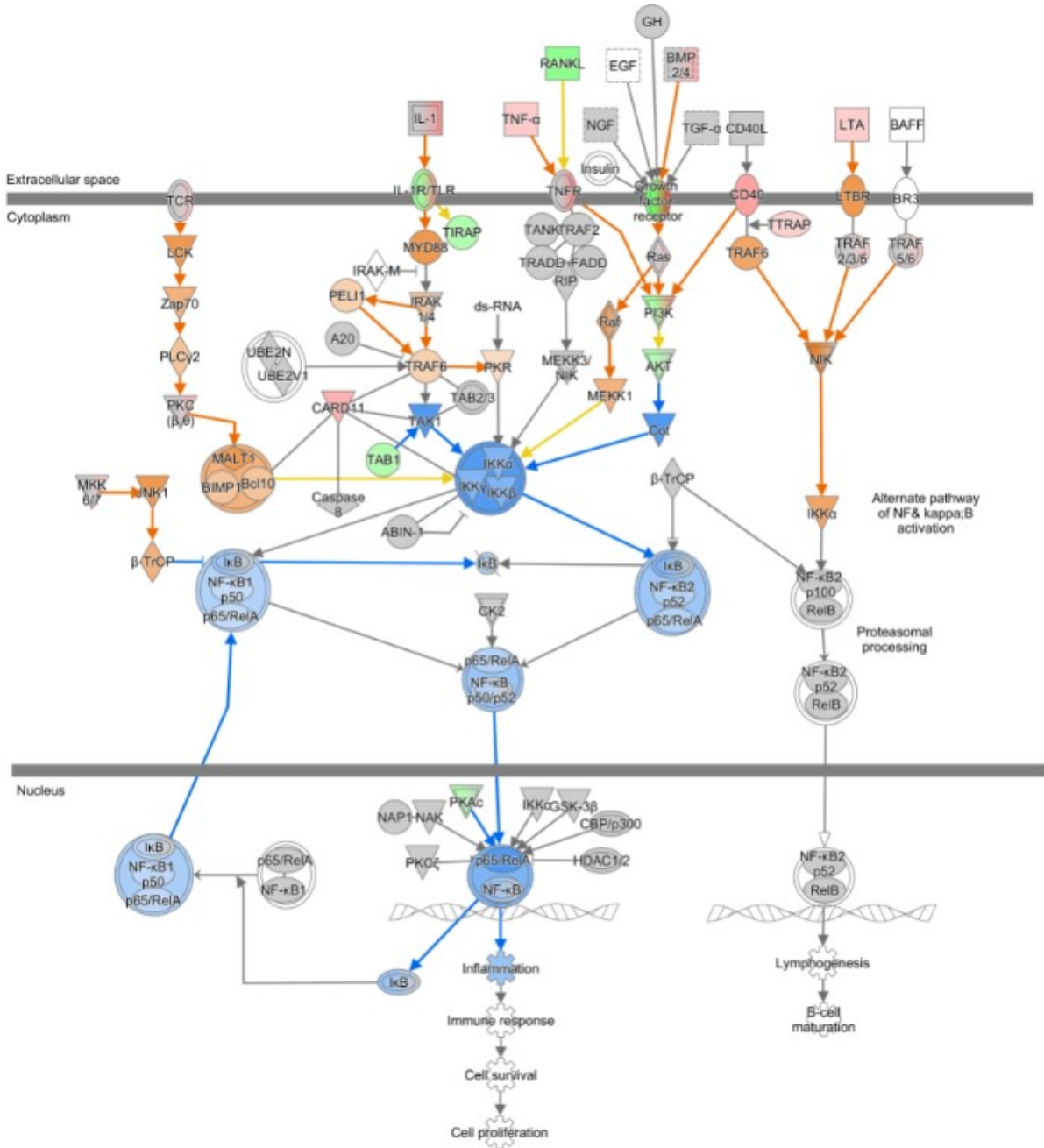
Figure 2.8 Inflammasome Signaling on d 1 relative to d 45

Molecule color key: Green = down-regulated, pink = up-regulated, blue = predicted inhibition, orange = predicted activation. Cows in 3rd or greater lactation (n = 8 per treatment) were orally administered anti-inflammatory treatment (SAL; 125 g/d sodium salicylate) or control (CON) from days 1 to 3 following parturition. Mammary biopsies were obtained on d 1 (prior to treatment initiation), d4, and d 45, and analyzed for gene expression. Canonical pathway enrichment for Inflammasome Signaling was obtained through Ingenuity Pathway Analysis of data analyzed by DESeq2.
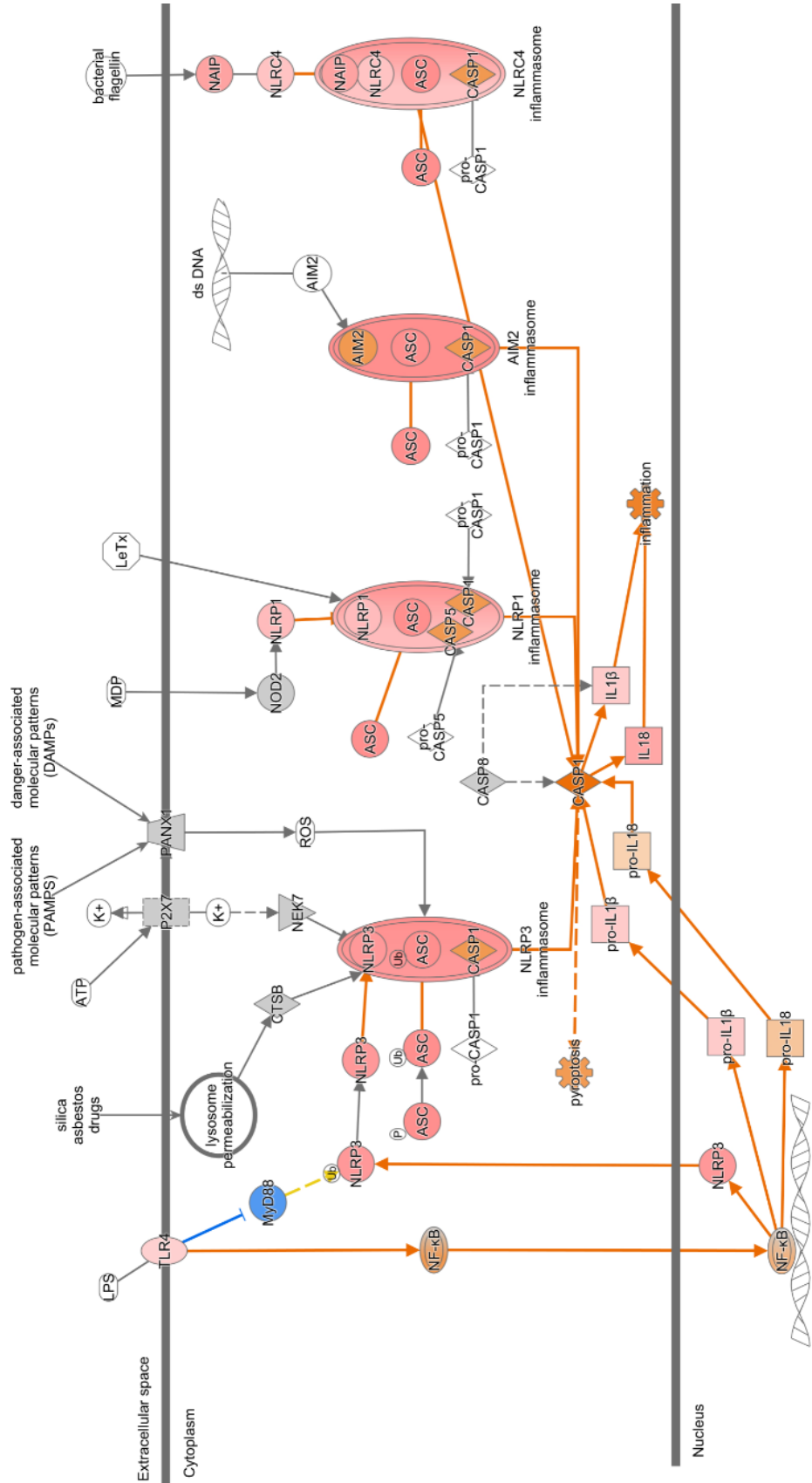
Figure 2.9 Acute Phase Response on d 1 of lactation relative to d 4

Molecule color key: Green = down-regulated, pink = up-regulated, blue = predicted inhibition, orange = predicted activation. Cows in 3rd or greater lactation (n = 8 per treatment) were orally administered anti-inflammatory treatment (SAL; 125 g/d sodium salicylate) or control (CON) from days 1 to 3 following parturition. Mammary biopsies were obtained on d 1 (prior to treatment initiation), d4, and d 45, and analyzed for gene expression. Canonical pathway enrichment for Acute Phase Response was obtained through Ingenuity Pathway Analysis of data analyzed by DESeq2.
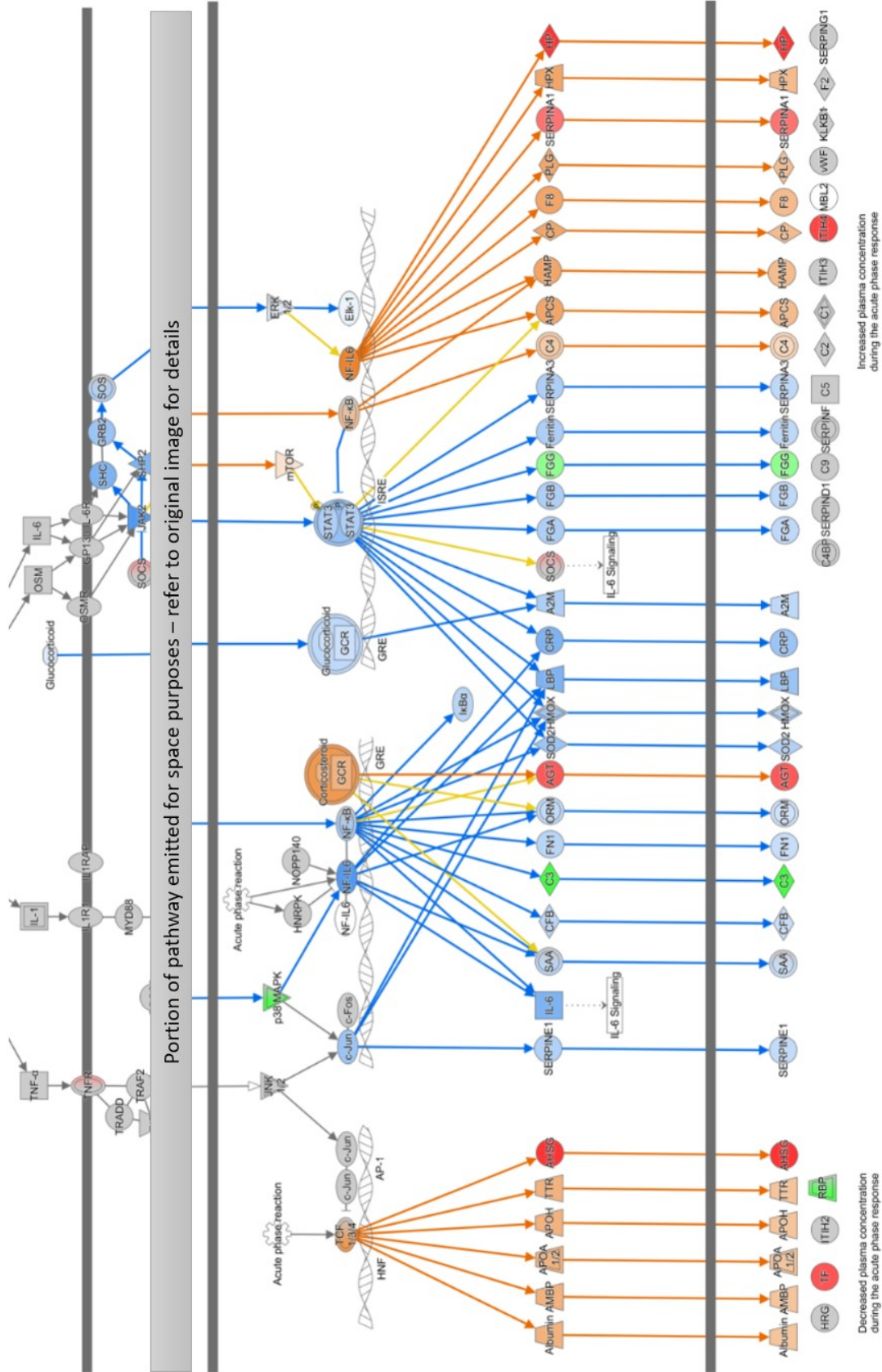
Portion of pathway emitted for space purposes – refer to original image for details

Chapter 3 - Associations between body condition score at parturition and microRNA profile in colostrum of dairy cows as evaluated by paired mapping programs

C. M. Ylioja, M. M. Rolf, L. K. Mamedova, and B. J. Bradford

ABSTRACT

MicroRNA (miRNA) are abundant in milk, and likely have regulatory activity involving lactation and immunity. The study objective was to determine the miRNA profile in colostrum of over-conditioned cows compared to cows of more moderate body condition score (BCS) at calving. Multiparous cows with either high ($\geq$4.0 on a 1-5 scale; $n = 7$) or moderate BCS (2.75-3.50; $n = 9$) in the week prior to parturition were selected from a commercial dairy herd. Blood and colostrum were sampled within 24 h after calving. Blood serum was analyzed for free fatty acids (FFA). MicroRNA was isolated from colostrum samples after removing milk fat and cells. MicroRNAs were sequenced and reads were mapped to the bovine genome and to the existing database of miRNA at miRBase.org. Two programs, Oasis 2.0 and miRDeep2, were employed in parallel for read alignment, and analysis of miRNA count data was performed using DESeq2. Identification of differentially expressed miRNA from DESeq2 was not affected by the differences in number of miRNA detected by the two mapping programs. Most abundant miRNA included miR-30a, miR-148a, miR-181a, let-7f, miR-26a, miR-21, miR-22, and miR-92a. Large-scale shifts in miRNA profile were not observed; however, colostrum of cows with high BCS contained less miR-486, which has been linked with altered glucose metabolism. Colostrum from cows with elevated serum FFA contained fewer miR-885, which may be connected to hepatic

function during the transition period. Potential functions of abundant miRNA suggest involvement in development and maintenance of cellular function in the mammary gland, with the additional possibility of influencing neonatal tissue and immune system development.

INTRODUCTION

Timely consumption of colostrum by the dairy calf is crucial for survival, as colostrum conveys immune protection to the calf in the first weeks after birth, when the calf's own adaptive immune system is yet under-developed. Literature to date indicates that immunoglobulins present in colostrum are responsible for the bulk of its immunoprotective effect, and the concentration of protein in colostrum, which correlates with IgG content, is used as an indicator of colostrum quality (Bielmann et al., 2010). However, there is a plethora of other signaling components in colostrum, including cytokines, growth factors, immune cells, and exosomal proteins (Samuel et al., 2017; Hagiwara et al., 2008; Langel et al., 2015) that may also contribute to tissue development, provide immune protection, and stimulate maturation of the neonatal immune system.

Non-coding RNAs, especially microRNAs (miRNA), have been detected in the milk of multiple species, and those miRNA with predicted function related to immune response are especially abundant in colostrum (Chen et al., 2014; Izumi et al., 2012). Associations between miRNA and extracellular microvesicles such as exosomes or apoptotic bodies, or with Argonaute proteins or HDL particles (Turchinovich et al., 2012), protect against degradation. Milk-derived microvesicles containing miRNA can withstand simulated gastrointestinal conditions (Benmoussa et al., 2016) and be taken up via endocytosis by intestinal cells (Rani et al., 2017)

and vascular endothelial cells (Kusuma et al., 2016). From these observations, it is plausible that miRNA in milk may survive gastrointestinal conditions of the calf to regulate function of neonatal tissues. Researchers studying miRNA in milk have often focused on those miRNA contained in exosomes. Although a large fraction of the miRNA in milk may be contained in microvesicles, the ultracentrifugation methods often used for exosome isolation yield only a subset of the miRNA present in the original sample and can result in selection bias of certain miRNA (Benmoussa et al., 2017, 2016).

The miRNA found in milk may be unique to the mammary gland or may reflect those found in circulation from other tissues or cells (Kosaka et al., 2010). Altered mammary miRNA profiles are associated with both mammary-specific conditions, such as mastitis (Sun et al., 2015), and with less localized treatments, such as dietary changes (Li et al., 2015b). It is unknown whether alterations in metabolic phenotype, such as those observed in dairy cows during the transition from gestation to lactation, would also affect the miRNA found in milk.

Dairy cows in the periparturient period exhibit immune suppression (Ingvartsen and Moyes, 2015). Those cows with excessive body condition prior to calving undergo a greater degree of adipose tissue mobilization, have greater circulating free fatty acid (FFA) and ketone concentrations, and are predisposed to fatty liver and metabolic disease after calving (Sordillo and Raphael, 2013). These cows also display greater systemic inflammation as well as a suppressed immune response (Sordillo et al., 2009). In humans, obesity is associated with an altered profile of circulating miRNA (Manning et al., 2019), and differentially expressed miRNA

are associated with human diabetes and metabolic syndrome (Karolina et al., 2012; De Candia et al., 2017), which have characteristics in common with periparturient dairy cows.

Our first objective in this study was to identify the miRNA most common in colostrum; this has been done by other groups but sample size has been small or samples have been pooled prior to sequencing (Chen et al., 2010; Gu et al., 2012). Additionally, we sought to examine the colostrum miRNA profile of cows divergent in adiposity, classified by either body condition score (BCS) or plasma FFA at the time of calving. We hypothesized that excessive adiposity of the dam would affect the profile of miRNA in colostrum, which could have implications for colostrum supporting the health of the bovine neonate.

Many programs and pipelines exist for bioinformatic processing of miRNA data, such as those compared by Li et al. (2012). Programs differ in data processing (sequence mapping, pipeline tools used) as well as allowed user inputs (reference files, customizable settings), which may affect results of read alignment. The miRDeep2 program is widely used by researchers analyzing miRNA sequencing data, while Oasis 2.0 is a more recently developed web-based program. These use Bowtie (Langmead et al., 2009) and STAR (Dobin et al., 2013), respectively, for initial alignment of mapped reads. As variation in this step can influence results, our second objective was to compare results of these two programs for alignment of raw reads, followed by a common method for analysis of mapping results.

METHODS

Multiparous (average parity 3.1; range, 2-5) Holstein cows were selected from a commercial dairy herd based on body condition score the week before calving. Body condition of cows in a maternity pen was evaluated weekly; scores by 3 trained individuals were averaged. Each cow with BCS of at least 4 (using a 1 to 5 scale; Ferguson et al., 1994) at the time of calving was paired with a cow that exhibited BCS between 2.75 and 3.50, calving within 5 d of each other. Samples were collected from a total of 16 cows ($n = 7$ for high BCS and $n = 9$ for moderate BCS) between October and November 2017. Samples from 2 selected high BCS cows were missed, resulting in the imbalance between groups.

According to farm protocol, newborn calves were removed from the calving pen immediately after birth and fed thawed colostrum previously tested for quality. All fresh cows calving within a 24 h period were milked for initial colostrum collection around 1300 h daily. Colostrum was collected using an automated milking system (Astronaut A3, Lely Ltd., Maassluis, the Netherlands). Blood was also collected from each cow via coccygeal venipuncture into 10-mL Vacutainer serum tubes (Becton, Dickinson and Co., Franklin Lakes, NJ) for analysis of circulating fatty acids. Colostrum samples were tested using a Brix refractometer to estimate IgG content at the time of sample collection. All methods were approved by the Kansas State University Institutional Animal Care and Use Committee (protocol #3807).

Sample processing and analysis

Blood samples were allowed to clot at 4°C before centrifugation (2,800 × g at 20°C for 15 min); serum was removed and stored at -20°C until FFA analysis (NEFA-HR; Wako Chemicals USA

Inc., Richmond, VA) was conducted. Colostrum was collected into a 50 mL sterile vial and kept on ice during transport (< 3 h) to the laboratory for processing. Phenylmethylsulfonyl fluoride (100 µM) was added to inhibit proteolysis. Colostrum was centrifuged (4,000 × g at 4°C for 30 min); milk fat was removed and the cell pellet discarded (modified from Blans et al., 2017). The aqueous supernatant was then diluted 1:1 with sterile Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY) to reduce viscosity and centrifuged again at 4,000 × g at 4°C for 20 min to pellet residual cells. Supernatant was again removed and stored at -80°C until RNA isolation.

RNA was isolated using the miRNeasy kit for serum/plasma (Qiagen, Hilden, Germany). This kit has been widely used in other milk miRNA and milk exosome miRNA studies and successfully isolates miRNA from exosomes as well as from supernatant (Izumi et al., 2015). Qiazol (1000 µL) was added to 200 µL of diluted colostrum and isolation was performed according to kit protocol. Samples were eluted into 30 µL of elution buffer and assessed for concentration and 260/280 absorbance ratio using a Take3 microvolume plate (Biotek Instruments, Inc., Winooski, VT) and SynergyHTX plate reader (Biotek Instruments, Inc.); 260/280 ratios of isolated RNA averaged 1.83 ± 0.09. RNA concentrations ranged from 20-94 ng/µL and averaged 49 ± 22 ng/µL. Samples were also analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using both RNA 6000 Nano (catalog no. 5067-1511) and Small RNA (catalog no. 5067-1548) chips to assess concentration and oligonucleotide size profile. Representative size spectra obtained from Bioanalyzer data with Total RNA Nano chips and Small RNA chips are shown in Figure 3.1.

Samples of isolated RNA were submitted to the University of Kansas Medical Center's Genome

Sequencing Facility (Lawrence, KS) for library preparation and sequencing. Sample libraries

were prepared using the TruSeq Small RNA sample prep kit (Illumina, Hayward, CA) and

sequenced on a HiSeq 2500 (Illumina) using a two-lane flow cell. Samples were run as single

reads with 65-cycle read length, and produced on average 5.87 ($\pm$ 0.49) million reads per sample.

Data processing and statistical analysis

Cutadapt (v1.16) was used for quality trimming, with a quality value threshold of 15 on the 5'

end and 10 on the 3' end, before trimming 3' adapters. Quality was assessed before and after

trimming with FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). The bovine

genome ARS-UCD1.2 (https://www.ncbi.nlm.nih.gov/assembly/GCF_002263795.1/) was

indexed with Bowtie (v1.2.2) before mapping reads with the mapper script of the miRDeep2

program (Friedlander et al., 2012; v2.0.0.8), allowing read lengths greater than 17 nucleotides

(nt) and with a maximum of 50 mapping positions in the genome (options -l 18 -r 50). The

miRDeep2 script was used to match mapped reads to known miRNA in the miRBase database

(Kozomara and Griffiths-Jones, 2014; v22; mirbase.org/ftp), and to predict novel miRNA using

additional known miRNA from human and mouse species.

Output files of miRNA counts were analyzed for differential expression using the Bioconductor

package DESeq2 (Love et al., 2014; R v3.5.1; DESeq2 v1.20.0). A total of 509 miRNA were

detected. After removing those with 10 or fewer total reads across all samples, 343 miRNA

remained for analysis. Read counts were analyzed for effects of BCS, FFA, and time elapsed

between calving and colostrum collection. Analysis of BCS as either a descriptive (High vs.

Moderate group) or continuous variable produced identical results. Overall effects of BCS and FFA were analyzed controlling for differences due to collection time. Differentially-expressed (DE) miRNA were those with an FDR-adjusted P-value < 0.1.

Raw fastq files were also uploaded to the sRNA Detection module of Oasis 2.0 (Rahman et al., 2018; https://oasis.dzne.de/) to validate mapping results. According to the program pipeline, reads were trimmed for quality and 3' adapters (cutadapt v1.7.1; FastQC v0.10.1) before mapping to known bovine miRNA in miRBase (v21) using STAR (Dobin et al., 2013; v2.4.1d). Only reads between 15 - 50 nt were used, mismatches were allowed at 5% of total read length, and reads mapping to more than 5 locations in the genome were discarded. Oasis used miRDeep2 (v2.0.0.5) to map reads to the bovine genome (assembly UMD3.1) for prediction of novel miRNA. A total of 779 known miRNA were detected, and after filtering out those with 10 or fewer total counts across all samples, 457 miRNA were analyzed for differential expression using DESeq2, as described earlier.

To test for variables that could potentially confound the effect of BCS group, statistical analysis on the effects of the BCS group on serum FFA, BCS, Brix values, and time elapsed between parturition and colostrum collection were conducted using the mixed procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Effects of collection time on Brix values were also assessed.

RESULTS AND DISCUSSION

miRNA identification

Using the mapper script of miRDeep2, 3.3 (± 0.34) million reads successfully mapped to the bovine genome, and 899,705 (range: 460,561 - 1,939,191) reads mapped to existing bovine miRNA in the database at mirbase.org. In comparison, Oasis 2.0 identified 1,547,114 (range = 984,337 - 2,567,670) miRNA reads mapping to previously identified miRNA in the bovine genome. Upon closer inspection, many of these miRNA were categorized as predicted miRNA, defined as novel miRNA previously detected by Oasis and contained in their internal database, but not yet incorporated into miRBase (Rahman et al., 2018). Although information on predicted sequence and structure are available through Oasis' search function, it is difficult to draw meaningful conclusions regarding the validity and potential functions of these predicted miRNA. For instance, Oasis mapping reported p-bta-miR-20 (note: unique nomenclature; not the same as miR-20) to be the most abundant miRNA detected, but submission of the mature sequence to NCBI's BLAST function resulted in only partial matches to multiple predicted (but uncharacterized) non-coding RNA species.

Disregarding these predicted miRNA, the number of detected miRNA was similar between miRDeep2 and Oasis mapping, which use Bowtie and STAR alignment software, respectively. Some differences were apparent regarding rank of miRNA in order of abundance, and also regarding identification of miRNA. For instance, miR-3600 was detected by Oasis but not by miRDeep2, while miR-26a, miR-22-3p, and let-7b were only detected by miRDeep2 (Figure 3.2). Examination of the miR-3600 sequence with NCBI's BLAST function revealed an identical sequence for miR-3600 and miR-22-3p, which may partially explain mapping differences.

However, Oasis detected 7,243 reads mapping to miR-3600, while miRDeep2 detected 28,610 reads for miR-22-3p, leaving room for other sources of mapping variation as well. Oasis also detected triple the number of miR-200b reads compared with miRDeep2. This miRNA has high similarity with sequences of miR-200c and miR-429, but neither had enough read counts with miRDeep2 mapping to be able to entirely attribute differences to the algorithm settings for handling ambiguous sequences.

In addition to the alignment algorithms used by the 2 programs, discrepancies in miRNA identification between Oasis and miRDeep2 could be due to version differences in the software used, the annotated reference genome used, the database version of known miRNA, or other mapping settings. For miRDeep2 mapping, we used the most recent assembly of the bovine reference genome (ARS-UCD1.2, released April 2018), while Oasis 2.0 used UMD3.1 (released December 2009). Oasis discarded any reads mapping to more than 5 genome locations, while miRDeep2 allowed those mapping up to 50 locations. Oasis and miRDeep2 used the miRNA database versions 21 and 22, respectively, which were released in 2015 and 2018. To estimate the extent to which these may have contributed to the alignment differences between the programs, we re-aligned the raw data of 2 of the cows using miRDeep2 but with the settings used by Oasis (3.1 UMD reference genome, miRBase version 21, and no more than 5 mapping locations). Surprisingly, the resulting miRNA counts were identical to those of our original alignment using miRDeep2, indicating that differences in read alignment between the 2 programs were unaffected by that particular combination of settings.

Effects of maternal adiposity

In this study, we sought to examine whether the miRNA profile of colostrum produced by the dairy cow is associated with measures of adiposity at the time of parturition. As is described below, we found very few statistical differences between cows characterized by circulating FFA or by BCS, regardless of the sequence alignment program used. Nevertheless, examination of the few miRNA that were DE as well as the miRNA most abundant in colostrum may shed light on the potential for colostrum components to regulate function at the transcriptional level.

BCS. In accordance with study design, BCS was significantly different between high and moderate BCS groups (4.12 vs. 3.28, SEM = 0.06; $P < 0.001$, Figure 3.3a). Time between calving and colostrum collection was 10.0 ± 6.2 h (mean $\pm$ SD), with no difference between BCS groups (8.0 and 11.6 for high and moderate groups, respectively; SEM = 2.2, $P = 0.27$). BCS group was unrelated to Brix values of colostrum (24.9 vs. 24.3 for high and moderate BCS groups, respectively; SEM = 1.6, $P = 0.82$).

Initially, global differences in colostrum miRNA profiles of different cows were assessed by principle component analysis. The two derived principle components explained 42% of the variation across samples; however, samples did not cluster by BCS group (Figure 3.4). The only DE miRNA for effect of BCS was miR-486, which was less abundant for cows with BCS ≥ 4.0 in comparison to cows with moderate BCS (FDR-adjusted $P = 0.06$; Figure 3.5a). Results were irrespective of mapping program utilized to produce miRNA count data, with similar fold-change and P values produced for miR-486 (fold change of -2.24 and FDR P-value of 0.06 for miRDeep2, and fold change of -2.28 and FDR $P = 0.07$ for Oasis).

MiR-486 may play an important role in regulating lactation through its inhibitory effect on phosphatase and tensin homolog (PTEN) protein (Li et al., 2015a). This protein constrains proliferation and differentiation of mammary epithelial cells (MEC) by downregulating the PI3k-Akt pathway in mice (Chen et al., 2012), and overexpression of PTEN in bovine MEC reduced synthesis of milk components *in vitro* (Wang et al., 2014). In heifers, elevated BCS has been linked to impaired mammary development and lactation performance (Silva et al., 2002), though the role of mammary miRNA regulation (and miR-486 in particular) has not been evaluated in the context of altered body composition. Additionally, although BCS is reflective of peripheral adipose depots, alterations in the mammary gland also occur in early lactation, such as the regression of mammary adipocytes to allow for epithelial cell growth and milk secretion (Zwick et al., 2018). Alterations of miRNA in either cell type may contribute directly to variation in milk miRNA content; in this case, one study detected miR-486 in bovine mammary epithelium but not in mammary adipose tissue (Li et al., 2015a). Adipokines also are important regulators of mammary function (Palin et al., 2017); although there are no studies yet specifically evaluating miR-486, altered adipokine production could conceivably be responsible for indirect effects of adiposity on miRNA regulation.

The function of miR-486 may also be tied to glucose metabolism. Circulating miR-486 was upregulated in obese vs. non-obese children, as well as in adults with type 2 diabetes (Cui et al., 2018). In the same study, overexpression of miR-486 increased proliferation of human pre-adipocytes and increased glucose uptake by C2C12 myoblast cells *in vitro* (Cui et al., 2018). Similar to people with type 2 diabetes, periparturient dairy cows also exhibit altered insulin

sensitivity during adaptation to lactation. Observed differences in miR-486 may be related to more pronounced changes to insulin resistance or glucose utilization in over-conditioned cows compared with cows of more moderate BCS.

Presence of miR-486 in colostrum may have implications for neonate development. Examination of miRNA expression throughout the calf gastrointestinal (GI) tract over the first 6 weeks of life showed both temporal and regional differences in miRNA expression in gut tissue, including that of miR-486 (Liang et al., 2014). Functional analysis by those authors predicted that miR-486 is involved in development of gut epithelium and immune cells. Additionally, studies show exogenous miRNA can be taken up by intestinal cells *in vitro* and *ex vivo* (Arntz et al., 2015; Wolf et al., 2015). It is possible that both the ingested and the endogenously-produced forms of miR-486 could have similar effects on GI tissue.

FFA. Similar to BCS, circulating FFA levels have been used to predict the degree of immune suppression exhibited by individual cows in the transition period. Data collected by Tremblay et al. (2018) on day 5 of lactation showed cows with plasma FFA > 700 µM exhibited poor metabolic adaptation and greater risk for metabolic disease. One premise for this study was BCS at parturition could be used to predict subsequent body fat mobilization, which is associated with altered immune function (Lacetera et al., 2005). Since BCS values did not always correlate with analyzed FFA values, we analyzed miRNA data for associations with both BCS and FFA. Circulating FFA concentrations did not significantly differ between BCS groups ($P = 0.66$); however, box-and-whisker plots of FFA distribution within each BCS group (using the boxplot procedure of SAS) identified 2 cows in the moderate BCS group as outliers for FFA

concentration, with measured FFA values even greater than for those cows in the high BCS group (Fig. 3b). Analysis of data after removal of these 2 datapoints confirmed cows with high BCS to have elevated FFA relative to those cows with moderate BCS (873 vs. 476 μM, SEM = 99, $P = 0.01$; Figure 3.3b).

Plasma FFA concentration was negatively associated with miR-885 abundance in colostrum (Figure 3.5b). Both mapping approaches revealed a similar association; the calculated fold-change was -0.2 per 100 μM increase in FFA concentration for both Oasis (FDR $P = 0.05$) and miRDeep2 (FDR $P = 0.02$).

Previously reported differences in miR-885 abundance point to associations with liver function. Human patients diagnosed with various liver pathologies, including hepatocellular carcinoma, liver cirrhosis, Hepatitis B, acute liver injury, or drug toxicity featured elevated circulating miR-885 (Gui et al., 2011; Vliegenthart et al., 2015). Individuals confirmed to have fatty liver had elevated mir-885 in circulation, which was also associated with large circulating lipoproteins and expression of oxysterol-binding protein (Raitoharju et al., 2016). Additionally, plasma miR-885 was associated with serum levels of liver enzymes alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyltransferase, which are often used as markers of altered liver function (Raitoharju et al., 2016; Sandrim et al., 2016). While the available data are largely from human clinical studies, decreased feed intake and increased FFA in transition dairy cows is often associated with hepatic lipidosis (Gerloff, 2000), and it is possible that measured differences in miR-885 may stem from altered hepatic function. Suppression of this miRNA has

inhibited proliferation in colorectal cancer cells (Su et al., 2018), but function in the context of the early lactation mammary gland is yet unexplored.

Time of colostrum collection

Brix values of colostrum were not affected by time elapsed between parturition and colostrum collection ($P = 0.51$). We analyzed mapped miRNA for differences due to collection time and detected five DE miRNA according to Oasis, 2 of which were also DE according to miRDeep2 (Figure 6). Four of those (miR-10b, miR-3431, miR-127, and miR-184) became more abundant as collection time was delayed, and one (miR-2284x) decreased in abundance over time. Others have observed differences in miRNA profile between lactation stages (Do et al., 2017; Sun et al., 2013; Chen et al., 2010); the differences we observed may be associated with changes in mammary gland activity between late gestation, lactogenesis, and galactopoiesis.

Potential impacts of miRNA abundant in colostrum

While we detected few DE miRNA associated with adiposity, the miRNA highly abundant in colostrum also provide valuable data for their potential regulatory impact on the dam as well as the neonate. Figure 3.2 shows the most abundantly expressed miRNA, using the normalized count data produced by DESeq2, averaged across samples. Supplemental data (File S1) contains a complete list of detected miRNA. The 10 most abundant miRNA according to miRDeep2 mapping were miR-30a, miR-148a, let-7a, miR-181a, let-7f, miR-26a, miR-22, miR-92a, miR-21, and miR-30d; collectively, these accounted for 71 ± 1.4 % of miRNA reads. The most abundant known miRNA detected by Oasis aligned closely with miRDeep2 mapping results (Figure 3.2) and also largely aligned with results of others (Alsaweed et al., 2016; Do et al.,

2017; Li et al., 2016). Other studies have used functional analysis programs to predict functional pathways that are impacted by the most abundant miRNA. Results commonly include broad pathways related to cell function, physiological system development, macromolecule metabolism, and immune response and cell trafficking. One reason that so many biological processes are potentially impacted is that one miRNA may bind to many different mRNA transcripts; for example, TargetScan.org (Agarwal et al., 2015) lists 1466 different predicted target genes for the bovine miR-30-5p family. While this highlights the broad scope of biological processes that could potentially be impacted by miRNA regulation, it does little to narrow down which of these biological processes might be most relevant for a given physiological scenario. We therefore took the approach of exploring the most abundant miRNA in the context of their potential relevance for the newly-lactating dairy cow, or for the newborn calf exposed to miRNA through colostrum ingestion.

Lipid metabolism. Mammary miRNA have been associated with fatty acid and lipid metabolism (Chu et al., 2018; Zhang et al., 2016; Tang et al., 2017). One obvious application involves milk fat synthesis; in addition to miR-221 and miR-29 discussed earlier, miR-181a was plentiful in our colostrum samples and has been shown to regulate lipogenesis (Lian et al., 2016; Du et al., 2017). Lian et al. (2016) transfected primary bovine epithelial cells *in vitro* with a miR-181a mimic and observed downregulation of an acyl-CoA synthetase (ACSL1) and decreased intracellular lipid concentration. In contrast, in primary bovine hepatocytes, a miR-181a mimic induced an increase in triglyceride content (Du et al., 2017). Alterations in lipid metabolism may translate to net effects that differ according to tissue type or other factors.

Lipid metabolism processes are also active in the newborn consuming milk, including the digestion and absorption of nutrients as well as adipose tissue development. For example, brown adipose tissue (BAT) that is plentiful at birth is steadily depleted in favor of white adipose tissue (Symonds, 2013). Chen et al. (2017a) discussed some of the miRNA that regulate BAT development, including miR-26, -27, -30, -93, -155, -182, and -378, which are relatively abundant in our colostrum data. For instance, while ACSL1 is a target of miR-181a in regulation of milk fat synthesis (Lian et al., 2016), ACSL1 is also required for oxidation of fatty acid substrate during BAT thermogenesis in mice (Steensels and Ersoy, 2019). Although these studies focused on mice, most of the miRNA discussed are broadly conserved across species (Targetscan.org; Agarwal et al., 2015) and may have similar activity in the bovine. Consumption of colostrum at birth therefore may contribute to the activation of BAT thermogenesis and adipose tissue remodeling; however, the extent to which colostral miRNA contribute is unknown.

Immune function. The upregulation of immune-related miRNA in colostrum compared to mature milk has been described in multiple species (Izumi et al., 2012; Sun et al., 2013; Ma et al., 2017; Modepalli et al., 2014). Documentation linking specific milk miRNA to immune function has been described previously (Ma et al., 2017; Gu et al., 2012), including for miR-148a, -92a, -30a, -30d, -25, -182, -191, -21, -27b, and let-7a, which were all in the top 20 of our detected miRNA (Figure 3.2). Liang et al. (2015) provided a thorough review of the potential for miRNA to impact the development of the bovine mucosal immune system. In addition, miR-21 and miR-29 (both within top 10 in our data) can bind to endolysosomal toll-like receptors to instigate innate immune cytokine production (Fabbri et al., 2012; Young et al., 2017). In addition

to potential neonatal impacts, the miRNA present in the mammary gland may also contribute to mammary health. MEC challenged *in vitro* with either *S. aureus* or *E. coli* bacteria resulted in altered miRNA expression, including upregulation of miR-148a, -92, and -21, and downregulation of let-7a and mir-423 (Jin et al., 2014), which were highly abundant in our colostrum samples. In primary MEC infected with *S. uberis*, mir-22a was among those downregulated and predicted to be involved in pathways related to innate immune response (Lawless et al., 2013). Naeem et al. (2012) reported a downregulation of miR-181a with *S. uberis* infection of mammary tissue, suggesting a role in regulation of pro-inflammatory genes. Although in our study none of these miRNA were DE, their presence may allow for a role in regulation of mammary health.

Cellular differentiation and development. MiRNA that regulate cellular differentiation may have implications for the mammary gland adapting to the onset of lactation as well as for the newborn neonate. In the mammary gland, survival of mammary alveolar progenitor cells has been altered by knockdown of specific miRNA *in vitro* (Elsarraj et al., 2013), and the same miRNA have been detected in human milk but not in plasma (Kosaka et al., 2010), suggesting local origin. MiRNA with regulatory activity within MEC may be actively exported from cells into milk, or escape via leakage of cell membranes (Chen et al., 2012b). In our data, miR-30a was the most abundantly expressed miRNA in colostrum, and together with the other 5 members of the miR-30 family (miR-30b, -30c, -30d, -30e, and -30f), made up 35% of all mapped miRNA reads. Overexpression of miR-30b in the mammary tissue of transgenic mice reduced alveolar size and altered lipid droplet characteristics, resulting in impaired growth of offspring (Le Guillou et al., 2012).

The let-7 family was highly present in colostrum; let-7a, -7b, -7c, -7d, -7e, -7f, -7g, -7i, and miR-98 together accounted for 15% of all mapped miRNA reads. This miRNA family is highly conserved across species and regulates cell differentiation (Lee et al., 2016). In a transgenic mouse model, let-7 over-expression decreased animal growth rates and altered glucose metabolism (Zhu et al., 2011). Data from both cell and lipid fractions of human milk showed similar abundance of let-7 family miRNA (Alsaweed et al., 2016), and mammary tissue isolated from both lactating and non-lactating cows showed abundant let-7a and -7f across all samples (Wang et al., 2018). The presence of this miRNA family in milk may reflect involvement of miRNA regulation in maintenance of normal cell function in mammary tissue that is rapidly increasing metabolic activity and secretory capacity.

Some miRNA detected in our samples are thought to inhibit processes predominant at the onset of lactation. MiR-221 has been recently identified as a regulator of MEC proliferation, and is downregulated at the onset of lactation (Jiao et al., 2019). Similarly, miR-150 declines during lactogenesis, and forced expression resulted in an inhibition of MEC function (Heinz et al., 2016). Others, such as miR-29, are upregulated with the onset of milk production (Do et al., 2017). In cultured primary bovine MEC, siRNA knockdown of miR-29 impaired synthesis of casein, lactose, and triglycerides, and increased methylation of DNA near lactation-related genes (Bian et al., 2015), indicating an epigenetic role in milk synthesis. MiR-148, which was the second most abundant miRNA detected in our data, likewise targets DNA methyl transferases that add methyl groups to DNA (Duursma et al., 2008), which could have important implications

for either programming of the mammary gland for the ensuing lactation, or for development of the neonate.

MiRNA in colostrum may also regulate neonate tissue development. Evidence indicates that exosomes containing miRNA can be taken up by rat intestinal epithelial cells, human colon carcinoma (Caco-2) cells (Wolf et al., 2015), and human macrophages (Izumi et al., 2015; Lässer et al., 2011); the enclosed miRNA may then regulate transcription within the cell. Additionally, miRNA cargo taken up from the gut lumen may be released into circulation. Incubation of Caco-2 monolayers with bovine milk exosomes on the apical side resulted in the release of miR-29b and miR-200c on the basolateral side (Wolf et al., 2015). In this manner, miRNA ingested by the calf via colostrum may have local effects on intestinal epithelial tissue function as well as systemic effects. Using both human intestinal epithelial cells and Caco-2 cells, Peck et al. (2016) showed that miR-30 regulates differentiation and proliferation by modulating the activity of SOX9, a transcription factor that regulates intestinal epithelial cell proliferation and differentiation (Gracz and Magness, 2011). Liang et al. (2014) identified other miRNA that appeared to be important regulators of GI tissue development in neonatal dairy calves.

The general importance of miRNA signaling in GI maintenance and function was demonstrated through a Dicer knockout mouse model (Liu et al., 2016). Dicer is an RNase enzyme involved in miRNA biogenesis (Ha and Kim, 2014), processing cytoplasmic pre-miRNA to form an effector complex which binds to mRNA transcript targets to facilitate silencing. Elimination of Dicer activity inhibited all miRNA regulation of transcription in the GI tract, and resulted in altered gut microbe populations and compromised barrier function of the colon (Liu et al., 2016).

miRNA processing. Lastly, to reiterate the complexity of miRNA signaling and the challenges of data interpretation, let-7 and miR-103, which were both highly abundant in our samples, have demonstrated Dicer targeting (Tokumaru et al., 2008; Chaulk et al., 2014; Martello et al., 2010). Downregulation in Dicer production could affect a global and indiscriminatory reduction in miRNA processing and regulatory activity by miRNA. The abundance of miR-103 in milk samples collected by Do et al. (2017) was similar to our data, and it did not appear to be DE by stage of lactation. The importance of either of these miRNA in regulation of global miRNA processing in early lactation mammary function is unknown.

CONCLUSIONS

We hypothesized that cows with greater BCS would exhibit an altered miRNA profile in secreted colostrum compared with those cows with more moderate BCS. This hypothesis was based on immune suppression especially observed in over-conditioned cows, combined with the anticipated role that microRNAs play in regulation of immune cell signaling. Results failed to demonstrate broad effects; exceptions were that miR-486 was downregulated in colostrum of over-conditioned cows and miR-10b was downregulated for cows with elevated circulating FFA. Mapping of raw sequencing data using both miRDeep2 and Oasis 2.0 resulted in similar statistical results. Overall, we found little evidence of broad differences in colostrum miRNA in cows of high vs. moderate BCS, but documented the presence of numerous miRNA in colostrum with potential importance for both the lactating cow and the neonate.

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APPENDIX

Raw sequencing datafiles can be accessed at the National Center for Biotechnology Information (NCBI) Sequence Read Archive, under BioProject #PRJNA528768.

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FIGURES

Figure 3.1 Length distribution of RNA extracted from bovine colostrum.
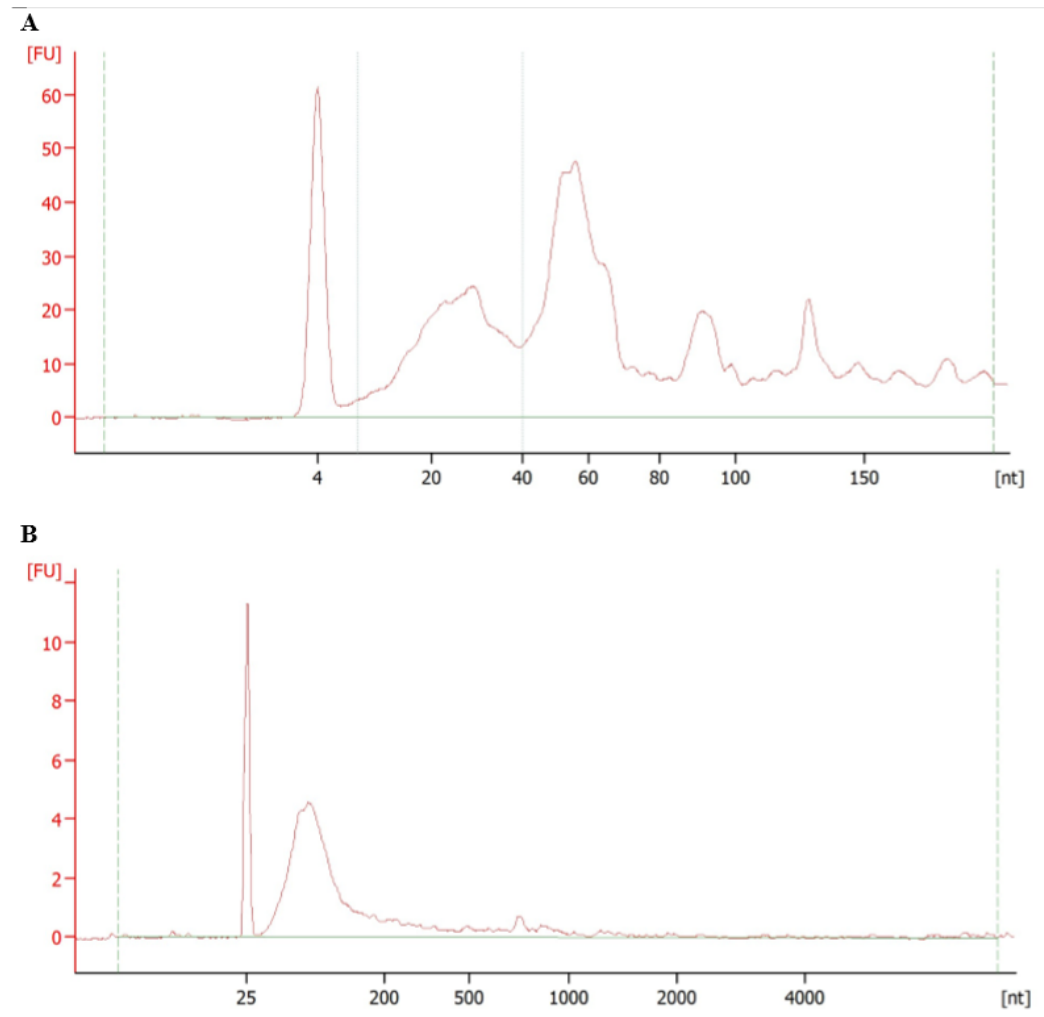
RNA extracted from de-fatted colostrum was analyzed for size distribution before sequencing.

Representative length distribution of RNA is shown plotted against fluorescence intensity,

derived from **(A)** Bioanalyzer Small RNA chip, and **(B)** Bioanalyzer Total RNA Nano chip. (FU

= fluorescence units; nt = nucleotides). RIN values were not measurable for our samples as the

ribosomal RNA used in calculating this value is contained largely in the cells and cytoplasmic

crescents in milk, which were removed prior to RNA isolation.

Figure 3.2 Most abundant miRNA in colostrum.
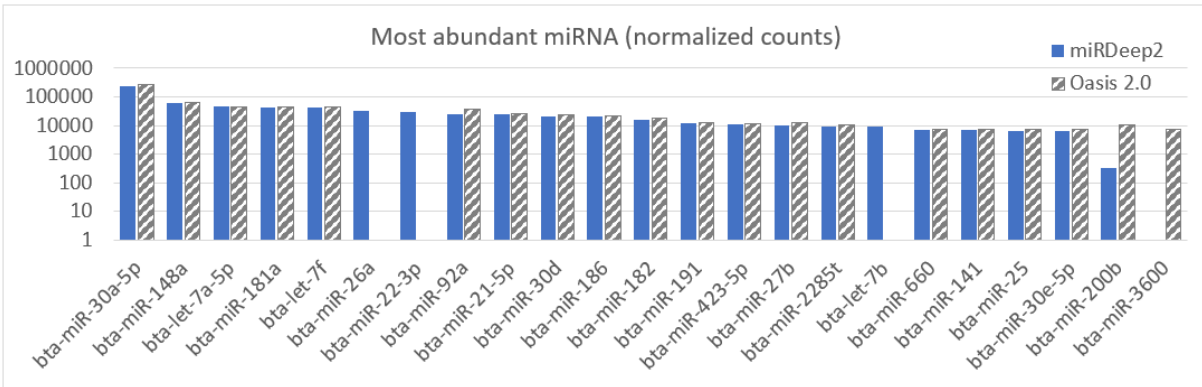
Most abundant miRNA in colostrum with comparison between miRDeep2 and Oasis 2.0 results. RNA was isolated from colostrum; raw reads from small RNA sequencing were aligned to the bovine genome and the database of known miRNA using either miRDeep2 or Oasis 2.0, and analyzed with DESeq2. The 20 most abundant miRNA for both programs (according to the mean of normalized counts of all samples) are shown here. Note the log scale on the y-axis; the top 10 most abundant miRNA accounted for 71 and 74 % of all reads, according to miRDeep2 and Oasis 2.0 mapping, respectively. The most abundant miRNA alone, miR-30a-5p, accounted for 30% or 34% of all reads, according to mapping by miRDeep2 and Oasis 2.0, respectively. MiR-148a accounted for 8% of all reads. Normalized counts are calculated by DESeq2 by first dividing raw counts for each gene by the geometric mean of that gene across all samples. Of the resulting ratios, the median value within each sample becomes the normalization factor (for that sample) by which raw counts are divided to produce the normalized counts (Love et al., 2014).

Figure 3.3 Body condition score and plasma free fatty acids of dairy cows at parturition.
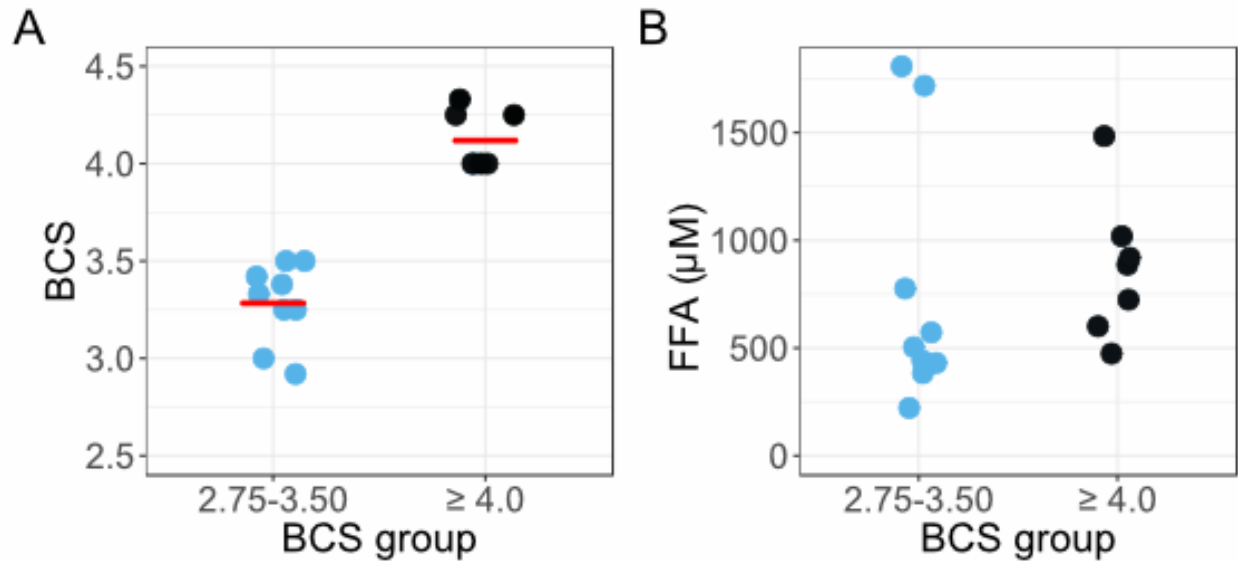
Cows of either moderate (2.75-3.50) or high (≥4.0) body condition score (BCS) at the time of parturition were enrolled. **(A)** Distribution of BCS at calving by group. Group means (3.28 and 4.12 for moderate and high BCS groups, respectively; SEM = 0.06, $P < 0.001$) are shown as horizontal red crossbars. **(B)** Distribution of serum free fatty acid (FFA) concentration within BCS groups on the day of parturition. A cutoff of 700 µM, shown as a horizontal dotted grey line, was used to differentiate between low and high circulating levels. Mean FFA did not differ between groups, except when 2 outliers in the moderate BCS group were excluded.

Figure 3.4 Principal Component Analysis plot of miRNA data, grouped by body condition score.
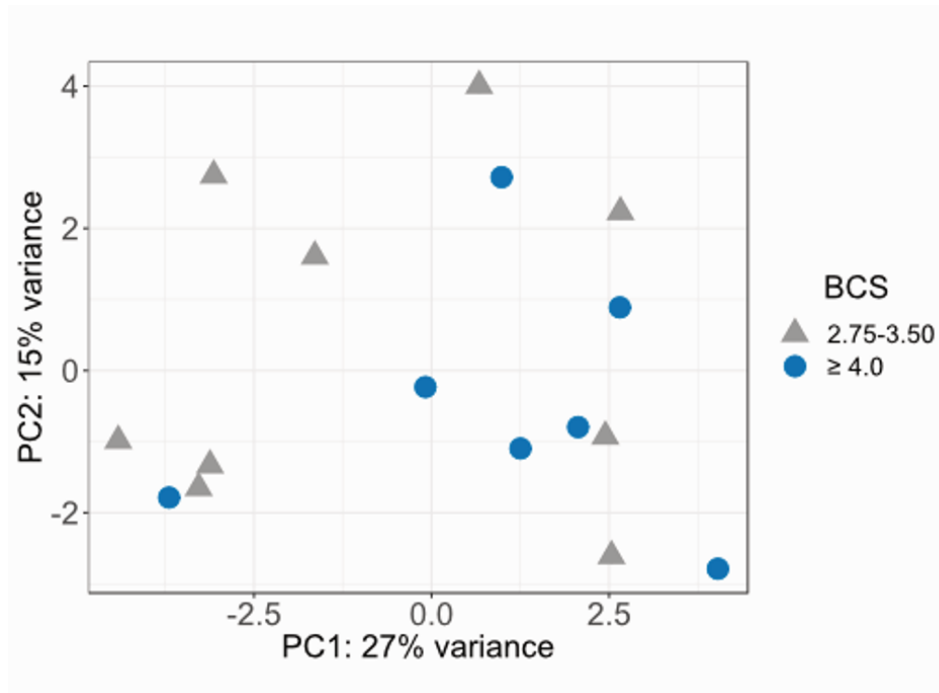
Principal Component Analysis (PCA) plot of miRNA data, grouped by body condition score

(BCS). RNA was isolated from colostrum of cows classified by BCS at calving; results of small

RNA sequencing were analyzed using DESeq2. Normalized counts were log-transformed and

values of first 2 principal components were used to construct PCA plot.

Figure 3.5 Differentially expressed miRNA associated with body condition score or plasma fatty acids.
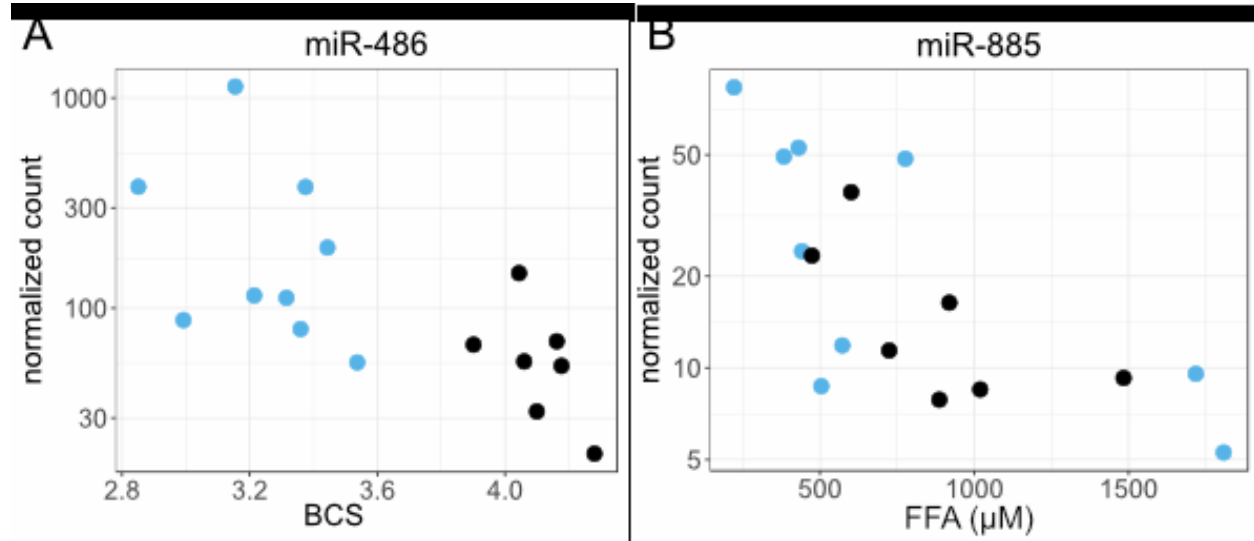
(A) miR-486 was downregulated in cows with BCS ≥ 4.0 (Fold change -2.15; FDR-adjusted P = 0.04). **(B)** miR-885 was downregulated for cows with elevated circulating FFA (Fold change - 0.002; FDR-adjusted P = 0.05). RNA from colostrum of cows with low (2.75-3.50) or high (≥4.0) body condition score (BCS) at calving was sequenced and aligned with known miRNA in the bovine genome using both miRDeep2 and Oasis 2.0, and analyzed using DESeq2. FFA was analyzed as a continuous variable; analysis of both BCS and FFA controlled for differences due to time elapsed before colostrum collection. MicroRNA identified as differentially expressed were similar for both mapping programs. Normalized counts are shown, which are calculated by DESeq2 by first dividing raw counts for each gene by the geometric mean of that gene across all samples. Of the resulting ratios, the median value within each sample becomes the normalization factor (for that sample) by which raw counts are divided to produce the normalized counts (Love et al., 2014).

Figure 3.6 Changes in colostrum miRNA abundance associated with time elapsed prior to milking.
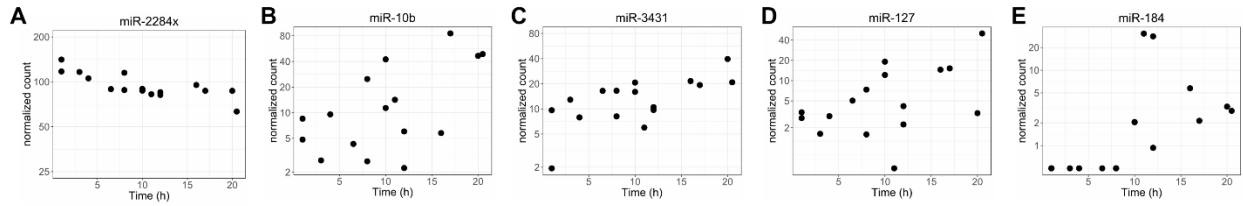
Colostrum samples were collected from the initial milking following parturition of 16 multiparous Holstein cows. RNA was isolated from colostrum, sequenced, and analyzed for differences due to time elapsed between parturition and sample collection. Oasis 2.0 identified 5 DE miRNA: miR-2284x (**A**; fold change -0.04 per hour), miR-10b (**B**; fold change 0.18 per hour), miR-3431 (**C**; fold change 0.09 per hour), miR-127 (**D**; fold change 0.18 per hour), and miR-184 (**E**; fold change 0.37 per hour; FDR P = 0.09 for all miRNA), which are shown as normalized counts by collection time. Normalized counts are calculated by DESeq2 by first dividing raw counts for each gene by the geometric mean of that gene across all samples. Of the resulting ratios, the median value within each sample becomes the normalization factor (for that sample) by which raw counts are divided to produce the normalized counts (Love et al., 2014). In contrast to results of Oasis 2.0, miRDeep2 mapping identified only miR-10b and miR-127 as DE miRNA (both FDR P = 0.07).

Chapter 4 - Effects of mammary nutrient demand on glucose kinetics of dairy cows in early lactation

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ABSTRACT

Early lactation is marked by increased peripheral insulin resistance, which allows for the shift in prioritization of nutrients towards milk production. Incomplete milking or reduced milking frequency in early lactation can improve energy balance of dairy cows and help facilitate a successful transition to lactation. We sought to measure effects of reducing nutrient demand by the mammary gland on insulin resistance, inflammation, and endocrine signaling in early lactation. Multiparous Holstein cows were enrolled at calving and randomly assigned to a milking frequency of either once ($1\times$) or three times daily ($3\times$), in combination with either sodium salicylate or control treatment, administered via drinking water for the first 5 d of lactation, in a 2×2 factorial design. Reduced milking frequency decreased milk yield without affecting feed intake, and energy balance was improved for cows milked once daily. More frequent milking decreased plasma glucose and insulin more over time compared to cows milked only once per day. Milking $3\times$ tended to increase the euglycemic glucose turnover rate but did not affect endogenous glucose production. Treatment differences in insulin sensitivity during the hyperinsulinemic-euglycemic clamp were not observed. Increased markers of inflammation were observed for $3\times$ milking, through increased plasma TNFα and increased expression of inflammatory genes in adipose tissue. Although differences in insulin sensitivity were not observed, alterations in endocrine signaling in multiple tissues likely contributed to altered metabolism associated with reduced milking frequency.

INTRODUCTION

The onset of milk production in dairy cows following parturition features a sharp increase in nutrient requirements by the mammary gland at a time when feed intake is often depressed. The inability of the dairy cow to consume enough nutrients through feed to meet lactation requirements increases her reliance on nutrient mobilization from body tissues, including adipose and muscle. Energy balance is quantified as the difference between energy intake and the energy required for maintenance and milk production. A more severe negative energy balance in early lactation is associated with impaired immune function and increased incidence of metabolic disease (Leblanc, 2010). Strategies to mitigate negative energy balance in early lactation can include increasing nutrient intake or decreasing nutrient output through milk (Grummer, 2007; Maltz et al., 2013; Patton et al., 2006). In addition to effects on energy balance, benefits of reduced milking frequency can include improved reproduction, reduced lameness, and reduced labor required for cow management (Stelwagen et al., 2013).

Increased insulin resistance in the periparturient period is an adaptive mechanism by which nutrient uptake by peripheral tissues is reduced, and tissue mobilization is increased, to prioritize the increasing nutrient requirements of the mammary gland (Giesy et al., 2012). Our objective was to evaluate the effects of altered milking frequency on insulin resistance and inflammatory signaling of dairy cows in early lactation.

METHODS

All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee.

Thirty-three multiparous Holstein cows were enrolled at parturition and were milked once for colostrum removal before being moved to a tie-stall barn, where they were housed for the duration of the study with individual access to feed and water. Cows were fed twice per day, at 0500 and 1700 h, for *ad libitum* intake. TMR contained a mix of corn and triticale silages, alfalfa hay, corn gluten feed (Sweet Bran; Cargill, Blair, NE), cottonseed, bypass soybean protein (Soyplus; Ralston, IA), and vitamins and minerals to meet or exceed NRC requirements (nutrient profile: 18.4% CP, 32.7% aNDF, 19.7% starch, 4.5% ether extract, 71.3% TDN, 1.67 Mcal NE_L/kg DM).

Experimental design and treatments

Cows were enrolled from October 2016 to September 2017 and blocked by calving date into groups of 4; cows were randomly assigned within block to one of four treatment combinations in a 2×2 factorial design. Treatments were milking frequency (1× or 3× daily milking) and anti-inflammatory treatment (sodium salicylate plus molasses: SAL, or molasses carrier alone: CON). The final block of cows enrolled contained an extra cow of the CON 1× milking treatment group.

Cows were milked with a vacuum pump-operated portable milking unit. Cows milked 3× were milked at 0400, 1200, and 2000 h, whereas cows milked once daily were milked only at 1200 h. All cows were released outside for exercise twice daily.

Anti-inflammatory treatment began at 0600 h on the day following parturition (12-36 h post-calving), which was denoted as day 1. Stock solution of sodium salicylate (Wintersun Chemical, Ontario, CA) was prepared by dissolving 1 kg of SAL and 88 mL of feed-grade molasses into 3 L (final volume) of water. Control treatment was prepared similarly but without

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the SAL. Prepared treatments were delivered through the drinking water using Dosatron injectors (Dosatron International Inc., Clearwater, FL) attached to the water line, at a rate of 0.68%, targeting a 2 g/L final concentration. Treatments were administered for the duration of the study, until biopsies were completed on d 5.

Daily sample collection

Daily feed and water intake were recorded. At each milking, milk samples were collected and milk yield was recorded. Milk samples were analyzed for fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), milk urea nitrogen (MUN; MUN spectrophotometer, Bentley Instruments), and somatic cell count (SCC) by MQT Lab Services (Kansas City, MO). Energy-corrected milk yield was calculated as ECM = (0.327 × milk yield) + (12.95 × fat yield) + (7.65 × protein yield). Samples of total mixed ration (TMR) were collected at 0400 on d 3 and 4, and refused feed was sampled before removal of feed on d 4 and 5. Energy balance was calculated as EB (Mcal/d) = NE_L intake - NE_L maintenance - NE_L output, where NE_L intake (Mcal/d) was calculated using feed intake and estimated energy content of the feed through lab analysis; NE_L maintenance or the maintenance energy requirement was calculated as 0.08 Mcal per kg $BW^{0.75}$ (Dairy NRC 2001); and energy required for milk output was calculated as NE_L output = (9.29 × fat yield) + (5.63 × protein yield) + (3.95 × lactose yield).

Daily blood samples were collected via coccygeal venipuncture at 0400 h prior to morning feeding, into 2 separate 7-mL tubes (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ) containing either potassium oxalate with sodium fluoride (for glucose analysis) or potassium EDTA. Samples were analyzed using sandwich ELISA for resistin (#SEA857Bo, Cloud-Clone Corp., Katy, TX; human serum was used to dilute standards; 99% linearity, 88-99%

spike recovery), glucagon (#10-1281-01, Mercodia, Uppsala, Sweden), and insulin (#10-1201-01, Mercodia, Uppsala, Sweden). Additional plasma analyses included glucose (colorimetric kit, Wako Chemicals USA Inc., Richmond, VA), TNFα (according to protocol described by Farney et al., 2011), and free fatty acids (NEFA-HR; Wako Chemicals USA Inc., Richmond, VA).

Assessment of insulin sensitivity

On d 3 following parturition, cows were fitted with bilateral jugular catheters. Before the procedure, cows were administered Excede (Zoetis, Florham Park, NJ) subcutaneously at the base of the ear, according to label instructions. After surgical scrubbing, lidocaine HCl was administered subcutaneously as a local anaesthetic, then Tygon catheter tubing (Saint-Gobain Performance Plastics, Akron, OH)) was introduced and sutured to the skin. Catheters were flushed at least every 12 h with sterile heparinized saline (20 IU/mL) to prevent clotting.

On d 5 following parturition, cows were offered 5 kg of feed (as-fed) every 2 h beginning at 0500 h to facilitate steady state conditions. Following the 1200 h milking, assessment of glucose kinetics proceeded in 3 phases, of 2 h duration each:

1) Basal phase: A priming dose of deuterated glucose tracer (D-glucose-6,6-d_2, Sigma-Adrich, St. Louis, MO; prepared by dissolving into 0.9% sterile sodium chloride followed by vacuum filtration sterilization) was administered (4.8 mg/kg BW). Glucose tracer was then continuously infused (dose: 0.08 mg/kg BW per min) for 2 h.

2) Euglycemic phase: Tracer infusion continued at the same rate. Additionally, dextrose (50% dextrose injection, USP; Hospira, Inc., Lake Forest, IL) was continuously infused to maintain a blood glucose concentration of 60 mg/dL. Blood was sampled every 10

min and analyzed immediately using a handheld blood glucose meter (Abbott Diabetes Care, Inc., Alameda, CA); the dextrose infusion rate was adjusted accordingly.

3) Hyperinsulinemic-euglycemic phase: The tracer infusion rate was adjusted to 0.12 mg/kg BW. Bovine insulin (#I0516, Sigma-Aldrich, St. Louis, MO) was infused (dose: 1 mU/kg BW per min) at a steady rate. Blood glucose was measured every 10 min and the infusion rate of 50% dextrose was adjusted to maintain blood glucose concentrations of 60 mg/dL.

Infusion rates were controlled using calibrated peristaltic infusion pumps (Baxter Flo-Gard 6201, Baxter Healthcare, Deerfield, IL). Body temperature was recorded prior to infusion commencement and monitored throughout the clamp procedure. Blood samples were collected prior to infusion and every 30 min through all 3 phases for analysis of insulin, resistin and glucagon. Additionally, samples for D2-glucose tracer analysis were collected prior to infusion as well as every 10 min during the last 30 min of each phase.

Tissue collection

Tissues biopsies were collected 2 h following the completion of the hyperinsulinemic-euglycemic clamp. Lidocaine HCl was administered subcutaneously as a local anaesthetic for each biopsied area. Muscle tissue was collected at the first lumbar vertebra. A 14-gauge biopsy needle (US Biopsy, Franklin, IN) was inserted perpendicular to the skin and 300 mg of the longissimus dorsi lumborum muscle was collected. Subcutaneous adipose tissue was collected from the tailhead region by first making a 3-cm long incision using a scalpel blade, and then removing adipose tissue using sterile forceps and scissors. Liver tissue was collected by first

making an incision with a scalpel blade in the 10th intercostal space, then approximately 200 mg of tissue was collected using a 14-gauge biopsy needle. Tissue samples for RNA analysis were immersed in Trizol Reagent (Life Technologies Corporation, Carlsbad, CA); all samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). Concentrations were assessed by measuring absorbance at 260 nm with a Take3 microvolume plate and SynergyHTX plate reader (Biotek Instruments, Inc, Winooski, VT), and quality was verified with a Total RNA 6000 Nano chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). PCR was performed using SYBR Green Supermix (Bio-Rad) and forward and reverse primers (250 nM each per reaction) on a 7500 Fast Real-Time PCR System (Applied Biosciences, Foster City, CA). Primer sequences and amplification efficiencies are listed in Table 4.2. Abundance of target genes were normalized using the geometric mean of *GAPDH* and *RPS9*, and differential expression was determined using the ddCt method (Schmittgen and Livak, 2008).

DNA was extracted from muscle tissue by crushing with mortar and pestle in liquid nitrogen, followed by extraction with Quick-DNA Miniprep Plus kit (Zymo Research Corp.). Samples were incubated with Proteinase K and solid tissue buffer (supplied with kit) for 1 hour at 55°C. RNase A (2 ug/uL; Zymo Research Corp.) was added and incubated for 2 min at 25°C, after which samples were centrifuged for 1 min at $10,000 \times g$, and the supernatant was used for the remainder of the kit protocol. DNA concentration was determined with a Take3 microvolume plate (Biotek Instruments, Inc.). Global DNA methylation was determined via colorimetric assay (5-methylcytosine Enzyme-Linked Immunosorbent Assay Kit; Zymo Research Corp.) using 100

ng of DNA from each sample, according to kit protocol. After horseradish peroxidase developer

was added, plates were incubated in the dark for 10 min before reading at 450 nm wavelength.

Calculation of glucose kinetics

Tracer enrichment of plasma samples was analyzed via gas chromatography - mass spectrometry

by a commercial laboratory (Metabolic Solutions, Inc., Nashua, NH). Glucose turnover rate was

calculated as the tracer infusion rate divided by the measured enrichment in plasma, adjusted for

background. Endogenous glucose production, assuming steady state, was calculated as the

glucose turnover rate less the rate of dextrose infusion.

Statistical analysis

Mixed models of SAS (version 9.4, SAS Institute Inc., Cary, NC) were used to analyze

data. All parameters were analyzed to assess the main effects of anti-inflammatory treatment,

milking frequency, and parity (2 or 3+) and their interaction, and cow was included as a random

effect. Measurements of daily plasma metabolites were analyzed using repeated measures with

the additional main effect of day, as well as all 2-, 3-, and 4-way interactions. Day 1 values were

used as covariates. Feed and water intake and milk yield were also analyzed as repeated

measures. To account for genetic variation in milk producing ability between cows, milk and

milk component models included genetic PTA values as covariates in the model. Plasma

metabolites during the infusion period were also analyzed for main effects as well as

interactions with treatment and milking frequency. Parity main effects and interaction terms were

included in analyses but are not reported here unless interaction terms were significant.

Interaction terms greater than $P > 0.25$ were removed from the model. DNA methylation was

analyzed using mixed models in JMP, with a random effect of cow and fixed effects of treatment, milking frequency, and their interaction. Abundance of gene transcripts were analyzed for main effects of milking frequency, anti-inflammatory treatment, and parity, and their 2- and 3-way interactions.

RESULTS

Although effects of anti-inflammatory treatment were analyzed, this chapter will focus on the effects of milking frequency, except where an interaction was detected with NSAID treatment.

Production parameters

As intended by study design, cows that were milked once daily produced 25% less milk than cows milked $3\times$/d ($P < 0.001$; Figure 4.1A). Results for milk component analyses are shown in Table 4.1. Milk fat content was increased for cows milked once daily (5.3 vs. $4.2 \pm 0.3\%$, $P = 0.01$; Figure 4.1C) and milk lactose content increased more rapidly for cows milked $3\times$ (interaction with time $P = 0.02$; Figure 4.1D). Protein content did not differ due to milking frequency ($P > 0.10$) but daily protein yield was greater for cows milked $3\times$ (Figure 4.1F). Milk urea nitrogen was decreased for cows milked more frequently (7.8 vs. 10.1 ± 0.7 mg/dL, $P = 0.04$). Similar to milk volume, energy-corrected milk was decreased for cows milked 1 $\times$/d (35.0 vs. 40.4 ± 1.6 kg/d, $P = 0.02$). Milk somatic cell content of cows milked more frequently decreased at a greater rate over the first 5 d of lactation than for those cows milked once daily (interaction with time $P = 0.03$; Figure 4.1E).

Frequency of milking had no effect on either water or feed intake ($P > 0.15$; Table 4.1), but more frequent milking did increase ECM production per unit of DMI (2.9 vs. 2.4 ± 0.1, $P <$

0.01). Negative energy balance was more pronounced for cows milked more frequently (-10.0 vs. -5.1 ± 1.3 Mcal NE_L/d; Figure 4.2A).

Plasma metabolites

Hormone and metabolite concentrations measured in plasma samples collected through the first 5 days of lactation are shown in Figures 4.2B and 4.3. Despite differences in energy balance, no treatment effect was detected for circulating plasma FFA ($P = 0.11$; Figure 4.2B). Plasma insulin concentration was decreased for cows milked 3×/d ($P = 0.02$). Milking frequency also interacted with time such that insulin values steadily decreased for cows milked 3×/d, while values for cows milked once daily were more stable over time ($P = 0.02$; Figure 4.3A).

Plasma glucose was affected by the 3-way interaction between milking frequency, anti-inflammatory treatment, and time ($P = 0.01$; Figure 4.3B). Differences between treatments became more pronounced over time, with analysis of slice effects on d 5 showing effects of both milking frequency ($P = 0.08$) and anti-inflammatory treatment ($P = 0.03$), as well as an interaction ($P = 0.07$). Cows milked once daily had greater glucose concentrations than those milked thrice daily, but plasma glucose was decreased for those cows treated with SAL, for both milking frequencies.

Glucagon also was affected by the 3-way interaction of milking frequency, anti-inflammatory treatment, and time ($P = 0.01$; Figure 4.3C), as well as by interactions between milking frequency and anti-inflammatory treatment ($P = 0.05$), and milking frequency over time ($P = 0.01$). The effects of milking frequency over time appeared to be largely due to those cows administered anti-inflammatory treatment. For SAL cows, plasma glucagon concentration of cows milked more frequently increased over time, while cows milked once daily showed the

opposite trend; glucagon concentration of CON cows appeared to be relatively unchanged over time regardless of milking frequency.

The daily mean of plasma resistin was not affected by milking frequency ($P = 0.56$). Cows milked more frequently also had greater circulating levels of plasma TNFα in plasma (56.1 vs. 34.1 ± 5.3 pg/mL, $P < 0.01$; Figure 4.3D).

Glucose kinetics

Glucose kinetics during basal, euglycemic, and hyperinsulinemic-euglycemic conditions are shown in Figure 4.4. Baseline plasma glucose concentrations at the beginning of infusions were affected by milking frequency, with more frequent milking resulting in reduced plasma glucose concentrations (49.6 vs. 56.0 ± 2.1 mg/dL, $P = 0.04$), but there were no differences in glucose turnover rate during the basal phase. During euglycemia, more frequent milking tended to increase glucose turnover rate (179 vs. 158 ± 8.3 g/h, $P = 0.08$, Figure 4.4A) but did not affect endogenous glucose production (Figure 4.4B). No effects of milking frequency were detected during hyperinsulinemia-euglycemia for either endogenous glucose production or glucose turnover rate ($P > 0.83$).

Parity affected circulating insulin concentration during euglycemia; cows in 3rd or greater lactation had elevated insulin compared to those in 2nd lactation (0.37 vs. 0.20 ± 0.05 ng/mL, $P = 0.04$; Figure 4.5A).

Interactions between milking frequency and parity were detected for circulating resistin (Figure 4.5B&C). During euglycemia, for 2nd parity cows, resistin was decreased for cows milked more frequently (76.0 vs. 135.2 ± 29.8 pg/mL), whereas for cows in 3rd+ lactation, those milked more frequently had greater resistin levels (93.9 vs. 57.2 ± 23.8 pg/mL; interaction $P =$

0.09). A similar pattern was detected during hyperinsulinemia-euglycemia (96.5 vs. 153.1 ± 27.5 pg/mL for 3× and 1× milking within parity 2, and 98.4 vs. 43.3 ± 22.1 pg/mL for 3× and 1× milking for parity 3+ cows; interaction $P = 0.03$).

Interactions between milking frequency and anti-inflammatory treatment were detected for circulating glucagon during both euglycemia and hyperinsulinemia-euglycemia (Figure 4.6). Glucagon was decreased for CON cows milked more frequently (24.1 vs. 29.3 ± 4.1 pg/mL), but for cows treated with SAL, those milked more frequently had greater glucagon levels (30.0 vs. 20.3 ± 4.2 pg/mL; interaction $P = 0.08$). The hyperinsulinemic-euglycemic phase showed a similar pattern (14.4 vs. 27.7 ± 4.3 pg/mL for 3× and 1× milking within CON treatment, and 23.1 vs. 17.1 ± 4.4 pg/mL for 3× and 1× milking for SAL treated cows; interaction $P = 0.03$).

mRNA abundance

Relative transcript abundance for liver and adipose tissue can be found in Table 4.3. In agreement with plasma TNFα results, *TNFA* abundance was increased in adipose tissue of cows milked more frequently (fold change 2.41; $P = 0.01$). Milking frequency interacted with parity to affect mRNA abundance in adipose tissue, such that relative expression of *IRS1*, *NFKB1*, *NEF2L2*, *MCP1*, and *TNFA* were all greater in 2nd parity cows milked 3× compared to 1× daily ($P < 0.05$; Figure 4.7), but no differences were detected for older cows. In liver tissue, no differences in mRNA abundance were detected due to milking frequency for *INSR*, *IRS2*, *PCK1*, *PC*, *PPARGC1B*, *NEF2L2*, *NFKB1*, *TNFA*, or *HP* (all $P > 0.40$).

DNA methylation in muscle tissue

Muscle DNA was less methylated for cows milked more frequently ($P < 0.01$). SAL treatment prevented the decrease in global methylation induced by 3× milking in the CON cows (interaction $P = 0.02$; Figure 4.8).

DISCUSSION

Effects of milking frequency on milk yield in early lactation

Effects on milk yield have varied largely due to variation in duration of the altered milking frequency. Decreasing milking frequency for 3 or 6 weeks (Phyn et al., 2014; Eslamizad et al., 2010) or 4 weeks (Schlamberger et al., 2010; Soberon et al., 2011) caused sustained decreases in milk production lasting well beyond the duration of treatment. Shorter periods of 5 days of incomplete milking (Morin et al., 2018; Carbonneau et al., 2012; Krug et al., 2018) showed no long-term effects on milk yield. Other than the decreased frequency of milk removal, mechanisms for reduced milk production include alterations in mammary epithelial cell proliferation (Murney et al., 2015) or apoptosis (Vetharaniam et al., 2003), decreased tight junction integrity between mammary epithelial cells caused by milk accumulation (Stelwagen and Singh, 2014), or altered capacity for nutrient uptake from circulation (Delamaire and Guinard-Flament, 2006). At least for the first week of lactation, any of these mechanisms triggered by altered milk removal apparently have minimal effects on long-term mammary function.

Few studies observed differences in nutrient intake when milking restriction occurred in early lactation. One study, however, combined either a low or high feed allowance with altered milking frequency, and saw that cows milked 1x/d yielded similar milk output regardless of

intake, but for those milked 2x/d, cows with greater feed intake produced more milk (O'Driscoll et al., 2012). This suggests that for the cows milked less often, mammary-related factors such as milk storage or rate of nutrient conversion were limiting for milk production, whereas for cows with increased milk removal, nutrient supply was the more limiting factor in early lactation. Glucose infusion over the first 12 d of lactation increased plasma glucose and tended to increase milk yield (Brown and Allen, 2013). This idea is supported by our data showing more pronounced hypoglycemia in cows milked more frequently, although we did not measure other nutrients such as circulating AAs.

Indicators of energy balance

One key motivation for decreasing milk removal in early lactation is to minimize the negative energy balance experienced by cows after parturition. Although not all studies calculated EB, circulating metabolites can help to characterize tissue mobilization associated with negative energy balance. As we observed, reduced nutrient requirements by the mammary gland allow for increased plasma glucose concentration. Although we did not observe a reduction in lipid mobilization, studies largely agree that reducing milking frequency consistently decreased circulating FFA and BHBA (Soberon et al., 2011; Loiselle et al., 2009; McNamara et al., 2008; Carbonneau et al., 2012), as well as increased plasma glucose concentrations, indicating less demand for mobilization of body stores to support milk production. Interestingly, these effects on plasma analytes were sustained beyond the duration of altered milking frequency, even in short studies with no long-term effects on milk yield (Loiselle et al., 2009; Carbonneau et al., 2012).

Although more frequent milking reduced blood glucose concentrations, we did not observe differences in gluconeogenesis due to milking frequency (measured during basal period on d 5), suggesting that reduced circulating glucose is at least partly due to increased uptake by the mammary gland for milk production. Additionally, endogenous glucose production decreased during euglycemia for all cows, though the effect was not modulated by milking frequency, indicating that liver sensitivity to circulating glucose was unaffected by treatment. Consistent with this, we also did not observe differences in expression for genes related to gluconeogenesis in liver tissue; this agrees with hepatic gene expression microarray data collected from 3 weeks into lactation (Grala et al., 2013). In contrast, reduced milking frequency in late lactation resulted in decreased feed intake, increased plasma glucose, and decreased expression of gluconeogenic genes in the liver (Grala et al., 2016). It is possible that gluconeogenic activity in the liver is already maximized in early lactation as a function of the transition to the lactating state, and that reducing milk output allows for attenuation of hypoglycemia without negatively affecting glucose output by hepatic tissues, although this is contrary to the reduction in hepatic glucose production observed during euglycemia.

Insulin sensitivity

Insulin resistance is thought to be a mechanism for the early lactation dairy cow to help meet her requirements for lactation through mobilization of body stores (Giesy et al., 2012). In this context, we hypothesized that increased nutrient demand by the mammary gland would result in more pronounced insulin resistance accompanying greater tissue mobilization.

Assessment of insulin sensitivity via the hyperinsulinemic-euglycemic clamp revealed no differences in glucose turnover due to frequency of milking. We expected that cows with greater mammary glucose demand for milk production would have the homeorhetic adaptation of greater insulin resistance by other tissues, which would be observed through reduced response to elevated insulin. However, during hyperinsulinemia-euglycemia, circulating glucagon was decreased to a greater extent for cows milked more frequently (at least for CON cows), suggesting that glucagon production by the pancreas was actually more responsive to insulin. This supports the concept that alterations in insulin sensitivity are tissue dependent in early lactation (Zachut et al., 2013), and pancreatic signaling could be altered by other factors associated with milking frequency, such as elevated circulating FFA (Bossaert et al., 2008).

Plasma resistin concentration is also reported to be positively associated with circulating FFA, lipid mobilization, and insulin resistance (Reverchon et al., 2014). Cows fed a high energy diet in late gestation tended to have increased expression of resistin in adipose tissue (Selim et al., 2014). Resistin measured in our study showed parity interactions with milking frequency, where reduced milking frequency was associated with increased resistin for parity 2 cows but decreased resistin for older cows during both euglycemia and hyperinsulimia-euglycemia. This suggests that alterations in resistin signaling due to parity or factors associated with older cows in the transition period may be dependent on the mammary glucose demand or mean levels of glucose in circulation. Resistin also may be involved in pancreatic function, as it regulates both insulin and glucagon secretion in rats (Sassek et al., 2016). Although in mice resistin is thought to be mainly produced by adipocytes, in humans it is secreted more by monocytes and macrophages (Tsiotra et al., 2013). In rats, resistin is also secreted by pancreatic islets (Sassek et al., 2016), and in dairy cows mRNA has been detected in both adipose and mammary tissue

(Komatsu et al., 2003). Further characterization of this adipokine is necessary to evaluate species and tissue differences during various physiological states. Nevertheless, it is possible that circulating resistin in early lactation is related to either adipose or pancreatic tissue function.

Cows with increased nutrient demand had elevated circulating concentrations of TNFα. Studies with ruminants (Kushibiki et al., 2001) have shown that acute TNFα administration increases insulin resistance, although others saw no differences in glucose turnover rate with repeated subacute administration (Yuan et al., 2013). Cows with fatty liver displayed increases in both insulin resistance and plasma TNFα (Ohtsuka et al., 2001). In rodents, TNFα administration has promoted lipid mobilization and increased circulating fatty acids and triglycerides (Ruan et al., 2002; Sethi and Hotamisligil, 1999). Although we did not observe large differences in insulin sensitivity, increased TNFα in cows with increased nutrient demand may help promote adipose mobilization.

Indicators of inflammation

Resistin has also been associated with inflammation and disease states (Filková et al., 2009). We did observe increased expression of inflammatory genes in adipose tissue for 3× vs. 1× cows in 2nd parity cows only. The main effects of parity, however, showed greater abundance of inflammatory transcripts in older cows vs. 2nd parity. The increase in pro-inflammatory gene expression in adipose tissues is consistent with increased plasma TNFα in 3× cows. In mice, TNFα administration increased expression of genes associated with inflammatory responses in adipose tissue (Ruan et al., 2002), and in dairy cows, anti-inflammatory treatment decreased circulating TNFα (Farney et al., 2013a). Reasons for the differential response due to nutrient demand are unclear. In humans, a high fat diet induced elevated plasma levels of TNFα, which

would be consistent with promotion of insulin resistance (Laker et al., 2017). Nutrient restriction of rats during lactation induced an elevation in serum TNFα in adult offspring (Silva et al., 2010). Evaluation of plasma TNFα concentrations in response to intramammary bacterial challenge revealed a greater response for midlactation cows during hyperinsulinemia-euglycemia compared to cows during hypoglycemia (De Matteis et al., 2016), indicating that inflammatory cytokine production is dependent on glucose supply. This is relationship was opposite in our data, however, as 3× cows displayed increased plasma TNFα but reduced circulating glucose. It is possible that the role of TNFα signaling is dependent on stage of lactation. Administration of TNFα in early lactation resulted in altered pituitary and thyroid signaling (Kushibiki et al., 2006), potentially reducing metabolic rate through reduced T3; it is possible that TNFα signaling in early lactation has a broader effect on metabolism than its traditionally described roles related to inflammation or insulin resistance (Bradford et al., 2015).

Milking frequency may have also influenced mammary immunity. We observed a greater decrease in SCC over time for cows milked more frequently. Reduced milk removal may cause increased somatic cells, simply as a function of greater concentration in a decreased total volume of milk, or because less frequent milk removal may allow for increased susceptibility to pathogen invasion. Soberon et al. (2011), however, saw no difference in overall SCC with altered milking frequency, and two methods of incomplete milk removal resulted in reduced SCC compared with control cows (Carbonneau et al., 2012). As milk accumulation in the mammary gland can promote apoptosis of MEC (Vetharaniam et al., 2003) and immune cells are recruited to dispose of dying cells (Monks et al., 2002), this may explain the increased number of cells present in mammary gland secretions with less frequent milking. Immune cell populations and function have also been affected by milking frequency. Polymorphonuclear cells (primarily

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neutrophils) isolated from cows milked less frequently had reduced phagocytic activity and reduced oxidative burst (Moya et al., 2008), but assessment of cell proliferation, migration, respiratory burst, antibody production, and TNFα or IL-4 production by PBMC revealed no differences due to milking frequency (Loiselle et al., 2009). Immune cell profile responses to milking frequency have been inconsistent; lymphocytes as a percent of total immune cells were increased with frequent milking (Carbonneau et al., 2012) but decreased with frequent milking in another study (O'Driscoll et al., 2012); neither group reported differences in total immune cell numbers in circulation. It is likely that immune cell abundance and activity are influenced more by other factors in early lactation than milking frequency alone.

Epigenetic alterations

Studies altering either supply or utilization of nutrients have been conducted to study impacts on epigenetic mechanisms, especially in human clinical settings. Changes in methylation of DNA regions related to muscle function have been observed following exercise intervention programs for type 2 diabetes (Nitert et al., 2012). In a human study altering nutrient supply, those consuming a high fat diet also experienced altered DNA methylation patterns in muscle tissue (Jacobsen et al., 2012). It is difficult to evaluate the longevity of these epigenetic changes, however, as even a single bout of exercise can apparently decrease global methylation in muscle tissue (Barrès et al., 2012). Carrió and Suelves (2015) described studies showing decreased global methylation in muscle tissue with aging phenotypes, or accompanying decreased proliferation and differentiation, and suggested that changes in DNA methylation may play a role in proteolysis. Since methylation decreases availability of DNA for transcription, this may shed light on our study results showing decreased methylation for cows with increased mammary

nutrient demand. Cows experiencing more severe energy balance in early lactation undergo increased nutrient mobilization from adipose as well as muscle tissue to support gluconeogenesis and milk production; it is conceivable that the homeorhetic adjustment to lactation could include epigenetic changes to muscle tissue to promote catabolism.

CONCLUSIONS

We assessed the effects of altered nutrient demand by the mammary gland on insulin sensitivity and energy balance of dairy cows. Although circulating glucose was decreased for cows with increased milk production, we did not observe alterations in glucose turnover, demonstrating greater extramammary adaptation to prevent hypoglycemia. Negative energy balance was attenuated for cows with reduced milk yield, along with decreased plasma TNFα and decreased inflammatory transcripts in adipose tissue of second parity cows. Future studies are needed to characterize epigenetic involvement in early lactation tissue metabolism.

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Table 4.1 Effects of milking frequency on milk production parameters during first 5 d of lactation.

	1× milking	3× milking	SEM	*P*-value	MF[1] × day *P*-value
Milk yield, kg/d	25.0	33.1	1.3	<0.01	0.05
Milk fat % [2]	5.2	4.1	0.26	0.01	<0.01
Protein %	4.87	4.55	0.16	0.18	0.15
Lactose %	4.47	4.56	0.06	0.29	0.02
Milk urea nitrogen, mg/dL	10.1	7.8	0.7	0.04	0.79
Milk fat, kg	1.38	1.42	0.09	0.73	0.01
Protein, kg	1.15	1.43	0.05	0.0006	0.19
Lactose, kg	1.13	1.52	0.06	<0.0001	0.29
logSCC[3]	5.02	4.91	0.19	0.67	0.03
ECM[4], kg/d	35.0	40.4	1.6	0.02	0.33
Water intake, L/d[6]	79.9	83.3	4	0.55	0.15
Dry matter intake (DMI), kg/d[6]	16.9	16.2	0.7	0.46	0.61
ECM/DMI[4,6]	1.96	2.47	0.11	<0.01	0.08
Energy balance (Mcal NE$_L$ /d) [5,6]	-5.12	-10.00	1.26	0.01	0.07

[1] MF = milking frequency
[2] Milk fat % values were subject to square root transformation before analysis; values shown are back-transformed
[3] SCC = somatic cell count; values were subject to natural log transformation before analysis
[4] ECM = Energy corrected milk; calculated as $(0.327 \times \text{milk yield}) + (12.95 \times \text{fat yield}) + (7.65 \times \text{protein yield})$
[5] Energy balance (Mcal/d) = NE$_L$ intake - NE$_L$maint - NE$_L$ milk, where NE$_L$ intake (Mcal/d) was calculated using feed intake and estimated energy content of the feed through lab analysis; NE$_L$maint or the energy maintenance requirement for lactation was calculated as 0.08 mCal per kg BW$^{0.75}$ (NRC, 2001); and NE$_L$ milk = $(9.29 \times \text{fat yield}) + (5.63 \times \text{protein yield}) + (3.95 \times \text{lactose yield})$.
[6] Analysis excluded values from d 5 of study

Table 4.2 Gene primers used to measure transcript abundance in bovine liver and adipose tissue

Gene Symbol	Primer Sequence (5' to 3')	Dilution curve		
		Slope	R^2	E^1
RPS9	F: GAACAAACGTGAGGTCTGGAGG	-3.5468	0.9985	0.914
	R: TTACCTTCGAACAGACGCCG			
RPS15	F: GGCGGAAGTGGAACAGAAGA	-3.2814	0.997	1.02
	R: GTAGCTGGTCGAGGTCTACG			
NFKB1	F: ACCTCCTTCCGCAAACTCAG	-3.5533	0.9985	0.912
	R: ATAGGTCCTTCCTGCCCGTA			
TNFA	F: AAGTAACAAGCCGGTAGCCCA	-3.5108	0.9803	0.927
	R: CTTCCAGCTTCACACCGTTG			
NFE2L2	F: CTCAGCATGATGGACTTGGAGC	-3.4494	0.9977	0.949
	R: AGTAGTTGGCAGACCCACTG			
AP1	F: GACTGCAGCAAGTTCGGTTG	-3.5254	0.9972	0.922
	R: TCAGCTTTCTGTCGGTGCAT			
INSR	F: CGGAGCTCAGAGATCACGACTAT	-3.4796	0.992	0.938
	R: AGGTTCACAGTTAAGTGCTCAGATGA			
IRS2	F: GTTCCAAGCTGTCCATGGAG	-3.5465	0.988	0.914
	R: CTCATGAGCACGTACTGGTC			
PDK4	F: GCTGGCTGGTTTTGGTTACG	-3.3225	0.9959	0.999
	R: CAGCCACTTTTTCCTTCGCC			
PEPCK1	F:	-3.3851	0.9984	0.974
	R:			
PC	F: CGAGGCGTGAAGACCAACAT	-3.3235	0.9787	0.999
	R: CTCTGGGTTCTCGTCGATGAA			
PPARGC1B	F: TGGGGTTCGTTTACAGTCGG	-3.3915	0.9858	0.972
	R: GGTGCCAAAGCCTGAACTTG			
MCP1	F: TCTCGCTGCAACATGAAGGT	-3.2297	0.996	1.04
	R: TATAGCAGCAGGCGACTTGG			

[1] E: Efficiency was tested with 5 serial dilutions and was calculated as $E = (10\wedge(-1/\text{slope})) - 1$

Table 4.3 Transcript abundance in liver and adipose tissues in response to milking frequency

	Liver			Adipose		
	Fold change			**Fold change**		
	(3x relative to 1x)	**SEM**	*P*-value	**(3x relative to 1x)**	**SEM**	*P*-value
AP1	0.84	0.25	0.67	1.51	0.76	0.30
GLUT4	-	-	-	1.11	0.56	0.72
HP	1.06	0.41	0.91	-	-	-
INSR	0.95	0.22	0.87	0.99	0.49	0.97
IRS1	-	-	-	1.29	0.65	0.50
IRS2	1.32	0.66	0.68	-	-	-
LPL	-	-	-	0.94	0.47	0.85
MCP1	1.05	0.23	0.88	2.18	1.09	0.08
NFE2L2	0.88	0.28	0.77	1.43	0.72	0.16
NFKB1	1.11	0.40	0.83	0.98	0.49	0.96
PC	1.17	0.46	0.78	-	-	-
PDK4	0.8	0.63	0.69	-	-	-
PEPCK1	1.09	0.75	0.92	-	-	-
TNFA	1.15	0.16	0.47	2.41	1.2	**0.01**

Multiparous cows were milked either once or thrice daily for the first 5 days of lactation. On d 5, tissue biopsies were performed 2 h after hyperinsulinemic-euglycemic clamp. Transcript abundance was measured relative to mean of *GAPDH* and *RPS9* abundance. Values are expressed as fold change of 3x milking relative to 1x.

FIGURES

Figure 4.1 Effect of milking frequency on milk yield, feed intake, and milk components

Multiparous cows were milked either 1× or 3× daily for the first 5 days of lactation. Milk samples were collected at all milkings; measured values for 3× cows were averaged within day before statistical analysis. Milk yield and milk fat data were analyzed using predicted transmitting ability for milk volume or milk fat as covariates.

A: Effect of milking frequency (**MF**) on milk yield $P < 0.01$; interaction with time $P = 0.05$.

B: Effect of milking frequency on dry matter intake (**DMI**) $P = 0.46$; interaction with time $P = 0.61$.

C: Effect of milking frequency on milk fat content $P = 0.01$; interaction with time $P < 0.01$. Values were subject to square root transformation for analysis; figure shows back-transformed data.

D: Effect of milking frequency on milk lactose content $P = 0.29$; interaction with time $P = 0.02$.

E: Effect of milking frequency on milk somatic cell count $P = 0.67$; interaction with time $P = 0.03$. Data were subject to natural log transformation for statistical analysis; values shown are back-transformed.

F: Effect of milking frequency on milk protein yield $P < 0.001$; interaction with time $P = 0.19$.
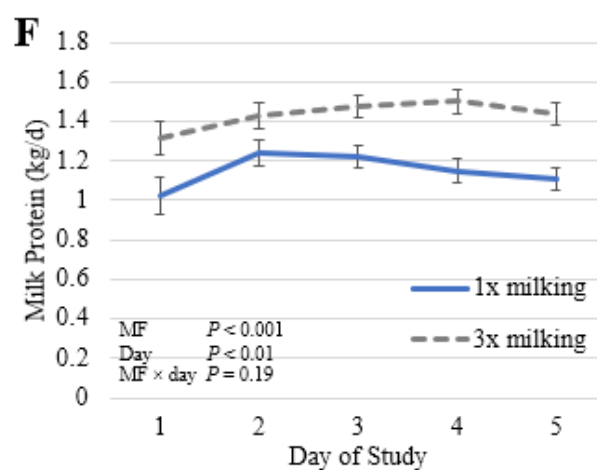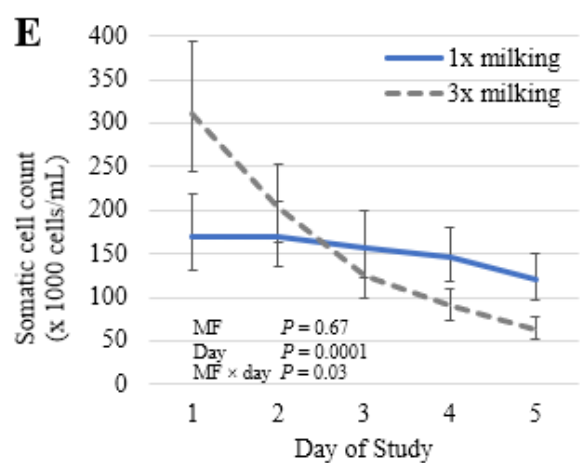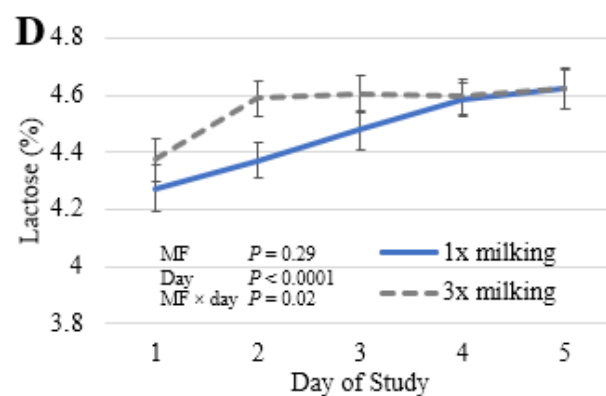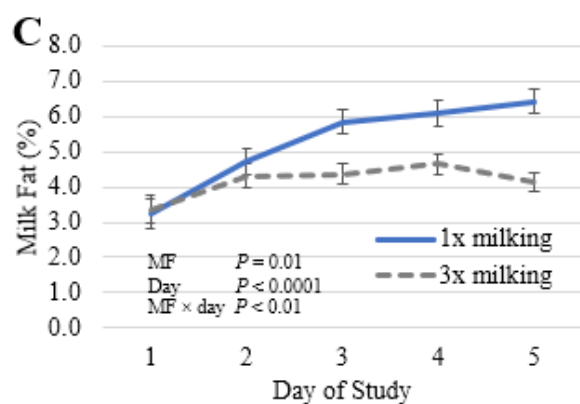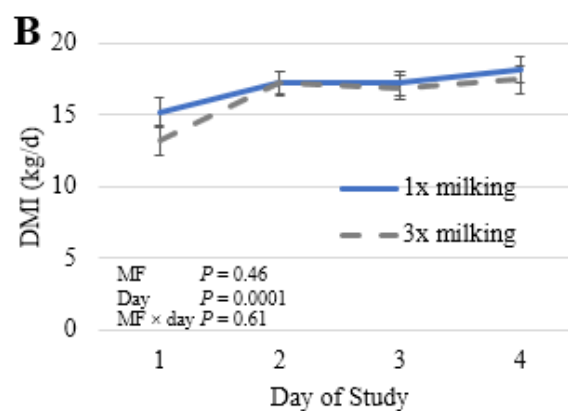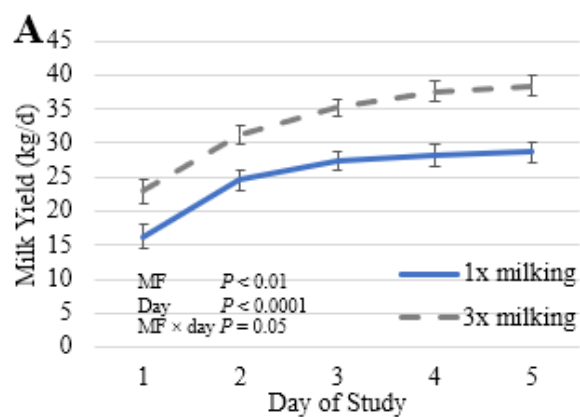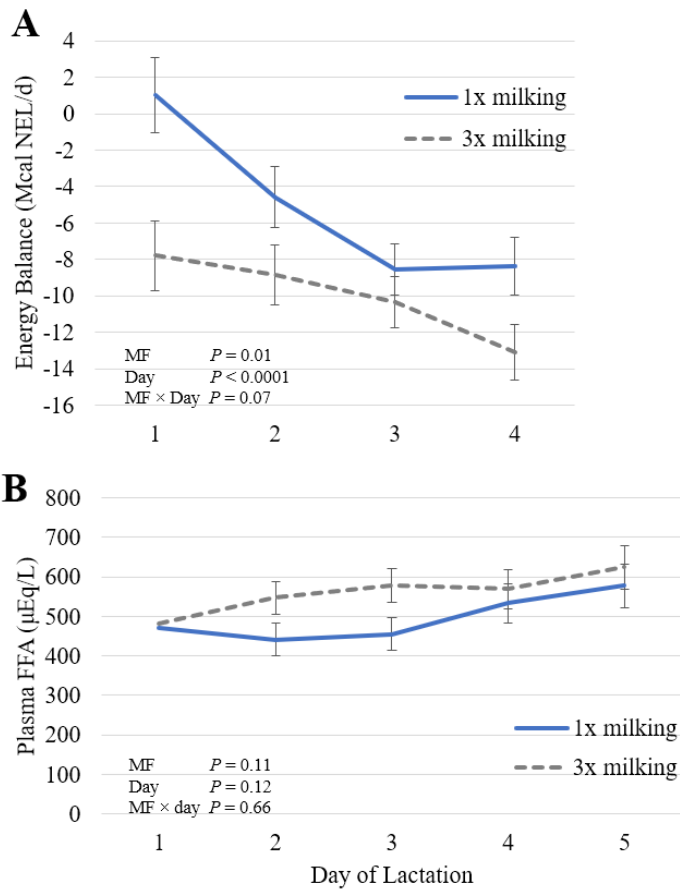
Figure 4.2 Effects of milking frequency on energy balance indicators in early lactation

Multiparous cows were milked either 1× or 3× daily for the first 5 days of lactation. Energy balance (panel A) was calculated as **EB (Mcal/d) = NE$_L$ intake - NE$_L$maint - NE$_L$ output**, where NE$_L$ intake (Mcal/d) was calculated using feed intake and estimated energy content of the feed through lab analysis; NE$_L$maint or the energy maintenance requirement for lactation was calculated as 0.08 mCal per kg BW$^{0.75}$ (Dairy NRC 2001); and energy required for milk output was calculated as NE$_L$ output = (9.29 × fat yield) + (5.63 × protein yield) + (3.95 × lactose yield). Effect of milking frequency (**MF**) $P = 0.01$; interaction with time $P = 0.07$. Day 5 values were excluded from energy balance analysis due to hyperinsulinemic-euglycemic clamp assessment of insulin sensitivity on d 5. Blood samples were collected once daily at 0600 h and analyzed for plasma free fatty acids (FFA; panel B) with d 1 value as covariate, $P = 0.11$; interaction with time $P = 0.66$.

Figure 4.3 Effect of milking frequency on plasma metabolites measured over first 5 d of lactation

Cows were milked either 1× or 3× daily, in combination with either sodium salicylate (SALS) or control (CON) treatment via the drinking water, for the first 5 days of lactation. Plasma samples were collected once daily prior to morning feed delivery. Metabolites were analyzed using d1 values as covariates, and included insulin (panel **A**; effect of milking frequency $P = 0.02$; interaction of milking frequency (**MF**) and time $P = 0.02$), glucose (panel **B**; effect of milking frequency $P = 0.27$; interaction of milking frequency with anti-inflammatory treatment (NSAID) and day $P = 0.01$), glucagon (not significant), resistin (panel **C**; effect of milking frequency $P = 0.27$; interaction of milking frequency and anti-inflammatory treatment $P = 0.08$), and TNFα (panel **D**; effect of milking frequency $P = 0.007$, interactions $P > 0.25$).
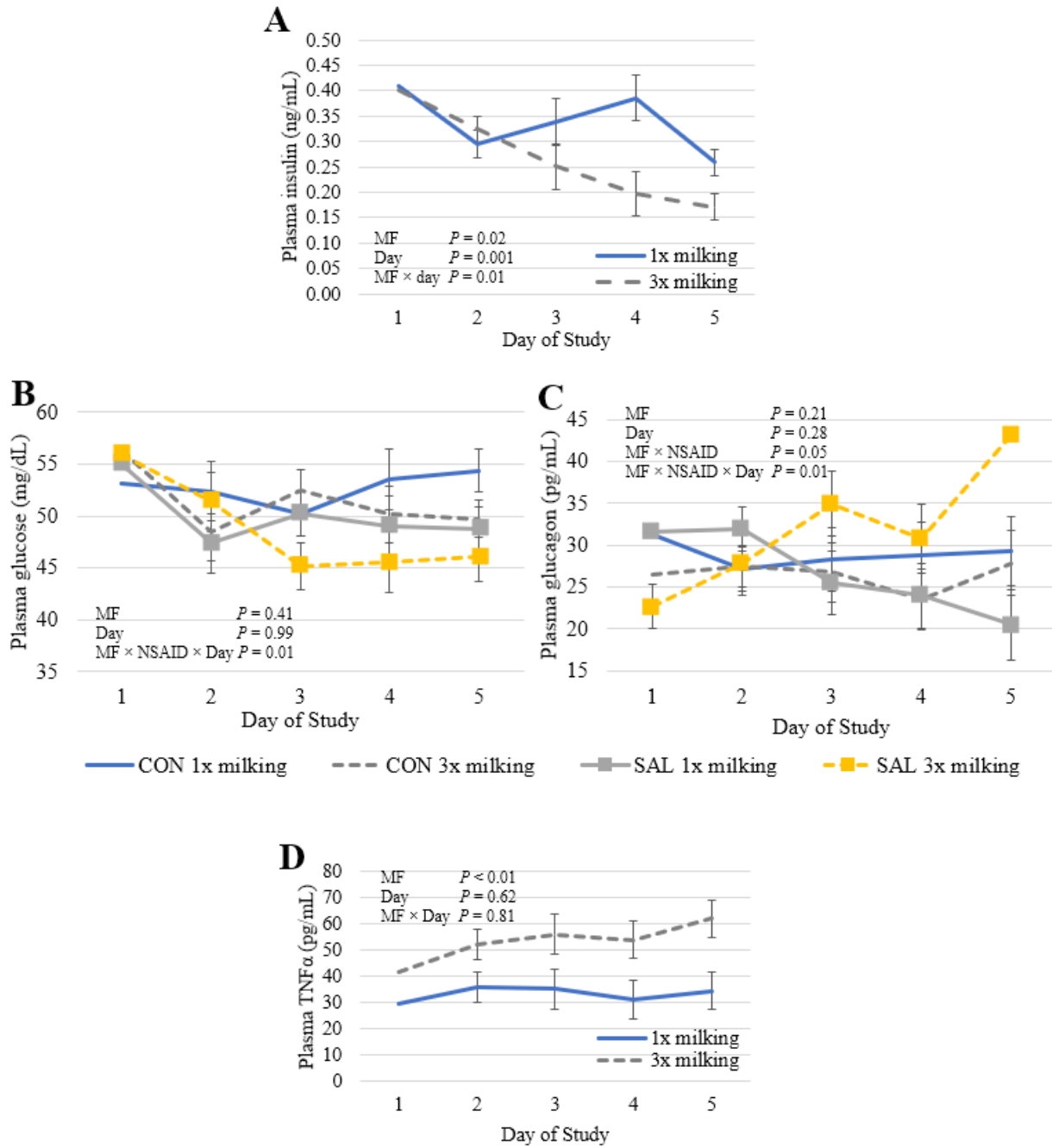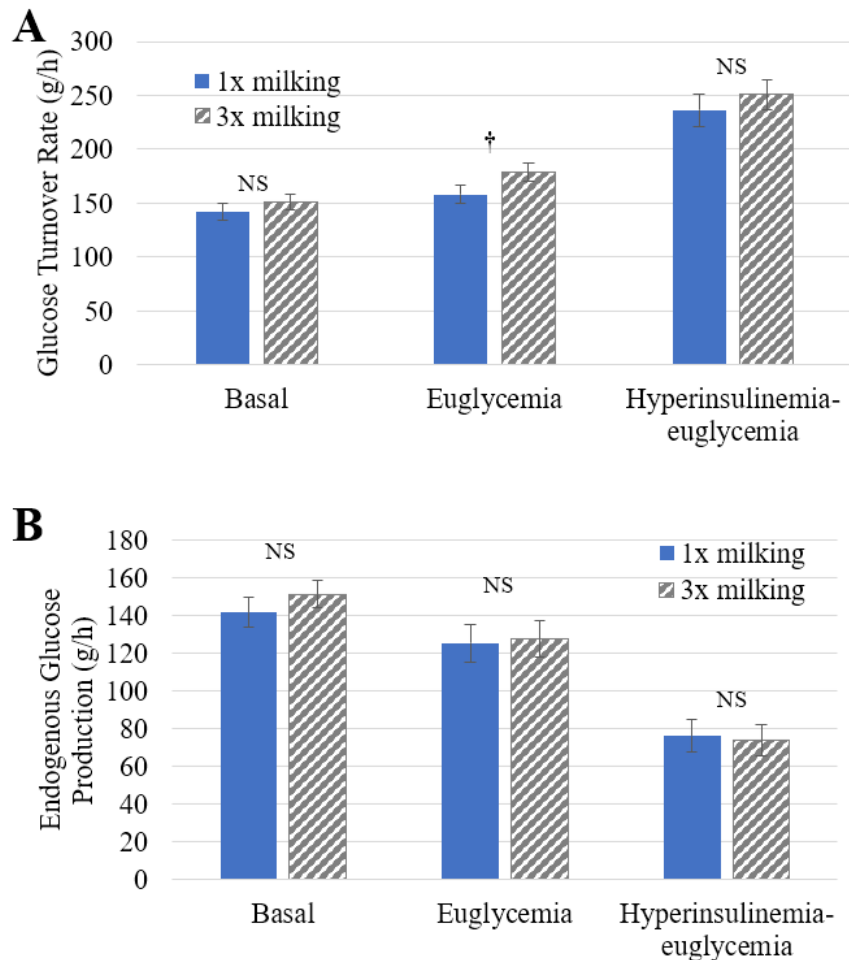
A

MF   *P* = 0.02
Day   *P* = 0.001
MF × day *P* = 0.01

— 1x milking
-- 3x milking

B

MF   *P* = 0.41
Day   *P* = 0.99
MF × NSAID × Day *P* = 0.01

C

MF   *P* = 0.21
Day   *P* = 0.28
MF × NSAID   *P* = 0.05
MF × NSAID × Day *P* = 0.01

— CON 1x milking   -- CON 3x milking   ■ SAL 1x milking   ■ SAL 3x milking

D

MF   *P* < 0.01
Day   *P* = 0.62
MF × Day *P* = 0.81

— 1x milking
-- 3x milking

Figure 4.4 Effect of milking frequency on glucose kinetics

Glucose kinetics were assessed on d 5 of lactation for cows milked either 1× or 3× daily. Basal measurements of plasma followed 2 h of tracer infusion. Measurements during euglycemia followed 2 h of continuous glucose infusion, fluctuating as required to achieve plasma glucose concentration of 60 mg/dL. Measurements for hyperinsulinemia-euglycemia followed 2 h of insulin infusion to achieve hyperinsulinemia, accompanied by glucose infusion to maintain blood glucose levels of 60 mg/dL. Glucose turnover rate (panel A) was calculated as the tracer infusion rate divided by the measured enrichment in plasma, adjusted for background (effect of milking frequency on glucose turnover rate during euglycemia only was $P = 0.08$). Endogenous glucose production (panel B) was calculated as the rate of dextrose infusion subtracted from the glucose turnover rate. No effect of milking frequency was detected for endogenous glucose production within any of the 3 phases ($P > 0.37$), but the rate of glucose production between phases was significantly affected by phase ($P < 0.01$).

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Figure 4.5 Effect of parity and milking frequency on circulating plasma metabolites during glucose clamp

Cows were milked 1 × or 3 × daily in combination with either sodium salicylate (SAL) or control (CON) treatment administered via the drinking water for the first 5 days of lactation. Plasma glucagon was assessed in samples drawn following 2 h of continuous glucose infusion, fluctuating infusion rate as required to achieve plasma glucose concentration of 60 mg/dL (euglycemia). Measurements for hyperinsulinemia-euglycemia followed 2 h of insulin infusion to achieve hyperinsulinemia, accompanied by glucose infusion to maintain blood glucose levels of 60 mg/dL. Insulin during euglycemia (panel A) was affected by parity ($P = 0.04$; effect of milking frequency $P = 0.19$, interaction $P = 0.14$). No main effects of milking frequency were detected for resistin but interactions with parity were detected during euglycemia ($P = 0.09$; panel B) and during hyperinsulinemia-euglycemia ($P = 0.03$; panel C).
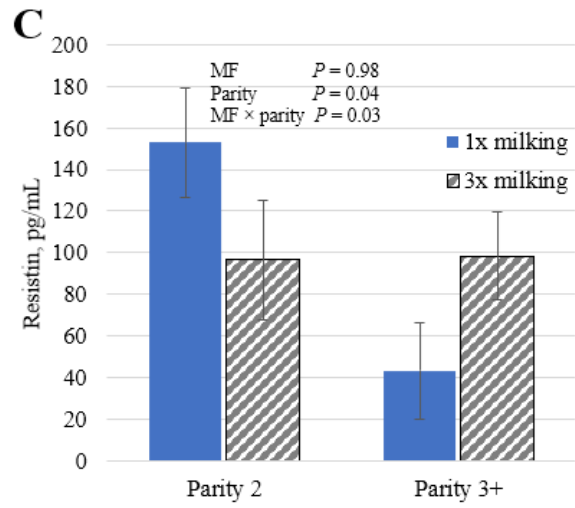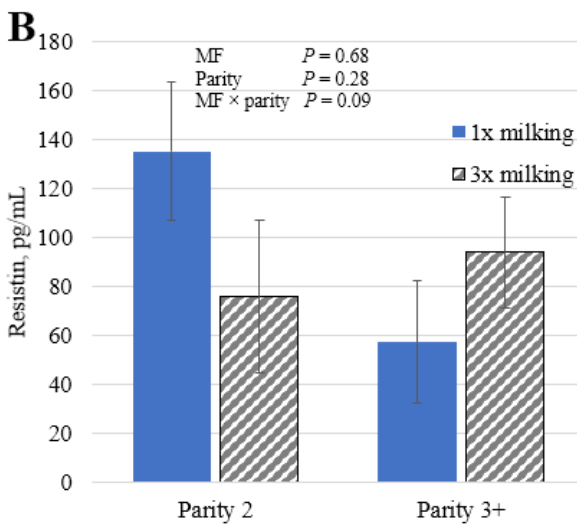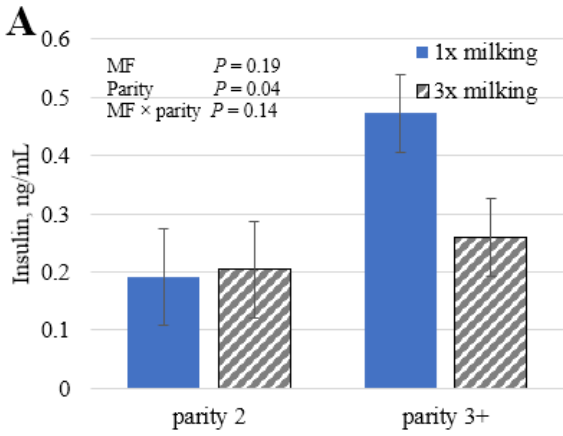
A

MF $P = 0.19$
Parity $P = 0.04$
MF × parity $P = 0.14$

■ 1x milking
▨ 3x milking

Insulin, ng/mL

parity 2   parity 3+

B

MF $P = 0.68$
Parity $P = 0.28$
MF × parity $P = 0.09$

■ 1x milking
▨ 3x milking

Resistin, pg/mL

Parity 2   Parity 3+

C

MF $P = 0.98$
Parity $P = 0.04$
MF × parity $P = 0.03$

■ 1x milking
▨ 3x milking

Resistin, pg/mL

Parity 2   Parity 3+

144

Figure 4.6 Effect of milking frequency on plasma glucagon during euglycemia and hyperinsulinemia-euglycemia

Cows were milked 1× or 3× daily in combination with either sodium salicylate (SAL) or control (CON) treatment administered via the drinking water for the first 5 days of lactation. Plasma glucagon was assessed in samples drawn following 2 h of continuous glucose infusion, fluctuating as required to achieve plasma glucose concentration of 60 mg/dL (euglycemia). Measurements for hyperinsulinemia-euglycemia followed 2 h of insulin infusion to achieve hyperinsulinemia, accompanied by glucose infusion to maintain blood glucose levels of 60 mg/dL. No main effects were detected but interaction of milking frequency and anti-inflammatory treatment was $P = 0.08$ during euglycemia, and $P = 0.03$ during hyperinsulinemia-euglycemia.
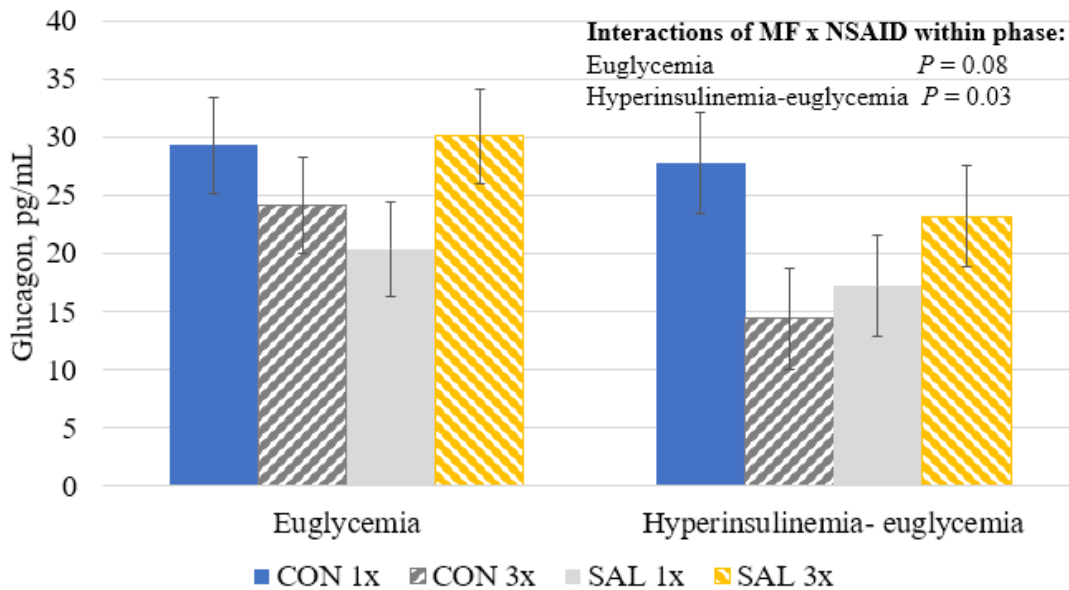
Figure 4.7 Effects of milking frequency and parity on mRNA abundance of adipose tissue on d 5 of lactation

Tissue samples were collected on d 5 of lactation from cows that were milked 1 × or 3 × daily.

mRNA abundance in adipose tissue was calculated relative to the mean of *GAPDH* and *RPS9*;

fold changes were calculated by the ddCt method. Milking frequency interacted with parity such

that genes shown here were differentially expressed for 2nd parity cows only (*$P < 0.05$; **$P < 0.01$); no differences were detected for cows of parity 3 and greater.
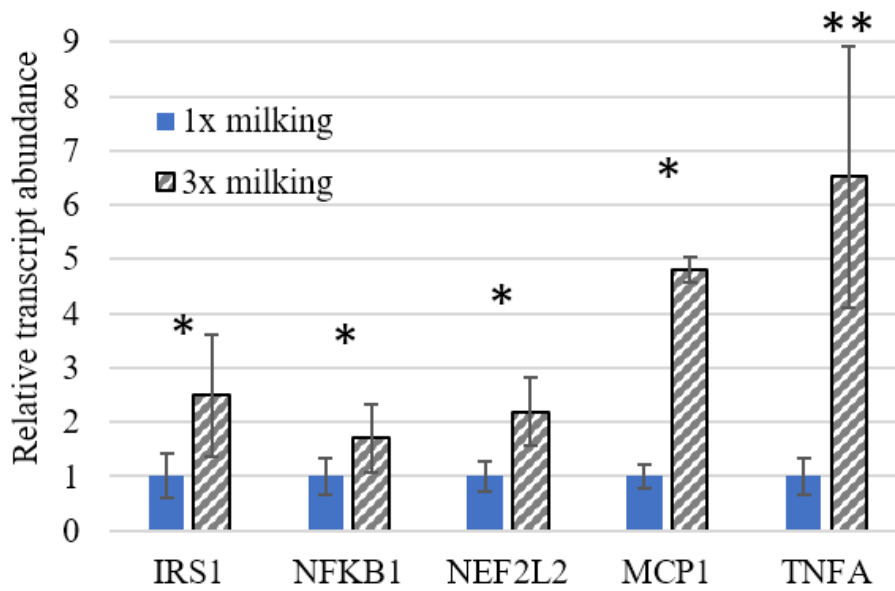
Figure 4.8 Effect of milking frequency and anti-inflammatory treatment on global DNA methylation of muscle tissue on d 5 of lactation.

DNA was extracted from muscle tissue collected on d 5 of lactation from cows milked $1 \times$ or $3 \times$ daily, and treated with either SAL (sodium salicylate; dose) or CON (control). Global DNA methylation was assessed via colorimetric assay. Effect of milking frequency $P < 0.01$, interaction of milking frequency and NSAID treatment $P = 0.02$.
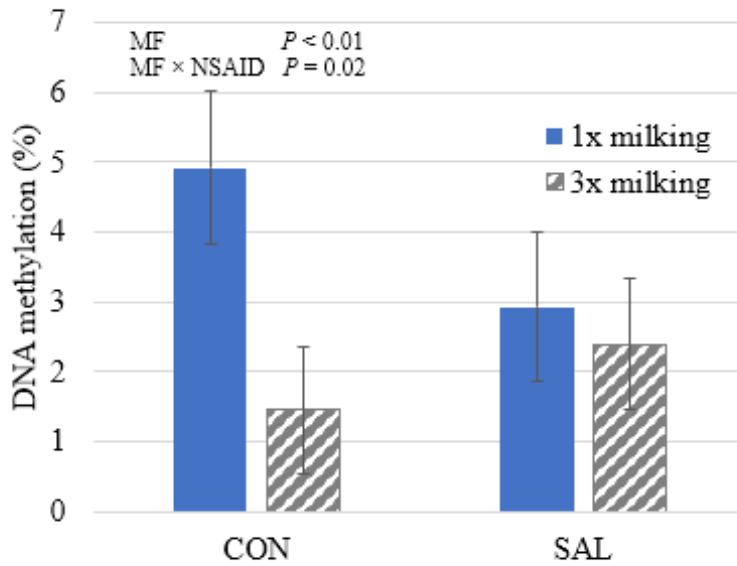
Chapter 5 - Implications and Future Directions

For decades, dairy producers and scientists have been seeking ways to further improve the health and performance of the transition cow. The physiological adaptations occurring during this period are required for the transition to a lactating state; however, exaggerated or prolonged deviations from homeostasis can lead to increased risk for metabolic disorders. In this dissertation, three factors were discussed that can be considered during management of transition cows: anti-inflammatory treatment, cow body condition prior to calving, and short-term reduction in milking frequency in early lactation.

Such is the nature of scientific inquiry that many of our working hypotheses were either disproven or inconclusive. However, negative results, or unexpected results, can also lead to valuable conclusions and new directions of inquiry. In chapter 2, we hypothesized that long-term changes in milk yield following anti-inflammatory treatment would be caused by increased mammary epithelial cell proliferation or transcriptional changes associated with milk component synthesis. Results did not support this, but were not conclusive as cows also did not exhibit increased milk production in response to anti-inflammatory treatment in this particular study. Transcriptional changes in mammary tissue over time produced surprising results in the enrichment of pathways related to immune activation in early lactation. In light of the literature reviewed in chapter 1 highlighting the immune cells and associated activity that are prominent in the mammary gland during development and remodeling, the transcriptional changes observed were consistent with the upregulation of immune signaling during the remodeling of the mammary gland that occurs around the time of lactogenesis.

We also detected changes in global DNA methylation in mammary tissue in response to NSAID treatment, suggesting that epigenetic changes could potentially be involved in the long-term milk production response. This presents an avenue for further inquiry; follow-up investigation of which specific regions of the genome are affected by NSAID treatment may clarify whether changes to methylation occur in promotor regions connected to milk component synthesis. Additionally, other anti-inflammatory strategies (i.e., plant extracts) have also produced similar effects on long-term milk yield, and evaluation of tissue samples from other such studies may show whether these treatments elicit their effects through similar mechanisms. Collectively, these investigations may lead to additional strategies for reducing excess inflammation in the transition period while positively affecting milk production.

In chapter 3, we hypothesized that cows of divergent adiposity would secrete distinct profiles of microRNA in colostrum, with the rationale that many of the microRNA in colostrum are predicted to regulate immune function, and that cows with excess adiposity at parturition are often more immune suppressed, have greater inflammation, and are at greater risk for developing metabolic disorders. However, we saw few differentially-expressed microRNA in colostrum associated with either cow body condition or circulating plasma free fatty acids. It is likely colostrum contains a core profile of regulatory miRNA that are involved in the onset of lactation. Future research could examine the importance of these microRNA during the first days of life for the calf ingesting the colostrum, as well as the content of other immune-regulatory components in colostrum and how they might be affected by improved metabolic health of transition cows. These questions could lead to more nuanced colostrum management strategies with benefits for both the dam and the neonate. Additionally, in chapter 3 we evaluated a tool for the

bioinformatics portion of small RNA analysis, in comparison with a similar tool that is widely used but can be challenging for those researchers with limited knowledge of computer programming. Our results were largely consistent between the 2 programs, suggesting that this is a valuable tool to increase accessibility of small RNA analysis to a broader population of researchers.

The study outlined in chapter 4 examined the effect of milking frequency, or nutrient demand by the mammary gland, on insulin sensitivity and energy balance in early lactation cows. We hypothesized that the degree of insulin resistance exhibited by these cows would depend on the intensity of negative energy balance, or the magnitude of the difference between nutrient intake and nutrient output. Results showed that insulin sensitivity was not affected by the improvement in energy balance due to reduced milking frequency, and differences in glucose uptake from blood were likely due to insulin-independent mechanisms (i.e., the mammary gland). This study also showed alterations in DNA methylation, this time in muscle tissue, which may be related to increased muscle mobilization to support the increased milk protein yield that was observed in the cows milked more frequently. This observation could lead to future research asking whether nutritional supplementation of methyl donors, or even dietary protein, could modulate methylation or catabolism of muscle tissue in early lactation and thus mitigate the tissue mobilization that occurs to offset negative energy balance.

These studies have contributed to the collective body of knowledge around transition cow physiology, especially related to inflammation and energy balance. Research in the 1990's already explored the importance of energy balance in the transition period, and studies in the

early 2000's highlighted inflammatory mediators and insulin resistance. Despite this, advancements in technology steadily present new tools and analysis methods such that we are now monitoring changes at the transcriptome and genome level related to some of these transition cow physiological phenomena. These tools allow for more in depth exploration and greater understanding of both old and new research questions. Moving forward, greater understanding of the adaptations occurring during the transition period, and greater clarification of which are necessary (and to what degree) vs. which should be mitigated, will allow for more successful management of dairy cow operations and improve the health, production, and longevity of our dairy cow population.