AN EVALUATION OF TISSUE MOBILIZATION AND CHRONIC CIRCADIAN DISRUPTIONS IN TRANSITION DAIRY COWS

by

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ACRONYMS

3-MH	3-Methylhistidine
ADF	Acid Detergent Fiber
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BCS	Body Condition Score
BEC	Before Expected Calving
BFD	Backfat Depth
BHB	β-hydroxybutyrate
BW	Bodyweight
CON	Control
CP	Crude Protein
CRE	Creatinine
CTS	Circadian Timing System
DA	Displaced Abomasum
DIM	Days in Milk
DMI	Dry Matter Intake
EBP	Empty Body Protein
HEC	Hypoinsulinemic Euglycemic Clamp
HM	High Muscle
HOT	Hepatic Oxidation Theory
IVGTT	Intravenous Glucose Tolerance Test
LDD	Longissimus Dorsi Depth
LM	Low Muscle
ME	Metabolizable Energy
MESOR	Midline Estimating Statistic of Rhythm
NAD	Nicotinamide Adenine Diphosphate
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NDF	Neutral Detergent Fiber
NEFA	Nonesterified Fatty Acid
NE_L	Net Energy of Lactation
PP	Postpartum
PS	Phase Shift
RQUICKI	Revised Quantitative Insulin Check Index
SAM	S-adenosyl methionine
THF	Tetrahydrofolic Acid
VLDL	Very Low-Density Lipoprotein

ABSTRACT

A successful dairy cow transition period is paramount to a productive lactation. Termed as the several weeks before and after calving, the transition period is characterized by several metabolic changes that occur in all tissues to support copious amounts of milk production in early lactation. Due to insufficient intake in early lactation, cows undergo tissue mobilization whereby they reduce their insulin sensitivity to liberate adipose and muscle tissue to meet nutrient requirements. However, physiological stresses during this period are responsible for an increased risk of disease, which animal welfare and thus productivity. Furthermore, a cow's metabolism is regulated by the circadian system, which integrates timing cues from the environment to coordinate the body's physiological systems to regular occurring daily events. Disruptions to the timing of events can negatively affect animal health and thus potentially lactation performance. Sixteen multiparous Holstein cows were subjected to either a control (CON) consistent timing of the light-dark cycle or a 6 h forward shift light dark cycle phase (Phase Shift; PS) every 3 d in the last thirty-five days before expected calving (BEC). Once animals calved, they were given CON timing of light through 60 days in milk (DIM). Secondly across two phase-shift studies, 48 multiparous Holstein cows had ultrasound measurements collected at the 12^{th} intercostal space and blood metabolite markers of tissue mobilized analyzed at various timepoints between 35 d BEC and 60 DIM. Cattle that had a longissimus dorsi depth (LDD) >4.10 cm at 35 d BEC were determined to be in a high muscle (HM) group whereas those below that threshold were relegated to a low muscle (LM) group.

Cattle that were exposed to the PS light pattern in the late gestational period were determined to have a disrupted, weaker circadian rhythm than CON animals as a result of a decreased internal temperature circadian rhythm amplitude and delayed start of the daily circadian

rhythm. These circadian disruptions resulted in PS cows developing greater insulin resistance than CON cows at 14 d BEC (4,303 vs. 2,386 mIU Insulin AUC/180 min) and at 7 DIM (1,053 vs. 697 mIU Insulin AUC/180 min) after the light timing treatment was removed. The PS timing of light was associated with reduced mammary development at 21 d BEC (5.22 vs. 12.44% epithelial proliferation) and those animals produced less milk compared to CON through 60 DIM (40.3 vs. 42.6 kg/d). Thereby demonstrating the importance of maintaining consistency in the timing of events during the dry period to maximize animal health and performance through early lactation.

HM cattle mobilized more muscle tissue than LM cows from 35 d BEC through 60 DIM (1.64 vs. 0.30 cm LDD mobilized) to have no difference in LDD at 60 DIM (3.41 vs. 3.09 cm). While there were no differences in milk production (40.6 vs. 42.0 kg/d), milk components, or intake between HM and LM cows, there was a tendency for HM cows to give birth to heavier calves (44.3 vs. 42.3 kg) and have greater 3-MH:CRE ratio (0.153 vs. 0.135) over the first three weeks of lactation. Overall, cattle mobilized muscle and adipose tissue reserves through 30 DIM relative to the amount of those reserves they had available at 21 d BEC (R^2=0.47 and 0.82, respectively). Thereby suggesting that cattle tissue reserves in the last few weeks of gestation affect the nutrient source that cattle primarily use to meet nutrient requirements from late gestation into early lactation. Continued research will need to address how alterations in the timing of events and quantity of tissue reserves through the lactation period affect cattle performance and heath in the postpartum period.

CHAPTER 1. LITERATURE REIVEW

1.1 Introduction

The non-lactating dry period of the dairy cow has been named the most critical period of the lactation cycle. This period determines lactation success, and includes mammary gland involution and subsequent mammogenesis and colostrogenesis in coordination with whole body metabolic changes in the cow (Bell, 1995; De Vries et al., 2010). External environmental factors such as heat stress, the length of daylight, and the timing of environmental events, can affect mammary gland development during this period and thus in turn impact milk yield in the subsequent lactation (Wall et al., 2005; Tao et al., 2011). At the systemic level, chronic episodes of exposure to these disruptors can affect the central clock circadian timing system (CTS), which influences the timing of physiological systems across the body.

Additionally, the efficiency at which cattle transition from the dry period into lactation is one of the main indicators of lactation success. Approximately 75% of the diseases that occur throughout a lactation cycle occur during the transition period, and so it is of interest for researchers and dairy managers alike to set cows up for lactation success (Leblanc, 2010; Roberts et al., 2012). One main reason for the development of disease is due to the dairy cow's inability to consume sufficient nutrients in early postpartum to meet her lactational nutrient requirements. This causes her to mobilize adipose and muscle tissues in early lactation to bridge this nutrient gap. An over-mobilization of tissue results in the development of metabolic disorders (Fronk et al., 1980; Overton and Waldron, 2004), whereas insufficient tissue reserves at the onset of lactation causes the animal to suffer low milk production and poor reproductive performance (Roche et al., 2009). Thus, the dairy cattle transition period should be under focus by farm managers through ensuring

consistency of the cow's environmental events and monitoring tissue reserves to maximize animal health and lactation performance.

1.2 Dairy Cow Transition Period

The non-lactating, dry period is the time in the lactation cycle when the cow prepares for parturition and the subsequent lactation. To do this, cows must coordinate their nutrient flow from the late-term development of the fetus to yielding high amounts of milk production in early lactation. Disruptions in the timing of nutrient availability and stores can alter cow's ability to have a successful transition to lactation and negatively impact health and production performance.

1.2.1 Late gestation requirements

On the typical dairy farm, milking is discontinued approximately 60 days before expected calving (Watters et al., 2008). This allows for the reconstruction of the mammary parenchymal tissue and remodeling of the mammary gland to prepare for the subsequent lactation (Capuco et al., 1997; DeVries et al., 2010). In the last three weeks of the dry period, cows are predicted to consume dry matter intake as modeled by a percent of body weight by the formula $1.97\text{-}(0.75\times \frac{e^{0.16\times(DaysPreg-280)}}{100\times BW})$ $(\mathrm{NRC}, 2001)$. Using this model, a 700 kg cow at 250 days pregnant would be expected to consume 13.8 kg of dry matter per day. As of the most recent NRC, it is hypothesized that dry cows require their diet density to contain a minimum of 12% crude protein (CP) along with an energy density of 1.25 Mcal NE_L/kg DM. However, if a diet is formulated for primiparous animals, energy and protein density should be increased above 12% and 1.62 Mcal NE_L/kg DM, due to primiparous animal's greater nutrient requirements to support their current pregnancy as well as the animal's growth.

As cows move further into the late gestation period, they increase the amount of nutrients they partition to the developing fetus as nutrient requirements of the fetus increase at an increasing rate (Bell et al., 1995). Likewise, dairy cattle's intake decreases dramatically in the last few days prior to calving as the cow prepares to go through the high stress event of parturition (Drackley, 1999). To overcome this, diet nutrient density should be increased for all cows in their last few weeks prior to calving so they can meet the developing fetus requirements and prevent the animal from sacrificing their own tissue reserves prematurely. However, it is important to not over provide energy too early in the late gestation period to avoid excessive body condition gain as will be discussed later.

Late gestation requirements for metabolizable energy (ME) are [(0.00318 x D-0.0352) × (CBW/45]/0.14 where D is the day of gestation between 190-279 days of pregnancy and CBW is calf birth weight (kg). To convert from ME to NE_L there is a coefficient of efficiency of 0.64. Therefore, a 250-d pregnant cow that is giving birth to a 45 kg calf would have an ME requirement of 5.43 Mcals and a NE_L requirement of 3.47 Mcals/day. On the protein side, the NRC suggests that close-up dry cows should be provided 850 g/d of metabolizable protein in a diet that provides rumen degradable protein to makeup 10-11% of the diet (NRC, 2001). In a study where cows were fed 14.4% crude protein during the dry period, they demonstrated a relative but no statistical difference in milk yield in the subsequent lactation compared to cows provided an 11.7% crude protein diet (Robinson et al., 2001). However, cows do not have a crude protein requirement but rather a requirement for individual amino acids. Several nutritionists believe dry cow diets should be formulated for more protein than the NRC at 1,300 g of metabolizable protein/d, which includes 35 g of metabolizable methionine and 90 g of metabolizable lysine to meet the majority of animal's requirements (French, 2016). This means that dry cows should be provided with diets that are

around 11-13% crude protein and have a MP density of 90-100 g/kg DM (van Saun and Sniffen, 2014). Given its ubiquitous role in multiple pathways as will be discussed later, feeding supplemental methionine during the dry period has shown benefits in milk yield, milk protein percentage, and immune function in the corresponding fresh period (Ordway et al., 2009; Osorio et al., 2013; Vailati-Riboni et al., 2017). Therefore, feeding the correct amino acids and not necessarily more crude protein during the dry period can have multiple benefits for lactation performance.

1.2.2 Early lactation requirements

At the completion of pregnancy and the beginning of lactation, a cow's nutrient requirements drastically increase in accordance with a NE_L requirement of milk using the equation 0.0929 × Fat% +0.0547 × CP% +0.0395 × Lactose % per kg milk to calculate the Mcal required per kg of dry matter (NRC, 2001). Milk production increases progressively throughout the first two months of lactation until cows reach peak milk yield, which can be more than 70kg/d in the highest producing herds and is generally around 50 kg/d in the average herd. Thus, a cow producing 70 kg of 3.5% fat milk, 3.2% CP, and 5.0% lactose per day would require approximately 48.8 Mcals of energy whereas, a cow producing 50 kg of similar components milk has an energy requirement of 34.9 Mcals of energy. In accordance with the coordination to achieve peak milk yield, dry matter intake of an early lactation increases through the predicted dry matter intake formula, kg/d=$(0.372 \times FCM + 0.0968 \times BW^{0.75} \times (1 - e^{-0.192 \times (WOF + 3.67)})$, where WOF=week of lactation and fat corrected milk is 4% corrected milk yield per day (NRC, 2001). There is an estimated protein conversion efficiency for lactation of 0.67, therefore an animal's requirements should be adjusted by the milk protein yield/0.67 to predict the amount of protein required for lactation. Therefore, a cow producing 45 kg of 3.2% true protein, which would yield

1.44 kg of milk protein, would require 2.15 kg of metabolizable protein. Thus, with appropriate adjustments in place to account for between animal variation in feed consumption, dairy farms readily feed diets that contain 16% CP and 1.70 Mcal/kg of dry matter intake to meet cows' respective protein and energy requirements (Rabelo et al., 2003; Linn et al., 2018).

1.2.3 Homeorhesis: The transition to a new metabolic state

To shift nutrient partitioning from the fetus in late gestation to high yielding milk production in early lactation, cows must undergo homeorhesis (Bauman and Currie, 1980). Unlike homeostasis, which regulates the animal to return to a set point or a marked normal, homeorhesis is the coordination of tissues to adapt to the conditions of a new normal such as the adaptation to lactation in mammals or annual hibernation observed in other species. As observed previously in the marked differential in nutrient requirements between the physiological states, several changes occur at the systemic and individual organ level to coordinate nutrient flows to support milk production.

Due to the heightened nutrient demand in early lactation for milk production, a greater proportion of nutrients are partitioned towards the mammary gland. The two tissues used for nutrient supplies include adipose and muscle tissue. These tissues reduce the amount of accretion and instead demonstrate increased rates of lipolysis and proteolysis, respectively (Drackley, 1999). Furthermore, cows reduce their peripheral insulin sensitivity, to facilitate nutrient liberalization from peripheral tissues and spare nutrients from the diet for the mammary gland. This insulin resistance phenomenon has been found to occur in all cows no matter their body condition score or the energy density of the diet offered in late gestation (Mann et al., 2016).

With the transition to lactation, there is an increased need for glucose for mammary epithelial cell maintenance and lactose synthesis; the key milk volume osmoregulator (Chaiyabutr

et al., 1980). To meet this enhanced glucose requirement, cows must convert propionate, produced in the rumen, into glucose in the liver through the process of gluconeogenesis. In addition to the liver's role in synthesizing glucose in early lactation, there is also an increased demand for the liver to oxidize mobilized fatty acids into ATP or partially oxidize them into ketone bodies. Therefore, in early lactation the cow's liver can grow in size by as much as 8% from 10 to 22 DIM, which corresponds with an upregulated expression of transcripts related to fatty acid metabolism (Reynolds et al., 2004; McCabe et al., 2012). To meet the cow's heightened milk yield, the mammary gland accordingly increases its mammary epithelial cell proliferation during the transition period (Capuco and Choudhary, 2020). The success at which cattle respond to their shifting nutrient requirements from providing for the fetus in late gestation to high milk production in early lactation can determine lactation and health outcomes (Zebeli et al., 2015). Failure to ensure a successful transition can result in negative health outcomes, which impact animal welfare, farm profit, and increase the likelihood that that animal will be removed prematurely from the herd.

1.2.4 Transition Health Outcomes

In the first few weeks of lactation, cows exhibit insufficient DMI, which is unable to provide the nutrients to meet the energy and metabolizable protein requirements for milk production (Grummer, 1995). While a cow's daily dry matter intake progressively increases every day in lactation, this phenomenon in early lactation has been labeled as the period of negative energy and metabolizable protein balance. In order to overcome this nutrient gap, cows mobilize their body's nutrient reserves in the form of protein and adipose tissue (Komaragiri and Erdman, 1997; Drackley, 1999). However, miscoordination between these nutrient reserves, nutrients acquired through dry matter intake, and nutrients required for milk production can result in the

development of metabolic disorders of hyperketonemia, fatty liver, displaced abomasum (DA), milk fever, and metritis.

The excessive mobilization of non-esterified fatty acids (NEFA) in the early lactation period are responsible for many of the observed metabolic diseases. As part of the homeorhetic transition to lactation, NEFA are mobilized from adipose reserves. They are subsequently taken up by tissues with increased metabolic output such as the mammary gland and the liver to increase milk fat output or to be oxidized, respectively (Aschenbach et al., 2010). At the level of the liver, NEFA has four fates of being completely oxidized, partially oxidized into keto-acids or ketone bodies, stored in the liver in the form of triglyceride, or exported from the liver in the form of very low density lipoproteins (VLDL) (Grummer, 1995). When the supply of mobilized triglyceride is enhanced during the early postpartum period, cows are unable to repackage all NEFA into VLDL and export them from the liver (Drackley, 1999). In this scenario, cows develop fatty liver and partially oxidize fatty acid substrates into ketone bodies such as β-hydroxybutyrate (BHB). An overproduction of these ketone bodies leads to the metabolic disorder ketosis and is defined at the plasma subclinical level as 1.2 mmol/L and clinically at 2.9 mmol/L (Ospina et al., 2013). Animals that are diagnosed with subclinical ketosis events during the early lactation are at an increased risk of developing further disorders such as a displaced abomasum (DA) and metritis (Ospina et al., 2010). Additional risk factors for the development of DA include diets with too many non-structural carbohydrates or too much fiber that leads to depressed intake. Non-structural carbohydrates lead to the production of propionate in the rumen, which is transferred across the portal wall into the liver where it is converted into glucose and subsequently oxidized into adenosine triphosphate (ATP). When the cow senses high ATP levels in the liver, these positive energy signals relay negative feedback to the brain to promote satiety and reduce the animal's

intake as is suggested by the hepatic oxidation theory (HOT) (Allen et al., 2009). Whereas when cows are fed diets that are too bulky with high amounts of fiber, the stretch receptors in the rumen are activated to provide negative feedback on feed intake (Shaver, 1997). When cattle reduce their intake due to any of the aforementioned reasons, it increases the chance the abomasum will fill with gas and become displaced to the top of the abdomen and lead to a corresponding DA.

Additionally, as cows transition to heightened nutrient demands of early lactation, the conversion of nutrients from feed and body tissues into milk outputs requires the upregulation of gluconeogenic and tissue mobilization pathways (Aschenbach et al., 2010; van der Drift et al., 2012). These pathways generate increased oxidative stress through the formation of reactive oxygen species (ROS) and can lead to negative inflammatory responses (Abuelo et al., 2015). An excess of inflammation leads to an overactive immune system, which can use valuable nutrients that could instead be used for milk production and animal health. Thus, poor immune function has been associated with development of metritis, retained placenta, and mastitis to make the transition period home to 75% of all disease development (Leblanc et al. 2006; Gilbert, 2016). Therefore, farms have looked at potential interventions to reduce the formation of these ROS by limiting the number of stressful events or sequester these ROS through the use of antioxidant treatments (Spears and Weiss, 2008; Deters and Hansen, 2020).

While these disorders are detrimental to animal welfare and production metrics, they also have a negative outlook on farm-level profitability. Although there is a cost to treat each cow back to normal health, the largest financial losses have been recorded in the form of lost milk production and lost reproductive performance for the subsequent pregnancy. Cattle that exhibited elevated NEFA and BHB the week before parturition exhibited reduced milk yield by 1.6 to 3.2 kg/d over the first four Dairy Herd Improvement Association (DHIA) test dates and had an increased risk to

be culled prematurely from the herd (Roberts et al., 2012). Furthermore, it has been estimated that each case of subclinical ketosis costs a farmer $289 (McArt et al., 2015). The majority of these costs (41%) are attributable to the reduced milk yield in the lactation with the remainder represented by an increased risk for the development of metritis (33%) and reduced reproductive efficiency as well as the increased potential for development of a DA (26%). Meanwhile other models have demonstrated a greater cost for the development of a DA at $432 and $640 for primi- and multiparous cattle, respectively (Liang et al., 2017). These costs result from increased labor, treatment, decreased milk, and increased likelihood for the animal to be involuntarily culled from the herd. Therefore, management strategies taken to minimize the occurrence of disease in an animal's environment can have a tremendous impact on both animal health and correspondingly farm profitability.

1.2.5 Solutions to Reduce Disorder Incidence

Previous research has defined key management areas to minimize the disorder development risk to ensure a successful homeorhetic transition to lactation. The three main areas are to optimize the amount of triglyceride mobilized, increase the liver's capacity to oxidize and export substrates, or implement disease and body condition monitoring systems to detect and treat energy related metabolic diseases earlier (Ingvartsen, 2006). A combination or integration of these management pieces can be implemented to ensure maximal animal health and avoid the costly and negative health outcomes observed from the development of these diseases in early lactation.

It is important to monitor the amount of energy cows consume during the dry period due to the risk of cows gaining body condition in late lactation (Douglas et al., 2006; Ingvartsen, 2006). Cows that gain significant body tissue during the dry period, are more apt to mobilize those tissue stores in early lactation, which can lead to the negative health outcomes previously mentioned

(Contreras et al., 2004; Douglas et al., 2006; Grummer, 2008). Cows that were fed a restricted level of energy intake from dry off through calving experienced greater DMI, had lower circulating NEFA concentrations, and lower prevalence of a DA in the postpartum period (Douglas et al., 2006). While this study did not demonstrate a milk yield difference between cows fed restricted or *ad libitum* intake during the first 16 weeks of lactation, the restricted cows demonstrated hypoinsulinemia during the gestational period and had reduced BHB concentrations in the postpartum period, which would suggest a decreased likelihood of developing acetonemia. Therefore, by controlling the diet energy density, managers can control the amount of fat cows store and subsequently mobilize during the lactation period.

While cattle naturally mobilize tissue in early lactation to meet nutrient requirements, environmental stressors can exacerbate the quantity of tissue cows mobilize. Managers can minimize stressors by reducing stocking density, allowing consistent feed availability, and reducing the number of pen moves in early lactation (Grant and Albright, 2001; Jensen and Proudfoot, 2017). Both increased stocking density and frequent pen moves in early lactation can result in subordinate cows being displaced from the feed bunk, disrupting their time for feed intake, while resulting in an increased likelihood to mobilize fat. Additionally, over conditioned cows are more likely to mobilize greater amounts of NEFA through the first four weeks of lactation compared to normal body condition cows (Fronk et al. 1980; Pires et al., 2013). Therefore, management steps must be implemented in the early lactation period to prevent cattle from mobilizing excessive amounts of adipose tissue.

In addition to management strategies to reduce adipose tissue mobilization, there are several dietary supplements that have been evaluated to alter the amount of tissue mobilized. Propylene glycol is the dairy industry's most common treatment for hyperketonemia events in

early lactation with the standard treatment being 300 mL of an oral drench solution provided for three days (Mann et al., 2017). Once supplemented, propylene glycol is transported across the rumen wall into the liver where it is converted into glucose and becomes readily available for the cow. This increased glucose availability decreases the animal's reliance on mobilized body tissues. However, previous studies that have evaluated providing cows with increased energy through the diet alone have failed, due to reduced intake potentially due to the HOT (Grummer, 2008; Allen, 2014).

Another opportunity in supplements is to reduce the amount of tissue mobilized through the usage of nicotinic acid, which acts through incorporating niacin into nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) (Niehoff et al., 2009). These are coenzymes that are responsible for oxidative phosphorylation, which could potentially help cows achieve a more positive energy balance through greater ATP production. This would ultimately make cows less susceptible to mobilize their tissue reserves. However, previous trials in transition cows have shown limited efficacy in health and milk responses to niacin supplementation (Skaar et al., 1989; Minor et al., 1998). An additional supplement that can reduce the mobilization of fatty acids is chromium, which has been shown to increase a cow's insulin sensitivity through increasing the number of insulin receptors on the target tissues (Anderson et al., 1987; Rockwell and Allen, 2016; Horst et al., 2018). This increase in insulin sensitivity makes the tissues more responsive to insulin production and reduces the likelihood that cattle will mobilize tissue in early lactation. Previous work has shown animals supplemented throughout the periparturient period with chromium led to increased DMI and milk yield in the corresponding postpartum period (Smith et al., 2005; Rockwell and Allen, 2016). Through feeding

supplements and minimizing the number of external stressors, farm managers can limit the amount of tissue mobilized and decrease the likelihood for developing metabolic disorders.

While the first step in preventing disorders is to reduce the mobilization of adipose tissue, the second approach should be to improve the cow's ability to handle the increased metabolic challenge through supplements. One of the main supplements found to improve liver function has been cobalt. While the method of action is still being determined, it is believed to improve the liver's ability to package fatty acids into VLDL and facilitate their export from the liver (Overton and Waldron, 2004; Grummer, 2008). Additionally, carnitine, which is involved in the rate limiting step in fatty acid transport from the cytoplasm into the mitochondria to be oxidized, can also be used to reduce fatty liver accumulation. Transition cows fed varying levels of carnitine supplement (low carnitine, 6 g/d; medium carnitine, 50 g/d; high carnitine, 100 g/d) were found to have reduced fatty acids and triglyceride in liver biopsies at 10 DIM compared to cows that were not given carnitine supplementation (Carlson et al., 2007). However, cows fed high carnitine supplement demonstrated reduced DMI and thus reduced milk yield compared to animals fed low and medium levels of carnitine supplement. This result suggests that the treatment cows had increased oxidation of fat in the liver, which may have caused reductions in cattle intake potentially due to the HOT (Allen, 2014).

Another micronutrient that plays a role in cattle metabolic health is the supplementation of choline, which is responsible for the production of vitamin B-12 (cobalamin) (Pinotti, 2012). Studies where cows have been given supplemental choline during the periparturient period have reduced liver fatty acid content and increased milk yield (Piepenbrink and Overton, 2003; Janovick Guretzky et al., 2006; Zom et al., 2011). Furthermore, two B-vitamins, B-9 (folate) and -12 (cobalamin) have also been found to improve the liver's function as work in small ruminants has

shown that they are involved in converting propionate into succinyl CoA through gluconeogenesis (Kennedy et al., 1994). These two vitamins work through the mode of action of 5,10-methylene tetrahydrofolic acid (THF/folate), which can be converted into 5-THF and this methyl group is then donated to vitamin B12 (cobalamin) and forms methylcobalamin (Morris et al., 2017). This production of methylcobalamin then transfers the methyl group to homocysteine to form methionine and leads to the regeneration of S-adenosyl methionine (SAM). This compound donates methyl groups for processes of DNA methylation and synthesis. Another role of vitamin B12 is through the vitamin B12 dependent enzyme methyl-malonyl-CoA mutase. This pathway converts propionate into succinyl-CoA. Succinyl-CoA can then feed into the Krebs cycle and lead to ATP production for the large energy requirements of lactation. Cows that were in a 2×2 factorial design of vitamin B9 and B12 supplementation demonstrated increased plasma glucose and a trend for reduced NEFA concentrations during the postpartum period under the supplementation of both vitamins (Graulet et al., 2007). While most of these vitamins and minerals are provided in the diet through premixes, there is a strong interest in making sure all cows receive the appropriate nutrients to prevent the development of metabolic disorders.

While there are several opportunities to prevent a cow's development of metabolic diseases in the transition period, there is a strong interest in technologies to diagnose cows before they become clinically sick. Cows that are subclinically ill will have reduced feed intake, decreased activity, and lower milk yield (Leblanc, 2010). The faster that a manager can identify and treat these cows before they become clinically ill, the greater likelihood the farm will avoid the associated costs of clinical disease. Some of the technologies that have been implemented to monitor these diseases include rumen collars, automated robotic milking systems, and pedometers to flag deviations from physiological normal levels (Maatje et al., 1997; Stangaferro et al., 2016;

King et al., 2017). These monitoring systems were able to identify deviations from normal in some cases by as many as six days prior to clinical diagnosis. Since most metabolic diseases occur during the transition period and the large costs associated with clinical disease, monitoring technologies can be an opportunity to maximize herd health.

1.3 Circadian Timing Systems

The CTS is an endogenous timing system that generates 24-hour rhythms of physiology and behavior in mammals. It is composed of molecular clocks distributed in every cell of the body, which are regulated hierarchically by the upper level of the suprachiasmatic nuclei, which then relay temporal signals out to peripheral clocks (Mohawk et al., 2012). The suprachiasmatic nuclei receive and integrate the timing of information from the environment, which include the timing of light, exercise, and nutritional status, among others. The CTS functions to maintain homeostasis and is tightly integrated with the metabolic system (Marcheva et al., 2010). Disturbances in the timing of regularly occurring events that clocks respond to results in chronodisruption. Following chronodisruption, the animal will attempt to regain homeostasis in the short-term and in the long-term they can reset their CTS (Roenneberg & Foster, 1997). Although under periods of prolonged chronodisruption, as is observed in shiftwork conditions or in circadian disruption models, alterations in metabolism can occur and result in the development of disease (Filipski et al., 2004; Kohsaka and Bass, 2007; Potter et al., 2016).

Chronic disruptions in the timing of animal's environmental cues can cause a relay disruption signal to the peripheral clocks. Previous research has recorded that mice that undergo a disrupted CTS have greater odds of developing obesity and greater likelihood to experience premature mortality (Turek et al., 2005; Casey et al., 2016). Similarly, 15% of the adult population in the US find themselves subject to shift work scenarios, defined as work outside the typical work

hours of 9-5 (Sleep Foundation, 2020). These individuals are more prone to develop insomnia and have increased prevalence of obesity and other metabolic disorders compared to those who work during the normal work day period (Buxton et al., 2012). In modeling shift work conditions, eight days of circadian misalignment demonstrated prediabetic elevated glucose and insulin in three out of eight relatively healthy adults (Scheer et al., 2009). This shows the importance of maintaining circadian rhythms in both the short- and long-term to drive forward metabolic health.

1.3.1 Evaluation of Circadian Rhythms

There are several ways to analyze circadian rhythm maintenance or attenuation in mammals. Evaluation of changes in 24 h measurements such as temperature, blood metabolites or milk production can be fitted to a cosine curve using the cosinor package of R (RStudio 1.1.453, Boston, MA) or analysis in SAS v9.4 (Cary, NC) (Niu et al., 2014; Salfer and Harvatine, 2020; Suarez-Trujillo et al., 2020). Cosine curves are then evaluated using a variety of statistics, which include: the midline estimating statistic of rhythm (MESOR), area under the curve (AUC), R^2 (coefficient of determination), amplitude, and acrophase, to determine characteristics of the circadian rhythm (Bourdon et al., 1995). The MESOR is an evaluation of the average of all the points of the cosine and AUC is the total amount of area under the cosine curve. Therefore, changes in these two metrics parallel each other and can understand metabolic changes. For example an increase in temperature MESOR has been suggested to signify increased metabolism (Gamo et al., 2013). The R^2 is a measurement of how much of the data explains the variation observed in a cosine rhythm. Whereas the strength of the animal's circadian rhythm can be assessed through evaluation of cosine amplitude or the distance between the peak or trough of the curve and the MESOR. Therefore, increases in amplitude indicate an animal has a stronger circadian rhythm. Meanwhile, acrophase is described as the start of the cosine circadian curve and a deviation of this

timepoint suggests a delay or shift in an animal's circadian rhythm (Burfeind et al., 2014). Evaluation of these metrics together can be used to determine alterations and shifts in circadian rhythms.

1.3.2 Circadian Disruptions Impact Lactation

Beyond the health outcomes, circadian disruptions have been observed to impact lactation performance among several species. Expecting mothers that were subjected to circadian disruptions as measured by inconsistent sleep cycles had a delayed onset of lactation (Casey et al., 2019). Whereas mice that have their clock genes knocked out had reduced lactation performance at the onset of lactation (Kennaway et al., 2004; Dolatshad et al., 2006). Outside of research in monogastrics, ruminant lactation performance has been evaluated in response to circadian rhythm disruptions.

Disruptions in the timing of light have shown mixed results on lactation performance. Cattle in a one-week crossover design of consistent vs. continuously shifting light resulted in a decrease in milk yield and milk component yields during periods of continuously shifting light compared to consistent timing of light (Casey et al., 2014). Furthermore, milk production is also impacted by the timing of feed availability. A crossover study design that had the cattle feeding period restricted to the night hours showed animals produced less milk in periods of night restricted feeding compared to the normal time of feeding during daylight hours (Salfer and Harvatine, 2020). The investigators observed no differences in DMI or lying and feeding time between treatment periods but observed major shifts in the timing of lying and feed intake events during night feeding periods. Furthermore, cattle were milked four times a day for milk yield, fat, and protein circadian rhythm analysis. The investigators found a lower amplitude, delayed start to the circadian cycle, and weaker circadian rhythm for milk production parameters during night feeding periods.

Conversely, cattle that were exposed to a forward shifting pattern of light during the late gestation and consistent timing of light through 60 DIM, demonstrated greater milk and fat yield (Suarez-Trujillo et al., 2020). These discrepancies demonstrate a need to further study the impact of circadian disruptions on lactation performance in dairy cattle.

1.3.3 Environmental Disruptions in Ruminants

Ruminants have shown responsiveness to circadian disruptions in the timing and length of light. Sheep rely on the timing and length of daylight for reproduction and previous work in rams that had their hypothalamo-pituitary relay disconnected had increased resting insulin concentrations (Lincoln and Richardson, 1998). This eventually led to the rams developing obesity over the two-year study. Furthermore, a cross over study design demonstrated ewes that were exposed to continuous forward shifting pattern of light had increased insulin sensitivity as measured by an intravenous glucose tolerance test (IVGTT) (Varcoe et al., 2014). Whereas another study that subjected sheep to a forward shifting pattern of light demonstrated insulin resistance in response to an IVGTT (Gatford et al., 2019). The latter study demonstrated differences in insulin resistance in dams lambing singles alone no difference was observed in animals lambing polytocous litters. While the amount of literature in this area is limited, more studies are needed to understand the impact of circadian disruptions on metabolic profiles and insulin signaling.

Although cattle are not as responsive to the length of photoperiod as sheep, which observe estrus during the short daylight length of the fall each year, there is substantial evidence demonstrating the effect of the length and timing of light on cattle health and performance. Analysis of annual lactation curves has demonstrated that milk component yields and milk production are driven by an annual rhythm in herds throughout the United States by the day length observed across the seasons (Salfer et al., 2020). Furthermore, the late gestation dry period has

been documented as the time for an increased remodeling of the mammary epithelium to prepare for the upcoming lactation. External environment events during this time can impact subsequent lactation performance. The environmental influencers of heat stress and length of light during this period have found to influence milk production in the subsequent lactation (Auchtung et al., 2005; Tao et al., 2011). Cattle provided with cooling systems during the dry period produced 4.0 kg/d more milk over the lactation than cattle not cooled when the ambient temperature surpassed 21.1ºC. Additionally, cattle exposed to a short-day photoperiod (8 h light; 16 h dark) during the dry period produce more milk over week 4 to 8 of lactation, than cows exposed to a long-day photoperiod (16 h light; 8 h dark).

1.3.4 Insulin Sensitivity

The body functions to maintain a consistent concentration of plasma metabolites to promote health and metabolism. The average blood glucose concentration for a lactating dairy cow is around 50-55 mg/dL and this fluctuates throughout the day in response to events such as eating, fasting, and milking (Lucy et al., 2013). In response to a meal, blood glucose concentrations will elevate as nutrients from the feed are absorbed. To regain the baseline level of plasma glucose concentration, cows release insulin from the β-cells in the pancreas to facilitate the uptake of glucose from the blood into the peripheral tissues. Insulin enhances the uptake of glucose through mediating the translocation of GLUT4 glucose transporters to the cell surface and allow for insulin-mediated uptake of glucose (Suzuki and Kono, 1980; Jaakson et al., 2018). A cow's insulin sensitivity is exhibited by the amount of insulin required to elicit the uptake of glucose from the blood into tissues. Cows that require greater insulin production to clear the elevated glucose from the blood are said to have reduced insulin sensitivity or increased insulin resistance. Meanwhile, insulin sensitivity should not be confused with insulin responsiveness, which is a measure of the

maximal concentration of insulin that can be produced by the cow in response to elevated glucose concentrations (Hayirli, 2006). Therefore, cows that exhibit reduced insulin responsiveness have a reduced ability to produce insulin in response to elevated plasma glucose concentrations whereas cows that exhibit insulin resistance will require greater insulin to clear the same amount of glucose (De Koster & Opsomer, 2013).

There are multiple methods that can be implemented to quantify insulin sensitivity in dairy cows, which include the Revised Quantitative Insulin Sensitivity Check Index (RQUICKI), IVGTT, and hyperinsulinemic euglycemic clamps (HEC). These methods are listed in ascending order of specificity for their ability to record differences in insulin sensitivity (Holtenius and Holtenius, 2007; De Koster et al., 2016; De Koster et al., 2017).

1.3.5 RQUICKI

RQUICKI is calculated using the following formula: $\frac{1}{\log(\text{glucose (mg/dL)})+\log(\text{Insulin (mIU/mL)})+\log(\text{NEFA (mmol/L)})}$ (Holtenius and Holtenius, 2007). The plasma concentrations of each of the metabolites or hormone in the formula are sampled after at least one hour of feed restriction. Cows that have a greater RQUICKI score are suggested to have lower insulin sensitivity as they will have greater resting NEFA and glucose concentrations compared to a more insulin sensitive animal. Other researchers have included the concentration of baseline β-hydroxybutyrate (BHB) in the calculation as well to create the formula: $\frac{1}{\log\left(\text{glucose}\left(\frac{\text{mg}}{\text{dL}}\right)\right)+\log\left(\text{Insulin}\left(\frac{\text{mIU}}{\text{mL}}\right)\right)+\log\left(\text{NEFA}\left(\frac{\text{mmol}}{\text{L}}\right)\right)+\log(\text{BHB}(\frac{\text{mmol}}{\text{ml}}))}$ (Balogh et al., 2008). Similarly, cows with lower RQUICKI_BHB are suggested to have lower insulin sensitivity.

1.3.6 IVGTT

An IVGTT challenges the body with an enhanced glucose dose and then measures the body's ability to clear that glucose from circulation. Cows that perform metabolically normal, clear the glucose at a relatively fast rate and return their blood glucose levels to the homeostatic normal range. Whereas those animals that take a prolonged period of time to clear glucose from the blood stream or need an additional quantity of insulin to clear the same amount of glucose, are characterized as having lower insulin sensitivity or insulin resistance (De Koster et al., 2016). The typical dosage for an IVGTT on a mature cow is 250-300 mg glucose/kg of bodyweight (Kerestes et al., 2009; Rico et al., 2016). In heifers, authors concluded that a glucose dose between 0.5 and 1 g/kg metabolic body weight is sufficient to initiate an insulin response without having a significant proportion of that glucose excreted from the animal in pathways besides the target adipose and muscle tissues (González-Grajales et. al, 2018). Another study in adult dairy cows at two different timepoints of lactation (74 and 221 DIM) observed that the minimum dose tested at 92 mg/kg still exceeded kidney glucose clearance rates (Malacco et al., 2020). Therefore, there is still the need for additional work in lactating adult dairy cows to determine a standardized glucose dose to understand the body's ability to uptake glucose while balancing the kidney's ability to filter it out of the blood.

After glucose is dosed, cattle blood samples are collected and plasma is analyzed over two to four hours to evaluate the animal's response to the glucose challenge (Mann et al., 2016; Marett et al., 2015). Immediately after the glucose challenge, cows observe a dramatic increase in glucose concentrations along with a corresponding increase in insulin concentrations to return to homeostatic normalcy. The rate at which glucose and insulin are selectively cleared from the body post-glucose dose via uptake into the peripheral tissues describe the cow's fitness to respond to a glucose challenge. One way to evaluate this is via area under the curve, which is measured as the

total area under the glucose or insulin response curve from glucose administrations through the end of the test. AUC measurements can be performed during the first 60 min of the IVGTT due to this period having the greatest metabolic response. Cattle that exhibit greater glucose AUC or take a longer time to clear the glucose from the blood are said to have glucose impairment. Whereas cattle that have greater insulin AUC in response to an IVGTT, experience insulin resistance as more insulin is required to store or clear the glucose from the blood (Huzzey et al., 2012). These phenomena are not exclusive as cattle can have both glucose impairment and insulin resistance in response to an IVGTT. Additional metrics for understanding IVGTT response include evaluating insulin increment or the difference between baseline insulin and peak insulin concentrations.

In response to the glucose infusion, NEFA concentrations in plasma decrease until they reach their nadir approximately 45-90 minutes post glucose dosage depending on the physiological state of the animal. Cattle in an elevated physiological state such as lactation achieve their NEFA nadir at an earlier timepoint than animals at a lower physiological state. Once the majority of glucose is taken up by the peripheral tissues, NEFA subsequently increases to exceed the pre-glucose infusion concentrations and is later corrected to baseline levels (Marett et al., 2015). The rate at which NEFA disappears from the blood and the time at which NEFA nadir is achieved can be used to evaluate peripheral tissue insulin responsiveness (Girard et al., 2019). Meanwhile there has been minimal investigation on the impact of BHB concentrations in response to glucose administration. Rather BHB has been evaluated by comparing baseline ketone concentrations when performing an IVGTT (Mann et al., 2016). In experiments where BHB has been recorded, the measurements exhibit a lag in response to the glucose infusion and has therefore made it better to monitor the metabolite over the transition period instead of over the few hour test.

1.3.7 Hyperinsulinemic-Euglycemic Clamp

The gold standard in determining insulin resistance or sensitivity in mammals is the use of the hyperinsulinemic-euglycemic clamp (HEC). In this test, animals have their feed withheld approximately one to two hours prior to the start of the test and are double catheterized via the bilateral jugular veins (Schoenberg, et al., 2012; Kvidera et al., 2017; Davis et al., 2019). One of these catheters is used to infuse insulin and glucose and the other is used for blood sample collection. Baseline blood samples are sampled as in an IVGTT, then the animals are challenged with a continued infusion of 2.5% insulin solution at a flow rate of approximately 1 µg/kg of BW for an infusion rate of 9.0 mg/h. Glucose concentrations are consistently monitored and when they begin to decline below baseline levels, glucose administration is initiated through the same catheter to maintain blood glucose and avoid severe hypoglycemia. The test time ranges from 180 to 720 minutes over which the researchers attempt to reach a steady state blood glucose concentration. The point at which a steady state is achieved is known as the "clamp" of the test as a stagnant concentration of glucose and insulin inflow is achieved. Cows that require more glucose to be infused over the length of the test are defined as being more insulin sensitive than their counterparts. Additional measurements that can be taken during an HEC include NEFA and BHB, but as observed in an IVGTT, they are minor contributions to evaluating the cow's metabolism.

Out of all three insulin sensitivity techniques, the HEC requires the most labor to perform through consistent monitoring of blood glucose levels and subsequent modifications of glucose infusion rate to ensure a euglycemia clamp. On the other hand, the RQUICKI technique requires the least amount of labor as animals only need to be fasted prior to sample collection. However, there is limited correlation between the three measurements in their ability to holistically determine the insulin sensitivity of the test animal (De Koster et al. 2016; Alves-Nores et al., 2017) This may be due to the fact that the RQUICKI calculations were first developed in a monogastric (humans)

and then translated into cows, which may offer little biological significance for ruminants (Holtenius and Holtenius, 2007). However, there is evidence that the first 60 minutes of an IVGTT glucose concentration curve is well correlated with an HEC in evaluating insulin sensitivity (De Koster et al. 2016). While RQUICKI has been shown as an unmerited form of measuring insulin sensitivity compared to the other techniques, the HEC remains the gold standard technique while an IVGTT remains an appropriate alternative if the HEC is not possible.

1.4 Tissue Mobilization in Dairy Cows

As previously mentioned, tissue mobilization occurs in almost all cows over the first month in lactation. There are many factors that can influence the amount and extent of tissue mobilization, which include the environment, previous tissue stores, and diet. However, to evaluate tissue mobilization between cows and herds, the available tissue reserves and ways to measure tissue composition changes must be determined.

1.4.1 Tissue Reserves in the Transition Dairy Cow

Dairy cows have been estimated to lose anywhere from 12-15% of their bodyweight between calving and 50 DIM when they reach their bodyweight nadir (McCarthy et al., 2007). Upon reaching this absolute minimum, which normally corresponds with peak milk yield, cows begin to reaccrete body tissues back to their gestation levels. However, farm geographical location and management systems have been found to influence the amount of bodyweight and BCS lost over the first part of lactation with New Zealand systems experiencing lower change than US based dairy systems (McCarthy et al., 2007). This change in bodyweight is mainly reflected in changes of two main tissue stores, adipose and muscle. The former can be used to meet energy requirements alone and the latter can be incorporated to meet energy as well as amino acid requirements. A late

gestation cow, approximately one week prior to calving, has been estimated to have an empty body fat and protein composition of 90 and 78 kg, respectively (Andrews et al., 1994). Empty body fat and empty body protein describe the contribution from that tissue type to the body that has the weight of the intestinal and fetal/reproductive contents removed. Cattle in this study weighed an average of 584 kg at slaughter, making adipose and protein's empty body weight carcass contribution 19 and 17%, respectively. Due to today's larger frame sized cows, as animal size has increased over the past 26 years, these reserves may have increased accordingly in size. However, it is not possible to gauge whether the tissue proportions have grown in similar carcass contributions due to management strategies that have shifted towards minimizing body condition in the late gestation period (Contreras et al., 2004). While the proportion of these two nutrient reserves in the cow are very similar by weight at the time of calving, much of the previous research in periparturient dairy cows focuses on adipose rather than protein reserves.

Adipose tissue is the largest source of energy in a cow's body at 9 kcal/g tissue, which cows can mobilize to overcome their negative energy balance. In the early lactation period, when an energy gap is present, cows reduce their insulin sensitivity and production to reduce the number of signals that suppress adipose mobilization. This allows for the phosphorylation of hormone sensitive lipase that promotes lipolysis and liberation of adipose tissue into NEFA and triglycerides (Egan et al., 1992). Given the hydrophobic properties of esters, they must be transported through the blood via a hydrophilic vesicle to serve as an energy source for various tissues. One of the main tissues that utilizes fatty acids is the mammary gland. The mammary gland can incorporate mobilized fatty acids from adipose tissue and fatty acid protein products in the form of VLDL, at an estimated conversion efficiency of 82-84% (Moe et al., 1971). Long chain fatty acids can be readily incorporated into milk fat whereas de novo fatty acids (four to sixteen

carbons in length) are synthesized from short chain fatty acids by fatty acid synthase within the mammary gland (Zhu and Luo, 2017). Therefore, early lactation cows will have their fatty acid profile biased towards long-chain fatty acids that are mobilized directly from adipose tissue.

The majority of adipose mobilization occurs during the first five weeks in lactation, with the second and third month of lactation contributing a minor portion to the total amount of tissue mobilized (Komaragiri and Erdman, 1997). From two weeks before calving through five weeks postpartum, cows mobilized between 32-38% of their empty body fat reserves. This was followed by an additional decrease by 10-12% of empty body fat from five through 12 weeks postpartum. This number may not capture the total amount of tissue mobilized, as cows may have lost more tissue during that time frame and reaccreted the adipose tissue by the time the measurements were recorded. Yet, the amount of adipose tissue that a cow mobilizes in early lactation is dependent on the amount of reserves they have present in late gestation (Pires et al., 2013). Researchers quantify the amount of reserves through the use of body condition scoring (BCS) system, which assigns a score from 1-5 to cows based on subcutaneous fat levels (Wildman et al., 1982; Edmonson et al., 1989). While this is the most widely used technique to measure adipose reserves, a cow with a score of 1 is considered emaciated whereas an animal with a score of 5 would be considered obese. On average, each BCS point on the 1-5 point scale is equivalent to 50 kg of BW with a corresponding range of 39-66 kg depending on the stage of lactation and parity of the animal (Berry et al., 2011). Pires et al. (2013) found that cows with greater amounts of body condition at four weeks prior to calving (BCS $\geq$ 3.75) had higher NEFA concentrations, greater milk fat, and a greater negative energy balance through the first seven weeks in milk compared to medium (BCS $\leq$ 3.5-$\geq$2.75) and low body conditioned cows (BCS $\leq$ 2.50). While this study did not show health

outcomes, herd managers have shifted to minimize excessive body condition in late gestation animals to avoid large mobilization of adipose in early lactation.

While making up an almost equivalent amount of tissue mass in the late gestation cow, protein reserves can provide both energy and amino acids in early lactation. Besides the negative energy balance, the amino acid deficit in early lactation has been approximated at 500 g/day (Bell, 1995). Previous research with cows that were fed protein restricted diets to achieve a N balance of 0, has estimated that 27% of dairy cows protein reserves are labile and thus can be mobilized to meet nutrient requirements (Botts et al., 1979). Other researchers who have studied protein mobilization in cattle fed higher dietary crude protein (CP) levels have reported a lower percentage of total protein reserves mobilized at 22 and 18%, respectively (Komaragiri and Erdman, 1997; Van Der Drift et al., 2012). However, it is important to note that different techniques were utilized to measure protein mobilization in these additional studies, which may influence interpretation as will be discussed later. Komaragiri and Erdman (1997) demonstrated that 756 kg cows at two weeks prior to calving mobilized at least 21 kg or 22% of their empty body protein (EBP) reserves over seven weeks from two weeks prepartum through 5 weeks postpartum on a 16 or 19% CP diet. The authors found there was no difference in protein mobilization between 5 and 12 weeks, suggesting that dairy cows do not mobilize protein after the first five weeks of lactation or that minimal muscle was mobilized and then accreted between these two timepoints. Although muscle is a smaller energetic reserve within the body, its use as an energy and amino acid substrate makes it a rapidly used source of nutrients in the first few weeks postpartum.

1.4.2 Health and Economic Impacts of Tissue Mobilization

As has been previously discussed, many of the diseases and disorders in the transition period have been tied to excessive adipose tissue mobilization. Management and supplement strategies

attempt to overcome these challenges in periparturient dairy cows and one of the best strategies has been to minimize adipose reserve accumulation during late lactation and gestation through the incorporation of reduced-energy dry cow diets. While protein mobilization is lessor in the amount mobilized by weight compared to adipose, the quantity of tissue mobilized between cows is highly variable with some animals even gaining empty body protein between calving and eight weeks of lactation (Tamminga et al., 1997). Therefore, future research will need to address epidemiological approaches to assess if there is a threshold at which protein mobilization affects animal performance.

1.4.3 Ways to Quantify Tissue Reserves

To assess dairy cattle tissue mobilization, there are multiple ways to directly and indirectly quantify changes in tissue composition. The first and most obvious predictor is monitoring of changes in bodyweight over the periparturient period. It has been predicted that dairy cows undergo 12-19% change in bodyweight from calving through the first seven weeks of lactation (McCarthy et al., 2007; Pires et al., 2013). However, it is important to note that at high levels of body condition, body weight alone was not a good marker of respective amount of tissue mobilized, as cows with high body condition (BCS $\geq$ 3.75) tend to mobilize greater amounts of fat than average conditioned cows (BCS $\leq$ 3.50-$\geq$2.75) of similar weight four weeks prior to calving (Pires et al., 2013). Rather cattle bodyweight changes should be monitored within each individual animal to understand changes in tissue reserves due to variation in cattle mature bodyweight. While changes in bodyweight cannot detect the exact tissue reserves being mobilized, it can be used as an index for determining when to breed back cows for their subsequent pregnancy (Buckley et al., 2003). The timepoint at which cows reach their nadir bodyweight is suggested to be the timepoint when they surpass their negative nutrient balance. Once cows are beyond their negative nutrient

balance, they will have sufficient nutrient balance to support a subsequent conceptus. However, managers may use other energy status quantifiers such as body condition in making breed back decisions as will be mentioned later.

A more finite way to monitor tissue composition is through ultrasonography to measure respective tissue depths. Ultrasound images can be implemented at several locations on the cow. Two common locations used for benchmarking tissue depth include the dorsal side of the 12th intercostal space and the dorsal side of the pelvis between the tuber coxae and tuber ischia (Schröder and Staufenbiel, 2006; van der Drift et al., 2012). The former captures images of the longissimus dorsi and backfat depth, while the latter evaluates gluteus medius muscle depth and rump fat depth. The depth of muscle and adipose in these two locations are highly correlated with total body muscle and total body adipose reserves ($r>0.6$) from beef cattle slaughter studies (Greiner et al., 2003; Peña et al., 2014). Therefore, monitoring changes in the dairy cattle muscle and fat depth via ultrasonication can be utilized to demonstrate changes in total body muscle and adipose over the periparturient period.

While ultrasonication can give approximations of the total body muscle and adipose, the most accurate way to determine changes in muscle and adipose depth is to perform dairy cow slaughter studies at different timepoints in the transition period. Historically slaughter studies were performed to determine nutritional requirements for growth and pregnancy (Bell, 1995; Bartlett et al., 2006). However, additional studies have since been performed to determine changes in cattle tissue composition over the periparturient period and lactation cycle (Andrews et al., 1994; Reynolds et al., 2004). While slaughter studies are the most accurate quantifier of tissue composition, they have the downside that they can only assess each animal once and the extreme cost associated with animal sacrifice makes this technique prohibitive in most scenarios.

A fourth method to determine tissue composition, which is performed in research settings alone, is measuring tissue mobilization via D_2O water displacement. This technique infuses cows with deuterium oxide at a rate of 150 mg/kg of BW (Komaragiri and Erdman, 1997; Komaragiri et al., 1998). Subsequent frequent blood samples in the three hours post-infusion and less frequently over the following three days are collected to determine the proportion of the water in blood labeled by D_2O. Analysis assumes a consistent mixing of the water fractions together, which is then able to determine the empty body water content. This can then be implemented in a series of equations to determine the empty body protein, empty body ash, gastrointestinal DM fill, and fetal DM if the animal is pregnant. Empty body fat is subsequently determined by difference between bodyweight and the other measured components. This method would need to be repeated over the transition period to detect differences in tissue composition, but it gives researchers a more accurate result compared to bodyweight and ultrasonication measurements. However, this method may not be sensitive enough to detect small changes in body composition and thus may need to be performed at distinct timepoints that can measure macro differences in tissue type and mass. Not to mention dosing cows with 250 mg D_2O per kg of BW would cost more than $500 per animal making it cost prohibitive for large animal research settings alone (Komaragiri et al., 1998).

1.4.4 Quantifiers of Adipose Mobilization

While the above methods have mentioned how to determine changes in body composition, for both fat and muscle tissue, there are a set of techniques unique to measure adipose mobilization alone. While the BCS system has already been mentioned, the target BCS for a cow in her entry to lactation has been recorded as a 3.25-3.50 (Roche et al., 2009). Animals that carry more fat at calving will be more likely to mobilize their adipose reserves and have reduced postpartum DMI and subsequent metabolic disorders (Zachut et al., 2013). Whereas animals that have a BCS $\leq$2.50

at calving will suffer from poor milk production and reduced likelihood of being bred back for the subsequent pregnancy (Domecq et al., 1997; Buckley et al., 2003). Changes in subcutaneous fat can be monitored throughout the periparturient period and can be used to indirectly track adipose mobilization. Cows continue to mobilize adipose tissue until they reach their peak milk production, at which point their BCS should ideally minimize at 2.75 (Roche et al., 2007). This study found that if cows were below this 2.75 cutoff, they had reduced persistency of lactation and if they were above this threshold, they were more likely to suffer from metabolic diseases in early lactation. Tissue reserves implemented for energy requirements in early lactation are then reaccreted through mid and late lactation to achieve a similar BCS to the previous late gestation levels. However, previous research has shown that cattle BCS and ultrasound measurements are not highly correlated and BCS is subjective to scorer error (Jaurena et al., 2005). Therefore, multiple techniques may need to be implemented to holistically monitor changes in adipose reserves.

While BCS is a widely used technique for measuring changes in adipose reserves, NEFA in the blood can be monitored to evaluate changes in adipose mobilization. Cows that are above 0.7 mmol/L of plasma NEFA at more than one week postpartum are suggested to be in this negative energy balance and are susceptible to developing metabolic diseases (Ospina et al., 2013). On commercial farms in upstate New York, 65% of farms were found to have at least 15% of their early lactation animals to be over this 0.7 mmol/L threshold (Ospina et al., 2010). Excessive adipose tissue mobilization can subsequently lead to several negative health outcomes.

Unfortunately, to this point there has been no investigation between NEFA concentration, and the amount of adipose tissue mobilized. Calculations of NEFA area under the curve over the periparturient period could logically determine the amount of adipose tissue mobilized. However, there is diurnal variation in NEFA concentrations (Overton and Waldron, 2004) as well as variation

depending on the time the sample is taken relative to the animal's previous meal and it would require more frequent screening than is possible in most on-farm settings. Therefore, plasma NEFA concentrations have been predominantly used in research settings alone and can serve as a marker of adipose tissue mobilization.

1.4.5 Quantifiers of Protein Mobilization

Similarly, to monitoring changes in adipose tissue via blood NEFA concentrations, changes in muscle tissue can be evaluated by its own respective metabolites of creatinine (CRE) and 3-methylhisitidine (3-MH). Creatinine is a waste product formed during the degradation of muscle mass, specifically creatine and phosphocreatine. Since cows are constantly turning over muscle mass through the process of protein breakdown and synthesis, CRE has been recognized as a blood marker for indexing an animal's total muscle mass (Wyss and Kaddurah-Daouk, 2000). Previous studies have shown that cows decrease in CRE concentration throughout the early lactation period with second lactation animals having greater CRE concentrations compared to third and greater lactation animals (Pires et al., 2013). However, other studies showed no difference in CRE concentration between primiparous and multiparous animals, which describes inconclusive evidence of lactation number on CRE concentration (Cozzi et al., 2011). Previous research has also shown that CRE is not affected by BCS and is therefore a marker of muscle reserves alone (Pires et al., 2013).

The metabolite 3-MH is a known byproduct of protein breakdown that cannot be reincorporated into muscle tissue through protein synthesis, which makes it an ideal marker of muscle mobilization (Doepel et al., 2002). The greatest concentrations of 3-MH are observed in the week directly before and after calving in correspondence with the greatest changes observed in muscle mobilization via ultrasound (van der Drift et al., 2012). Measurements of 3-MH decrease

accordingly as cows decrease the amount of protein they mobilize up until five weeks post calving. However, while 3-MH will greatly reduce after most protein mobilization occurs in the first month of lactation, it will never reach close to 0 as cows continue to turn over protein throughout lactation. When plasma samples are analyzed for 3-MH, it is imperative to differentiate 3-MH from 1-methylhistidine (1-MH) (Houweling et al., 2012). Although both have a similar molecular weight, 1-MH remains with the potential to be reincorporated into protein synthesis and is not representative of muscle degradation alone.

To standardize the amount of muscle cows' mobilize based on these metabolites, implementation of a ratio of 3-MH to CRE allows for the creation of an index to compare cows that have high versus low body protein mass. Therefore, this analysis would indicate if cows with a small amount of muscle mass are mobilizing a relatively greater amount of muscle compared to animals with greater empty body protein at any given timepoint (Pires et al., 2013). The authors of the paper found a trend between low body conditioned cows to have a greater 3-MH:CRE molarity ratio than medium and high conditioned cows grouped four weeks prior to calving (0.111 vs. 0.088 vs. 0.088). This shows that while the low conditioned cows had lower amounts of body fat, they trended to mobilize a greater amount of their muscle reserves as a proportion of total muscle reserves compared to those animals that had greater body condition.

To determine the optimal amount of tissue mobilization, the amount of tissue mobilized must be quantified via the various techniques that have been discussed. To this point there are a limited number of studies that discuss the sensitivity of these quantification techniques and which is the most optimal to understand differences in tissue mobilization in herds of different genetic makeup and across management systems (Friggens et al., 2004; McCarthy et al., 2007). Future research is necessary to determine the relationships between various techniques and sensitivity of

the measurements to changes in cattle body composition over the transition period. Greater knowledge of the timing and amount of tissue type dairy cattle mobilize can improve management decisions and nutritional strategies to avoid rapid tissue mobilization and improve animal health and nutrition.

1.5 Conclusion

The dairy cattle transition period is not only the most metabolically challenging time in the lactation cycle but likewise the period of the greatest disease development. The homeorhetic transition from a non-lactating, gestating state to a heightened nutrient demand in early lactation requires cows to shift their nutrient partitioning to meet the nutrient requirements for milk production in early lactation. One characteristic of this period is the negative nutrient balance, which results in the mobilization of tissue to compensate for insufficient nutrient intake. It has been documented that cows on average mobilize approximately 20% of their muscle and 35% of their adipose reserves from one week prior to calving through nadir tissue composition at approximately two months in lactation. However, there is significant variation in the quantity of respective mobilized tissue between cows over early lactation, depending on nutrition, environment, and body reserves available to mobilize. Many of the diseases observed in the early postpartum period are associated with an increased amount of tissue mobilization in early lactation. Various nutrition and management strategies to minimize the amount of tissue mobilized and increase the cow's metabolic efficiency from vitamin and micronutrient supplementation to animal body condition management have been evaluated in research trials and on-farm studies. However, one of our knowledge gaps remains being able to determine the quantity of body reserves cows use through the periparturient period.

Additionally, the timing of environmental events, such as the timing of feeding and milking, during the transition period should be held as constant as possible to promote a strong circadian timing system. The CTS is driven by circadian clocks, which function by relying on the timing of environmental cues to prepare cattle to anticipate events such as feed intake and milking, among others. However, chronic disruptions in the timing of environmental events can alter the expression of core clock genes, which can dysregulate the timing of the anticipation and lead to negative health and milk production outcomes. While there are a minimal number of studies identifying the impact of circadian rhythm disruptions on cattle health and performance, it is known that these environmental stressors such as pen moves and overcrowding should be minimized to promote animal health. If managers can eliminate these outside stressors, the daily consistency will promote the endogenous circadian timing system to facilitate a successful transition to lactation.

Moreover, to quantify the amount of tissue cows mobilize, there are several ways to evaluate body composition changes. However, there is minimal research looking at how these techniques correlate with one another to determine the timepoints at which cows mobilize tissue and how the cow's tissue composition changes over the duration of the lactation. Although techniques such as BCS are well recorded, they are subject to scorer variability and do not have the ability to precisely quantify tissue reserves as has been recorded via D_2O displacement and empty body composition measurements taken at slaughter. If managers and researchers know the timing of tissue mobilization and re-accretion in cows, cattle diets could be formulated to promote either of these processes and influence animal health and production outcomes.

The homeorhetic transition period requires cows to undergo metabolic changes to a new normal to support the elevated nutrient requirements of lactation. Tissue mobilization is one of the key adaptations that facilitates this transition to a new metabolic state and allows cows to bridge

their negative nutrient gap. Meanwhile chronic circadian disruptors in cattle have been found to affect animal health, milk production, and compromises their ability to maintain homeostasis. Although research in this area is limited, in the transition from late gestation to early lactation, cows must balance maintaining homeostasis and undergoing homeorhesis to drive forward milk production. To support a cow's transition to lactation, managers should minimize disruptions to maximize lactational yield and animal health.

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CHAPTER 2. CHRONIC PREPARTUM, DRY PERIOD LIGHT-DARK PHASE SHIFTS IN CATTLE DISRUPT CIRCADIAN CLOCKS, DECREASE INSULIN SENSITIVITY AND MAMMARY DEVELOPMENT, AND ASSOCIATE WITH LOWER MILK YIELD THROUGH 60 DAYS POSTPARTURITION

2.1 Abstract

Circadian and metabolic systems are interlocked and reciprocally regulated. To determine if the circadian system regulates glucose homeostasis and mammary development, the function of the circadian system was disrupted by exposing cattle to chronic light-dark cycle phase shifts from five wk before expected calving (BEC) to parturition. Multiparous Holstein cows were exposed to 16 h of light and 8 h of dark (CON, n=8) or phase shifting (PS, n=8) the light cycle 6 h every 3 d beginning 35 d BEC. After calving, both treatments were exposed to CON lighting. Mammary biopsies were taken at 21 d BEC and 21 DIM, and histological analysis indicated PS treatment decreased the ratio of lumen to alveolar area and percent proliferating epithelial cells in the prepartum period. Intravenous glucose tolerance test was performed at 14 d BEC and 7 DIM by administering 50% dextrose. Blood glucose, β-hydroxybutyrate, insulin, and non-esterified fatty acids were consequently measured over 3 h. At 14 d BEC there were no treatment differences in baseline glucose or insulin. There was no effect of treatment on blood glucose or glucose area under the curve (AUC) at 14 d BEC and 7 DIM. Insulin AUC was higher in PS versus CON at 14 d BEC and 7 DIM. PS cows produced less milk than CON cows through 60 DIM (40.3 vs. 42.6 kg/d). Exposure to chronic light-dark PS in late gestation decreased mammary development and increased insulin resistance in periparturient cows, which may have caused subsequent lower milk yield.

2.2 Introduction

The most metabolically demanding time for dairy cattle is the transition from late gestation to early lactation. During this time, major shifts in nutrient partitioning occur to support fetal growth in late gestation and then to support the metabolic demands of milk synthesis in early lactation. Physiological changes in response to the increased metabolic demands are reflected in changes in plasma metabolite and hormone levels, which are marked by decreased levels of glucose and insulin, and increased non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) (Bauman and Currie, 1980). To facilitate this transition, cows undergo natural insulin resistance by reducing insulin sensitivity in the peripheral tissues to spare glucose for late-term fetal growth and milk production (Bell et al., 2000; De Koster and Opsomer, 2013). To maintain health cows must sustain homeostasis while coordinating changes in metabolism in almost every tissue of the body during late pregnancy and early lactation. Minimization of the number of disturbances through the transition period is a strong predictor of the health and production performance of a dairy cow lactation (Duffield et al., 2009).

The transition period commonly overlaps with the 45-60 d nonlactating dry period in which the mammary gland undergoes significant remodeling to replenish mammary parenchymal tissue (Hurley, 1989; Capuco et al., 1997; DeVries et al., 2010). The first half of the dry period is marked by high levels of programmed cell death and extracellular matrix remodeling. Whereas the second half of the dry period is characterized by high levels of proliferation of mammary epithelial cells (MEC) in the glands in preparation for the ensuing lactation (Zhao et al., 2019). Mammary remodeling during the dry period is critical as the number and metabolic activity of MEC directly corresponds with the cow's milk production capacity (Knight, 2000), and dry period management and environmental factors, such as photoperiod and heat stress, are known to impact mammary development during this time (Collier et al., 2006; Dahl, 2008; Mabjeesh et al., 2013).

The circadian timing system (CTS) is a homeostatic system that functions to coordinate the timing of internal physiological processes across the body through the generation of 24 h rhythms of behavior and physiology (Nakagawa and Okumura, 2010). In mammals, the CTS is regulated hierarchically, such that the master clock in the suprachiasmatic nuclei of the hypothalamus integrates temporal cues from the environment and physiological systems, and in turn, sends out neural and hormonal signals to synchronize the timing of peripheral clocks across the body (Mohawk et al., 2012). Circadian rhythms enable animals to anticipate and prepare physiology for precise and regular environmental changes, with the 24 h light-dark cycle serving as a primary environmental cue (Stenvers et al., 2019).

Disruptions in the normal timing of temporal cues in the animal's environment can disrupt the CTS (Reppert and Weaver, 2002). Since circadian clocks and metabolic systems are interlocked and reciprocally regulated, disruptions of the CTS disrupt metabolic systems and can affect health (Gangwisch 2009; Buxton et al., 2012). Therefore, it is plausible that management factors that cause chronodisruption may also affect the metabolic health of cattle during the transition period, which is supported by studies in other species. For example, epidemiological studies of pregnant women reported that working nights or rotating shift work, which disrupt circadian clocks, was associated with an increased risk of preterm delivery, intrauterine growth restriction, and preeclampsia (Valenzuela et al., 2015). Sleep disruption, which also disrupts circadian clocks, during pregnancy was associated with increased risk of hyperglycemia and gestational diabetes mellitus (Herring et al., 2014; Facco et al., 2017). Studies conducted in sheep during pregnancy found that exposure to chronic light-dark phase shifts similar to rotating shift-work light exposure increased the likelihood of a delayed lambing and reduced lamb weights (Gatford et al., 2019). The same study demonstrated ewes exposed to chronic shift work model

had decreased insulin sensitivity and increased peak glucose in response to a glucose tolerance test in single lamb dams. However, there was no effect on glucose tolerance or insulin sensitivity in dams that lambed twins. Conversely a crossover study design in non-pregnant, non-lactating ewes, decreased circulating glucose levels with no difference in insulin sensitivity under a glucose tolerance test with a rapid alternating timing of the light cycle (Varcoe et al., 2014).

Genetic studies conducted to understand the role of circadian clocks in reproduction found the *ClockΔ19* line of mice, which have a germline mutation resulting in loss of rhythmicity and lower expression of CLOCK regulated gene expression, found the mutation decreased lactation competence (Kennaway et al.,2004; Dolatshad et al., 2006; Hoshino et al., 2006). *ClockΔ19* mice exhibit altered glucose homeostasis and impaired mammary development in late pregnancy (Turek et al., 2005; Casey et al., 2016). Sleep disruption during pregnancy in women was associated with hyperglycemia and delayed onset of lactogenesis (Casey et al., 2019). A cross-over design study of dairy cattle found that exposure to chronic light-dark phase shifts during an established lactation decreased milk yield (Casey et al., 2014). Whereas late pregnant non-lactating dry cows exposed to chronic light-dark phase shifts over the last five weeks of gestation had reduced plasma glucose concentrations and yielded a greater amount of milk during the following two months of lactation compared to a group under a consistent timing of light (Suarez-Trujillo et al., 2020). Restricting feeding time to normal times of rests disrupts the circadian timing system. A cross-over design study found cattle restricted to feed availability during dark hours produced more milk as compared to periods when feed was available during periods of light exposure with no differences in dry matter intake (Salfer and Harvatine, 2020). Thus, alterations in the CTS on glucose metabolism and milk production metrics in ruminants has shown mixed results.

Based on these previous findings, we hypothesized animals with a disrupted CTS would have reduced insulin sensitivity in response to a glucose challenge, compromised mammary development, and reduced milk yield in the subsequent lactation. Therefore, our objective was to measure the effect of disrupting the CTS by exposing late gestation, non-lactating dry cows to chronic light-dark phase shifts on insulin sensitivity, mammary development, and milk yield through 60 DIM.

2.3 Materials and Methods

2.3.1 Animal Management and Experimental Design

The experiment was performed from February-June 2019 in the tie-stall barn at the Purdue University Animal Sciences Research and Education Center (ASREC) dairy. All procedures described were approved by IACUC protocol #1701001523 prior to beginning the study. Sixteen multiparous Holstein cows were enrolled into one of two treatments, control (CON) or phase shift (PS) and blocked by lactation number (2.88 ± 0.64 vs. 2.88 ± 0.64) and previous lactation yield (12,087 ± 2,486 vs. 12,467 ± 2,407 kg). Animals enrolled in a previous study [(n=6) in Suarez-Trujillo et al. (2020)] conducted the previous year were enrolled to the opposite treatment (n=3/treatment). Cows were dried off at 60 d before expected calving (BEC) and moved to the experimental barn at 35 d BEC. From 35 d BEC to calving, all cows received 16 h of light and 8 h of darkness. Light phase for CON cows was from 0500 to 2100, and dark phase was from 2100 to 0500 daily (Figure 2.1). Whereas for the PS group the timing of the start of a 16 h to 8 h light-dark cycle phase was shifted forward by 6 h every 3 d. Exposure to light-dark phase shifts was from 35 d BEC to approximately 3 d BEC when all cattle were moved to box-stalls in the maternity barn for calving. From 3 d BEC to 60 DIM all animals were on the CON light-dark schedule.

As detailed by Suarez-Trujillo et al. (2020), in the prepartum period, all animals were housed in the same experimental tie-stall barn, which was partitioned to separate treatments with floor to ceiling draping of two layers of fire-retardant tarps (Tarps Plus, Abadak Inc., Georgetown, TX). Outside sources of light were blocked by covering windows with black plastic and caulking was used around doors to block natural light. The light source consisted of bright white light-emitting diode (LED) lights (Smart Electrician 5000 lm 46 x 6 LED Tread Plate Shop Light, Menards Inc., Eau Claire, WI), that were positioned for 150 lx of light to be present at the eye level for all cows. The lights were synchronized to turn on at the aforementioned times using an automatic timer.

2.3.2 Circadian Rhythm Assessment

At 23 and 9 d BEC and 14 DIM vaginal temperature was measured in each cow every 30 min to determine internal temperature circadian rhythm over a 48 h period as described by Burdick et al. (2012). Briefly, internal temperature loggers (iButton DS1921H- F5#, iButtonLink Technology, Baulkham Hills, Australia) were secured onto the plastic skeleton of a controlled internal drug release device (EAZI- Breed CIDR Cattle Insert, Zoetis Inc., Parsippany- Troy Hills, NJ), and inserted into the vagina. Loggers were programmed to begin temperature recordings at 0430 and continued every 30 min for 48 h.

2.3.3 Dry Matter Intake

Feed ingredient samples were collected every two wk and analyzed for nutrient composition. Samples were dried at 60°C for 48 h and ground through a 1 mm mill (Retsch GmbH, Haan, Germany). Feed samples were measured for neutral detergent fiber and acid detergent fiber (Van Soest et al., 1991) (Ankom, Macedon, NY). While percent ash content was determined by

contents remaining after a 24 h oven cycle at 600ºC. Crude protein was determined on a pure nitrogen basis by combustion analysis (LECO, St. Joseph, MI) and then multiplied by 6.25 to determine crude protein percentage.

Animals were offered *ad libitum* feed daily at 1600, by dispersing feed at 110% of the previous day's intake. Daily dry matter intake of individual cows was calculated by weighing the feed offered and subtracting refusals from 35 d BEC until 21 DIM when cows were moved to the free-stall barn. The delivered as fed diet was determined via TMR Tracker (Topcon Agriculture Group; Fort Atkinson, WI). To calculate diet dry matter, each individual feedstuff was multiplied by their respective percent dry matter. Feed intake was then multiplied by the ration percent dry matter to determine dry matter intake. Cows were fed a prefresh diet (52.9% dry matter, 15.9% crude protein, 37.5% NDF, 24.4% ADF, and 5.7% ash; Table 2.1) from enrollment at 35 d BEC until calving. Once calving occurred, cattle were fed a lactating diet (51.8% dry matter, 15.3% crude protein, 27.2% NDF, 18.4% ADF, and 5.1% ash) through 60 DIM.

2.3.4 Milk Sampling and Analysis

Animals were milked twice daily at 0500 h and 1600 h. A homogenous milk sample was collected in the parlor from both milking timepoints at 7, 14, 21, 30 and 60 DIM. Milk samples were sent to Dairy One (Ithaca, NY) for analysis of protein, fat, somatic cell count (SCC), milk urea nitrogen (MUN), and total solids using infrared technology (MilkoScan 7 RM, Foss, Hillerrød, Denmark). Component yield for the day was calculated by multiplying the component percentage by the milking weight as recorded by the Afifarm Software (Afimilk USA Inc.) and then summing the yield from the morning and afternoon milk weights. Component percentages were determined by dividing the component yield by the total milk yield for that test day. Somatic cell count data was transformed by a $\log_{10}$ scale and subsequently analyzed.

2.3.5 Intravenous Glucose Tolerance Test (IVGTT)

At approximately 14 d BEC and 7 DIM all cows underwent an IVGTT. The 14 d BEC and 7 DIM timepoints were selected to ensure that all cattle would have a test performed while under treatment and to assess cattle insulin sensitivity during the period of expected greatest insulin resistance, respectively (De Koster and Opsomer, 2013). One CON cow did not complete the IVGTT at the 14 d BEC timepoint, due to complications with catheterization. Animals were weighed either the morning before or the morning of test date following the morning milking to the nearest half kg. Without the use of local anesthetic, an indwelling jugular catheter was inserted in either the right or left jugular vein the morning of IVGTT (0600 to 0800). At 1100, feed was removed from all animals for one h prior to dextrose administration. Feed was not made available at any time throughout the test, but water was available at all times. Baseline blood samples were collected at 15 (t = -15) and 5 (t = -5) min prior to dextrose administration via blood sample through catheter lines using a 12 mL syringe (Covidien; Dublin, Ireland). A dose of 250 mg/kg BW of glucose was administered intravenously via 60 mL syringes in the form of a 50% dextrose solution (Vet One; Boise, ID), at time point 0 (t=0). The dispensing time to infuse the entire dextrose dose ranged from 3-13 min, with a mean of 7.5±2.2 min. Immediately following administration of dextrose, catheter lines were flushed with 20 mL of saline, which was followed by blood sample collection from the same catheter. Subsequent blood samples were taken from the jugular catheters at time 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 min relative to the completion of the glucose infusion.

2.3.6 Blood Metabolite Analysis

An aliquot of whole blood was used to measure glucose and BHB concentrations using the Centrivet GK Blood Glucose & Ketone Monitoring System (Acon Laboratories, San Diego, CA).

The remaining 10 mL of whole blood was transferred into a 10 mL EDTA tube (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 4,000 g for 15 min at 4°C. Three aliquots of plasma were frozen in -20ºC until further analysis.

Plasma concentrations of insulin and NEFA were analyzed in duplicate using bovine insulin (ALPCO; Salem, NH) and NEFA kits (WAKO; Mountain View, CA). Intraplate CVs were 1.72% and 3.93% for NEFA and insulin, respectively. Interplate CVs were 8.00% and 15.74% for NEFA and insulin, respectively.

The area under the curve (AUC) was calculated for glucose and insulin concentrations from 0-180 min using the trapezoidal rule (Pires et al., 2007). Insulin increment was calculated by subtracting baseline insulin concentration from the peak insulin concentration. Insulin clearance rate was calculated as the slope between the peak insulin concentration and insulin concentration at 60 min post glucose administration. Whereas glucose clearance rate, was calculated using the formula: glucose clearance rate = $\frac{\ln([glucose]_0)-\ln([glucose]_{60})}{60}$ and the time to half-maximal glucose concentration was calculated using the equation $\frac{\ln(2)}{\ln(glucose\ clerance\ rate)}$ as detailed in (Kerestes et al., 2009). To calculate a cow's Revised Quantitative Insulin Sensitivity Check Index (RQUICKI; Holtenius & Holtenius, 2007) the following formula was used, RQUICKI = $\frac{1}{\log(\text{glucose})+\log(\text{Insulin})+\log(\text{NEFA})}$. The addition of baseline BHB was used to determine RQUICKI_BHB as detailed in Balogh et al., (2008) with RQUICKI_BHB = $\frac{1}{\log(\text{glucose})+\log(\text{Insulin})+\log(\text{NEFA})+\log(\text{BHB})}$. The greater the RQUCIKI and RQUICKI_BHB scores the greater the baseline insulin sensitivity.

2.3.7 Mammary biopsy

At 21 d BEC and 21 DIM, mammary gland biopsies were taken from the left-rear and right-rear quarter, respectively as described by De Vries et al. (2010). Prior to biopsy, cows were restrained in a squeeze chute, and sedated using xylazine via intravenous coccygeal vein injection (Rompun, Bayer HealthCare LLC, Animal Health Division, Shawnee, Kansas). The biopsy site was shaved and thoroughly cleaned with diluted betadine and 70% ethanol. Skin was locally anesthetized by injecting 10 mL of 2% lidocaine HCl subcutaneously (Vet One; Boise, ID). An incision was made through the skin using a scalpel to expose underlying mammary tissue. Approximately 1 g of tissue was collected using a stainless steel, retractable biopsy tool (AgResearch, NZ). Tissue was rinsed with sterile phosphate buffered saline (PBS), and divided into four sections. Tissue preserved for histology was placed in 10% buffered formalin and stored at room temperature. The remaining pieces were flash frozen in liquid nitrogen and stored at -80°C.

2.3.8 Histological analysis of mammary morphology and cell proliferation index.

Purdue University Histology Research Laboratory prepared and stained mammary tissue sections with hematoxylin and eosin (H&E) and immunostained for KI67 expression. Briefly, after fixation in 10% neutral buffered formalin for 24 h, tissues were placed in a Sakura Tissue-Tek VIP6 tissue processor (Sakura Finetek USA Inc.; Torrance, CA) for dehydration through graded ethanols, clearing in xylene and infiltration with Leica Paraplast Plus paraffin (Leic Microsystems Inc.; Buffalo Grove, IL). Using a Thermo HM355S microtome (Thermo Fisher Scientific; Waltham, MA), 4 µM sections were cut and mounted on charged slides and dried for 30-60 min at 60° C. After drying, all slides were deparaffinized through three changes of xylene and rehydrated through graded ethanols to water in a Leica Autostainer XL (Leica Microsystems Inc.; Buffalo Grove, IL).

Using the Leica Autostainer XL, slides were stained in Gill's II hematoxylin, blued, and counterstained in an eosin/phloxine B mixture (H&E stained section). Finally, slides were dehydrated, cleared in xylene and cover-slipped in a toluene-based mounting media (Leica MM24).

For immunostaining with KI67, deparaffinized tissues were subject to antigen retrieval by incubating in a TRIS/EDTA (pH 9.0) solution in a BioCare decloaking chamber (BioCare Medical; Pacheco, CA) at a temperature of 95 °C for 20 min. Slides were cooled for 20 min at room temperature and transferred to TRIS buffer with Tween 20 detergent (TBST), and for the remainder of the protocol, staining was carried out at room temperature using a BioCare Intellipath stainer (BioCare Medical; Pacheco, CA). Slides were incubated with 3% hydrogen peroxide in water for 5 min, rinsed with TBST and incubated in 2.5% normal goat serum (Vector Labs; Burlingame, CA) for 20 min. Excess reagent was blown off and KI67 (Cell Marque, 275R-16; Cell Marque; Rocklin, CA) was applied at a dilution of 1:100 (0.364 µg/mL) for 30 min. The negative control slide was stained with Rabbit IgG (Vector Labs, I-1000) at a concentration of 1:5000 (1 µg/mL) for 30 min. Slides were rinsed twice in TBST and a goat anti-rabbit secondary antibody (Vector Labs, MP-7451) was applied for 30 min. Slides were rinsed twice in TBST and Vector ImmPACT DAB (Vector Labs, SK-4105) was applied for 5 min. Slides were rinsed in water and transferred to a Leica Autostainer XL for hematoxylin counterstain, dehydration, and cover-slipping.

ImagePro Plus 5.1 (Media Cybernetics) was used to capture and analyze images. Mammary biopsies were taken from all cows at both timepoints, but good quality parenchymal tissue was only available from six cows per treatment at the prepartum timepoint. Five H&E stained images were captured per biopsy per cow (n=6/treatment prepartum; n=8/treatment postpartum) at 40 X (Nikon Eclipse 50i, Nikon Inc. NY; Evolution MP, Media Cybernetics Inc. MD). H&E stained

tissue was used to measure alveolar area and lumen area. The "Draw/Merge: Trace" tool was used to trace around the entire alveolus and area calculated using ImagePro (Figure 2.2A) and then the tool was used to trace around the lumen (Figure 2.2B) and area was calculated. Ratio was calculated by dividing the luminal area by the alveolar area.

Five KI67 immunostained images were captured per biopsy per cow at 200X. The proliferation index for MEC and intralobular stromal cells was determined by counting the number of proliferating (cells that were stained with KI67) and nonproliferating cells using count tool.

2.3.9 Statistical Analysis

A priori power analysis was performed using data from studies on the effect of different photoperiod length exposures during the dry period on epithelial cell proliferation at three weeks BEC. Using six animals per group the power of the study was found to be 100% if there was a 3-fold difference in cell proliferation rate, with a standard deviation of 0.5-fold (Wall et al., 2005). If the difference dropped to 2-fold, the calculated power was 96%.

Vaginal temperature data were analyzed for fit to a 24 h cosine curve using the cosinor package in R (RStudio 1.1.453, Boston, MA). MESOR, amplitude, and acrophase were program outputs and calculated for each animal at the three study timepoints of 23 and 9 d BEC and 14 DIM. Temperature data, proliferation indexes, lumen to alveolar ratio, milk yield, and dry matter intake were analyzed using the MIXED Procedure of SAS 9.4 (Cary, NC). The following model was used:

$$Y_{ijkl}=\mu+T_i+P_j+S_k+C(T)_{il}+e_{ijkl}$$

Where Y_{ijkl} is the dependent variable, μ is the overall mean, T_i is the fixed effect of treatment (i=CON or PS), P_j is the fixed effect of timepoint (temperature data j=23 d BEC, 9 d BEC, or 14 DIM; MEC proliferation and lumen to alveolar ratio j= 21 d BEC or 21 DIM; milk

yield j=1-60 DIM; dry matter intake j=35 d BEC-21 DIM), S_k is the fixed effect of physiological state (k=prepartum (BEC) or postpartum (DIM)); $C(T)_{il}$ is the random effect of cow nested within treatment, and e_{ijkl} is the residual error term. Due to the treatment only being applied during the prepartum period, pre and postpartum timepoints were run independently to assess treatment effects during the treatment period as well as any potential carryover effects into lactation. For temperature data, if physiological state was found to be significant, Tukey's HSD test was used to assess timepoint differences between treatments. Additionally, to assess the effect of physiological state, both prepartum and postpartum timepoints were included in the model.

IVGTT glucose, BHB, NEFA, and insulin data were analyzed using the MIXED Procedure of SAS 9.4 (Cary, NC). The following model was used:

$$Y_{ijk}=\mu+T_i+P_j+TP_{ij}+C(T)_{ik}+bl+e_{ijk}$$

Where Y_{ijk} is the dependent variable, μ is the overall mean; T_i is the fixed effect of treatment (i=CON or PS); P_j is the fixed effect of timepoint relative to glucose administration (j=0 to 180 min), TP_{ij} is the interaction effect of treatment × timepoint, C(T) is the random effect of cow nested within treatment (k=cow number), bl is the hormone or metabolite baseline covariate as average of t=-15 and -5 minutes relative to glucose infusion, and e_{ijk} is the residual error term.

Data was screened for influential outlier points where Cooks' D values were ≥ 0.5, data points above this cutoff were removed (n≤3 per analysis). Data was considered significant at $P \leq 0.05$ and trends were indicated if $P \leq 0.10$ to > 0.05. If there was a significant treatment or treatment × timepoint effect, the SLICE method was used to determine differences between treatments at different timepoints.

2.4 Results

2.4.1 Temperature Circadian Rhythm Data

The R^2 and *P*-value variables calculated from cosine fit analysis reflect the relative fit of the data to a cosine curve within a 24 h period. Across all time points, temperature rhythms in both treatments fit 24 h rhythms (all, $P<0.05$; Table 2.2). There was no significant difference in R^2 between treatments at 23 d BEC, however at 9 d BEC temperature data for CON cows fit a 24 h cosine curve better than PS cows ($P<0.05$; R^2, 0.48 vs. 0.23). There was no effect of treatment on R^2 or *P*-value at 14 DIM ($P>0.05$). All cows' temperature data had a stronger fit to a cosine curve at 23 and 9 d BEC compared to 14 DIM ($P<0.05$; 0.40 vs. 0.35 vs. 0.20).

Circadian rhythm temperature amplitude is a relative measure of circadian rhythm strength and was affected by treatment and physiological timepoint. Although, there was no difference in amplitude between treatments at 23 d BEC ($P>0.05$), PS cows had lower rhythm amplitudes than CON at 9 d BEC ($P<0.05$). At 14 DIM mean amplitude of core body temperature was not different between treatments. Temperature amplitude did not differ between the three physiological timepoints ($P>0.05$).

Acrophase, or the time of day the rhythm peaks, was not affected by physiological state or treatment. MESOR, the rhythm adjusted mean, and the area under the curve (AUC) of body temperature were not affected at any timepoint by treatment. Between 23 to 9 d BEC mesor and AUC increased ($P<0.05$; 39.04 vs. 39.30 ºC; 1894 vs. 1906 ºC/48 hr), but there was no difference in MESOR and AUC between 9 d BEC and 14 DIM.

2.4.2 Intravenous Glucose Tolerance Tests

At 14 d BEC there was no difference in baseline glucose, BHB, insulin, or NEFA between treatments (Table 2.3), and no difference between baseline insulin sensitivity, as indicated by RQUICKI. However, when BHB was added into the calculation (RQUICKI_BHB), PS cows had lower baseline insulin sensitivity at 14 d BEC compared to CON (P<0.05; 0.55 vs. 0.66). At 7 DIM, PS cows had higher baseline glucose (P=0.05; 67.63 vs. 61.50 mg/dL), lower BHB (P<0.05, 1.49 vs. 0.97 mmol/L), and higher NEFA (P<0.01, 0.71 vs. 0.43 mmol/L) concentrations, compared to CON. There was no difference in baseline insulin concentrations between the treatments, and no significant difference in RQUICKI or RQUICKI_BHB between treatments at 7 DIM. Blood glucose, BHB, insulin, and NEFA baseline concentrations were different (P<0.05) between the prepartum and postpartum periods, however, there was no difference in RQUICKI or RQUICKI_BHB between the two physiological states.

At 14 d BEC, there was no difference between treatments in glucose response curves (P>0.05; Figure 2.3A) glucose clearance rate, glucose AUC, and the time to reach half maximal glucose post-peak (Table 2.4). There was a treatment by time effect for the insulin response curve (P<0.05; Figure 2.3B), which resulted in the PS group having greater insulin concentrations at t= 5, 10, 15, and 20 min post glucose infusion. The CON treatment had a lower insulin AUC compared to PS (P=0.05; Table 2.4). PS cows tended to clear insulin at a faster rate than CON (P=0.08; 3.50 vs. 2.69%), which was calculated by the change per min in insulin concentration from the timepoint of peak insulin through 60 min post glucose administration.

Plasma NEFA concentrations over the IVGTT were not statistically different between treatments (P>0.05), nor were there treatment by time effects (P>0.05; Figure 2.4A). PS cows had a tendency for lower BHB concentrations over the IVGTT sampling period (P=0.08; Supp. Figure 1A).

At 7 DIM there was no difference between treatments in glucose concentration at any timepoint after dextrose infusion (Figure 2.3C & 2.3D). Similar to 14 d BEC, this resulted in no treatment differences in glucose AUC (Table 2.4), glucose clearance rate, and time to half maximal glucose). There was a treatment difference in insulin AUC measurement from 0-180 min (P=0.03; 697 vs. 1,053 mIU/mL; Table 2.4) as well as a difference in insulin increment between treatments (P=0.04; 28.8 vs. 43.5 mIU/mL). There was no difference in insulin clearance rate between treatments.

There was a trend for treatment by timepoint interaction for plasma NEFA concentrations (P=0.09; Figure 2.4B). Both treatments had similar NEFA concentrations at the time of glucose administration but NEFA concentration tended to be reduced in PS cows at t=30, 40, 50, and 75 min post glucose infusion and reduced NEFA concentrations at t=60. CON cows had greater baseline BHB concentrations, but there was no overall treatment difference for BHB in the 180 min post glucose administration (Supp. Figure 1B).

Comparison of IVGTT variables in cattle between 14 d BEC and 7 DIM indicated all cows had a greater glucose clearance rate, faster time to half maximal glucose timepoint, and lower glucose AUC in the postpartum versus prepartum period (Table 2.4). Similarly, for insulin metrics, cows in the postpartum period had a lower insulin increment, greater insulin clearance rate, and reduced insulin AUC relative to the prepartum time point.

2.4.3 Mammary development

PS cows had reduced lumen to alveolar area than CON cows at 21 d BEC (P<0.05; 0.086 vs. 0.16; Table 2.5). All cows had a greater lumen to alveolar ratio at 21 DIM compared to 21 d BEC (P<0.05; 0.21 vs. 0.13). The proliferation index of mammary epithelial cells, as indicated by percent of cells positively stained for KI67, was significantly reduced in PS compared to CON

cows at 21 d BEC (P<0.05; 5.2 vs. 12.4%). However, there was no significant difference in MEC proliferation at 21 DIM between the treatments (P>0.05; 0.77 vs. 0.78%). Rate of epithelial proliferation was significantly higher in the prepartum than postpartum periods. There was no treatment difference in proliferation index of mammary stromal cells, but levels were significantly greater in the prepartum versus postpartum sampling time points (P<0.05; 2.26 vs. 0.59%).

2.4.4 Dry Matter Intake

There was no difference in dry matter intake between treatments during the prepartum or postpartum period (Table 2.6; Supp. Figure 2). However, all cattle consumed more dry matter in the postpartum versus prepartum period (P<0.05; 17.1 vs. 12.5 kg/d).

2.4.5 Milk Production and Components

PS cows produced less milk than CON cows from 1-60 DIM (P=0.05; 40.3 vs. 42.6kg/d; Table 2.6).There was a trend for lower milk solids (4.87 vs. 5.30kg/d; P=0.10) and lactose (1.86 vs. 2.03kg/d, P=0.09) in PS cows compared to the CON group across the 5 d milk components were analyzed. There were no differences in percent fat and protein yield. The treatments had similar milk urea nitrogen values and somatic cell counts.

2.5 Discussion

Multiparous cattle exposed to chronic light-dark phase shifts in late gestation developed insulin resistance, which despite cessation of treatment was carried over into the postpartum period. Moreover, MEC proliferation at 21 d BEC and lumen to alveolar ratio was reduced in the PS treated cattle, indicating that exposure to circadian disruption negatively impacted dry period mammary development. PS cattle also had reduced milk yield in the first two months of lactation,

indicating that circadian rhythm disruptions in late gestation negatively impacted subsequent lactation performance.

2.5.1 Circadian Disruption Model Altered Internal Temperature Rhythm

The forward shifting pattern of light-dark phase was implemented based on previous studies in rodents that showed this chronic jetlag model disrupted the circadian timing system and resulted in alterations in metabolism (Reddy et al., 2002; Filipski et al., 2004). Similar to studies in rodents, we found that exposure to chronic light-dark phase shifts reduced the cosine rhythm of the 24 h body temperature rhythm. While there was no difference in fit of core body temperature after two wk of exposure to chronic light-dark phase shifts, after four wk of exposure, the body temperature circadian rhythmicity of PS cows was attenuated relative to CON. Although there was no treatment difference in amplitude at 23 d BEC, at 9 d BEC the PS cows had a lower amplitude at 9 d BEC compared to the CON group. These findings were similar to our previous study (Suarez-Trujillo et al. 2020) and support that the circadian timing system was disrupted in PS cattle.

Across all cows, temperature MESOR and AUC increased between 23 and 9 d BEC. These results are in agreement with Suarez-Trujillo et al. (2020), and likely reflect the increased metabolic output as cows transition to lactation (Gamo et al., 2013). At 14 DIM, there was no difference in R^2 or amplitude between treatments, and across all cattle R^2 was significantly lower than during 23 d and 9 d BEC recording periods. This finding suggests that the physiological adaptations that occur to support lactation may obscure or attenuate endogenously generated rhythms (Jilge et al., 2001).

2.5.2 Circadian Disruptions Affected Insulin Signaling

Exposure to chronic light-dark phase shift resulted in greater baseline insulin resistance in PS cattle at 14 d BEC as indicated by significantly lower RQUICKI-BHB. A similar discrepancy in RQUICKI-BHB and RQUICKI as indicators of baseline insulin sensitivity was observed by others (Balogh et al., 2008), and thus supports the use of both variables in analysis. In response to the IVGTT, PS cows produced greater insulin than CON cows, resulting in a treatment by timepoint interaction in insulin concentration, whereas there was no difference between treatments in glucose concentration response curves. The difference in insulin AUC with no difference in glucose AUC in response to the dextrose infusion, is consistent with insulin resistance in the PS treatment compared to the CON (De Koster and Opsomer, 2013). The finding that cattle develop insulin resistance in response to circadian rhythm disruption is consistent with studies of other species (Turek et al., 2005; Kennaway et al., 2013; Gatford et al., 2019), although it is important to note that the response may be variable (Varcoe et al., 2014).

At 7 DIM, the PS cows exhibited greater insulin production compared to CON cows in response to IVGTT, indicating that the effect of treatment on insulin resistance was carried over into the postpartum period. While the phenomenon of insulin resistance in early lactation enables cows to mobilize adipose tissue stores to support the energetic demands of milk synthesis, very high levels of insulin resistance are postulated to lead to the development of metabolic disorders such as ketosis and fatty liver due to overloading of NEFA, and likewise rapid body condition loss (Zachut et al., 2013). Health indices were not different between treatments in the study (data not shown), although a larger sample of cows would likely be needed to evaluate this outcome.

It is important to note, other studies have observed minimal correlation between baseline indices of insulin sensitivity and IVGTT AUC calculations (De Koster et al., 2016; Alves-Nores et al., 2017). This can be due to a number of factors which include the time relative to last meal,

physiological state, and these baseline indices were created to measure human insulin sensitivity and adapted for cattle (Holtenius and Holtenius, 2007; De Koster et al., 2017; González-Grajales et al., 2017). There is also the concern that insulin disappearance occurs through several insulin-independent pathways including renal filtration and fetal and mammary gland uptake depending on the physiological state of the animal (González-Grajales et al., 2018; Malacco et al., 2020). Although the gold standard of measuring insulin sensitivity in dairy cows has been the use of the insulin euglycemic clamp, there have been a number of studies evaluating insulin sensitivity using IVGTT and RQUICKI alone (Pires et al., 2007; Mann et al., 2016). Furthermore, the first 60 minutes of an IVGTT in evaluating insulin resistance have shown to be well correlated with the evaluation of a euglycemic clamp (De Koster et al., 2016). Therefore, while the insulin euglycemic clamp is the ideal measurement, there is sufficient data to suggest the IVGTT and RQUICKI alone can be used to characterize cattle insulin sensitivity.

In accordance with the glucose infusion, both groups demonstrated a marked reduction in plasma NEFA concentrations, which is consistent with findings of others (Schoenberg et al., 2012; Mann et al., 2016; Girard et al., 2019). At 7 DIM, the PS animals tended to have lower NEFA concentrations at 40-75 minutes post glucose infusion, which indicates a greater uptake or reduced mobilization of fatty acids in response to greater insulin production.

2.5.3 Circadian Rhythm Disruptions Compromised MEC Development and Milk Yield

The number of epithelial cells and metabolic activity of mammary epithelial cells is directly related to milk yield in dairy cattle (Knight, 2000; Capuco and Choudhary, 2020). At 21 d BEC, the PS animals had lower indexes of MEC proliferation compared to CON cows. Consistent with less epithelial cell proliferation at 21 d BEC, the lumen:alveolar ratio was lower in PS cattle at 21 d BEC and 21 DIM. Together supporting that exposure to PS treatment in late gestation decreased

dry-period mammary parenchymal replenishment. The insulin resistance observed in PS cattle prepartum may have played a role in compromised mammary development as insulin is essential for MEC development (Cohick, 2016).

The greater insulin resistance in the postpartum period of PS cattle may have also contributed to lower milk yield, as milk protein synthesis and mammary gland amino acid catabolism are insulin dependent (Menzies et al. 2009; Menzies et al. 2010). In addition, our cell culture and rodent studies demonstrated that circadian clock genes regulate mammary epithelial cell growth and differentiation (Casey et al., 2016). Previous chronodisruption studies of lactating dairy cattle, whether induced by chronic exposure to light-dark phase shifts (Casey et al., 2014) or restricted night feeding (Salfer and Harvatine, 2020), altered the dynamics of core clock gene expression in the mammary gland. The altered expression of core clock genes was related to changes in milk yield in both studies. Therefore, it is also plausible that the mammary clock was altered in the current study in a manner that negatively impacted gland development and therefore milk yield.

Also noteworthy is that significant alterations in circadian rhythms of the core body temperature were observed at 9 d BEC but not at 23 d BEC. This may be an important point to consider in interpreting the effect of treatment on metabolism and mammary development, as biopsies were taken at 21 d BEC and IVGTT was performed at 14 d BEC before differences in temperature circadian rhythm were observed. Factors regulated by circadian clocks exhibit variable response to factors that cause chronodisruption, with some becoming more sensitive or more resistant to changes in the external environment. Rhythms of core body temperature were more resistant to alterations when animals were exposed to factors aimed at forcing desynchrony between internal timing and external regulators (Cambras et al., 2007). There is currently little

data aimed at understanding the sensitivity of the mammary development to circadian disruption, however studies of metabolic systems suggest they are relatively sensitive to changes in external influences. For example, eight days of forced internal desynchronization to misalign circadian clocks in healthy humans, increased fasting glucose and insulin levels (Scheer et al., 2009). Nonetheless, half of the participants exhibited a pre-diabetic state during the period of circadian misalignment. Although the timing of the first mammary biopsy and IVGTT were held prior to the observed misalignment of core body temperature, studies in humans support that alterations in regulations of glucose homeostasis would be evident after two wk of exposure to chronic light-dark phase shifts.

Our previous study (Suarez-Trujillo et al. 2020), found exposure of cattle to chronic light-dark phase shifts during late gestation was associated with increased milk yield in the subsequent lactation. At this time, we cannot account for the differential response of cattle to treatments but provide the following to discuss similarities and differences. In the previous study, the overall metabolic response of cattle to the phase-shift treatment was decreased circulating glucose levels, without differences in insulin levels in non-fasted states. This relationship suggested treatment increased insulin sensitivity, which we did not observe in our present study. Other studies using sheep, also reported differential metabolic response in glucose homeostasis to shifting-light dark photoperiods (Varcoe et al., 2014; Gatford et al., 2019). Where the former showed no difference in insulin sensitivity and the latter demonstrated insulin resistance under chronic phase shift lighting conditions. Studies in humans and rodents have clearly showed that the circadian system regulates glucose homeostasis (Qian and Scheer, 2016). Model systems developed to study circadian regulation of glucose homeostasis found behavioral and sleep patterns contribute to the relative response to disruption, with sleep disruption and feeding pattern disruptions exacerbating

the insulin resistance response. Genetic studies have found deletion of the core clock gene *Bmal1* in various tissues elicits different effects on glucose homeostasis in rodents. For example, when *Bmal1* was ablated in the liver, rodents develop hypoglycemia, and whereas ablation in the pancreas resulted in rodents developing insulin resistance, with both responses found to be diet dependent (Qian and Scheer, 2016).

2.6 Conclusion

Multiparous cattle exposed to chronic light-dark phase shifts for the last five weeks of gestation had compromised mammary gland development and developed insulin resistance that carried over into the postpartum after the elimination of treatment. Metabolic alterations and compromised mammary development were associated with decreased milk yield in the subsequent lactation. This work indicates that the circadian timing system plays a role in regulation of glucose homeostasis and mammary development in cattle, and the importance of minimizing disturbances in day-to-day activities and environment of cattle and maintaining consistency during late gestation to ensure optimal milk production in early lactation.

2.7 Tables & Figures

Table 2.1. Study diet ingredients and nutrient composition for prefresh and lactating diets

Ingredients (g/100 g diet DM)	Prefresh	Lactating
Corn Silage	32.18	29.84
Mixed Mostly Legume Silage	5.31	8.35
Straw	15.30	6.93
Alfalfa Hay	-	9.29
Cotton Seed Hulls	7.18	-
Soybean Meal	6.61	5.82
Dry Ground Corn	10.46	9.59
High Moisture Corn	-	14.81
Amino Acid Blend[1]	9.48	5.31
Prefresh Supplement[3]	11.28	-
Lactation Supplement[2]	-	3.74
QLF 6343[4]	2.19	4.12
Palmit 80[5]	-	2.19
Water, % as fed	9.1	11.0
Nutrient Composition		
Dry Matter	52.9%	51.8%
Crude Protein	15.9%	15.3%
NDF	37.5%	27.2%
ADF	24.4%	18.4%
Ash	5.7%	5.1%

[1]AA Blend included 84.24% Blood Meal, 6.39% Smartamine (Adisseo, Alpharetta, GA), 6.28% Biocycle Plus (Agrarian Solutions, Middlebury, IN), and 3.09% AjiPro-L (Ajinomoto Animal Nutrition, Chicago, IL)

[2]Lactating Premix included 27.48% calcium carbonate, 21.03% sodium bicarbonate, 10.57% white salt, 8.46% calcium phosphate, 8.34% DCAD+ (Arm & Hammer Animal Nutrition, Princeton, NJ), 5.13% Magnesium Ox, 4.98% Diamond V XP (Diamond V, Cedar Rapids, IA), 4.65% Calcium Sulfate Dihydrate, 4.58% Fat yellow grease, 2.64% mineral premix, less than 2% ETX5 (Feedworks USA, Cincinnati, OH), and less than 1% Vitamin E, and Rumensin (Elanco Animal Health, Greenfield, IN)

[3]Prefresh supplement included: 63.12% Biochlor (Arm & Hammer Animal Nutrition, Princeton, NJ), 10.55% Calcium Carbonate, 7.51% Megalac (Volac Wilmar, Orwell, UK), 3.67% Magnesium Oxide, 3.49% Diamond V XP (Diamond V, Cedar Rapids, IA), 2.40% Monocalcium Phosphate, 2.24% Magnesium Sulfate, 1.93% White Salt, 1.77% Vitamin E, 1.60% Calcium Sulfate Dihydrate, 1.55% mineral premix, and less than 1% Rumensin (Elanco Animal Health, Greenfield, IN), and Vitamin A

[4]Quality Liquid Feeds, Dodgeville, WI

[5]ADM Animal Nutrition, Quincy, IL

Table 2.2 Cosine-fit analysis of core body temperature (ºC) at 23 and 9 d before expected calving (BEC) and 14 DIM in control (CON; n=8) and phase shifted (PS; n=8) cows[1]

(A)

	23 d BEC				9 d BEC				14 DIM			
Items	**CON**	**PS**	**SEM**	***P*-value[2]**	**CON**	**PS**	**SEM**	***P*-value[2]**	**CON**	**PS**	**SEM**	***P*-value[2]**
MESOR (ºC)	38.99	39.09	0.08	0.37	39.36	39.22	0.07	0.21	39.31	39.24	0.08	0.58
Amplitude[3] (ºC)	0.20	0.15	0.03	0.18	0.24	0.13	0.02	0.01	0.18	0.18	0.05	1.00
Acrophase[4] (h)	2142	0023	86	0.19	2232	0118	100	0.26	2326	2356	95	0.82
R^2 [5]	0.47	0.33	0.06	0.13	0.48	0.23	0.05	0.01	0.23	0.17	0.05	0.43
P-Value[6]	<0.05	<0.05	0.01	0.37	<0.05	<0.05	0.01	0.18	<0.05	<0.05	0.01	0.44
AUC[7] (ºC/48 hr)	1891	1896	3.79	0.37	1909	1902	3.61	0.21	1906	1903	3.87	0.59

Table 2.2 continued

(B)

	Physiological State				
Items	**23 d BEC**	**9 d BEC**	**14 DIM**	**SEM**	***P*-value[2]**
MESOR (ºC)	39.04[b]	39.30[a]	39.28[a]	0.06	<0.01
Amplitude[3] (ºC)	0.17	0.18	0.18	0.02	0.95
Acrophase[4] (h)	2303	2354	2340	67	0.85
R^2 [5]	0.40[a]	0.35[a]	0.20[b]	0.038	<0.01
P-Value[6]	<0.05	<0.05	<0.05	0.008	0.61
AUC[7] (ºC/48 hr)	1894[b]	1906[a]	1905[a]	2.66	<0.01

a,b Indicate differences between treatments by Tukey post-hoc analysis at $P<0.05$

[1]CON cattle were exposed to consistent cycles of 16 h: 8 h dark. Whereas PS cows were exposed to 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC

[2]P-value assessed between treatments at each timepoint or physiological state

[3]Amplitude is the difference between the MESOR and peak value, calculation was determined as half the difference between the peak and trough of the curve

[4]Acrophase is the time of the peak (h)

[5]Variability of the data that is explained by the model: temperature=MESOR +amplitude x [2π x (hour-acrophase)/24]

[6]Calculated between the observed data and the fitted curve data points

[7]Area under the curve

Table 2.3. Baseline energy metabolite, hormone, and insulin sensitivity indexes at 14 d BEC and 7 DIM for control (n=8) and phase shifted (n=8) dairy cows1

	14 d BEC				7 DIM				Physiological State			
Item	**CON**	**PS**	**SEM**	***P*-value[2]**	**CON**	**PS**	**SEM**	***P*-value[2]**	**BEC**	**DIM**	**SEM**	***P*-value[2]**
Glucose (mg/dL)[3]	79.14	80.88	2.94	0.67	61.50	67.63	2.01	0.05	80.06	64.56	0.33	<0.01
BHB (mmol/L)[3]	0.70	0.84	0.07	0.18	1.49	0.97	0.14	0.02	0.76	1.23	0.07	<0.01
Insulin (mIU/mL)[3]	5.25	7.90	1.86	0.32	0.92	1.54	0.46	0.35	6.57	1.67	0.97	<0.01
NEFA (mmol/L)[3]	0.24	0.23	0.07	0.90	0.43	0.71	0.080	<0.01	0.23	0.61	0.05	<0.01
RQUICKI[4]	0.60	0.52	0.04	0.12	0.39	0.20	0.28	0.63	0.56	0.30	0.14	0.17
RQUICKI_BHB[5]	0.66	0.55	0.03	0.03	0.57	0.22	0.25	0.31	0.60	0.40	0.12	0.21

[1]CON cattle were exposed to consistent cycles of 16 h: 8 h dark. Whereas PS cows were exposed to 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC

[2]P-value assessed between treatments at each timepoint or physiological state

[3]Baseline levels were determined by measuring concentrations in blood samples taken at 15 min and 5 min prior to dextrose infusion, and averaged between time points

[4]RQUICKI was calculated as $\frac{1}{\log(\text{glucose})+\log(\text{Insulin})+\log(\text{NEFA})}$). Greater RQUICKI indicates greater insulin sensitivity

[5]RQUICKI_BHB = $\frac{1}{\log(\text{glucose})+\log(\text{Insulin})+\log(\text{NEFA})+\log(\text{BHBA})}$. Greater RQUICKI_BHB indicates greater insulin sensitivity

Table 2.4. Intravenous glucose tolerance test (IVGTT) glucose and insulin clearance rates, time to half maximal glucose, and insulin increment at 14 d BEC and 7 DIM for control (n=8) and phase shifted (n=8) dairy cows1

	14 d BEC				7 DIM				Physiological State			
Item	**CON**	**PS**	**SEM**	***P*-value[2]**	**CON**	**PS**	**SEM**	***P*-value[2]**	**14 d BEC**	**7 DIM**	**SEM**	***P*-value[2]**
Glucose Clearance Rate (%/min)[3]	1.50	1.61	0.11	0.51	2.16	2.16	0.15	0.97	1.56	2.16	0.094	<0.01
Time to Half-Maximal Glucose (min)[3]	47.5	41.0	2.7	0.12	32.6	31.4	2.0	0.12	46.3	33.3	2.4	<0.01
Glucose AUC (mg/dL/180 min)	19113	18849	365	0.61	14800	15018	439	0.73	18977	14909	289	<0.01
Insulin Increment (mIU/mL)[4]	79.9	141.3	32.9	0.20	28.8	43.5	4.8	0.04	111.8	49.7	18.7	0.027
Insulin Clearance Rate (%/min)[5]	2.69	3.50	0.32	0.08	6.04	5.34	0.78	0.52	3.09	5.69	0.41	<0.01
Insulin AUC (mIU/mL/180 min)	2386	4303	638	0.05	697	1053	108	0.03	3356	1100	350	<0.01

[1] CON cattle were exposed to consistent cycles of 16 h: 8 h dark. Whereas PS cows were exposed to 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC.

[2]P-value assessed between treatments at each timepoint or physiological state

[3]Calculated from glucose concentrations at timepoint t=0 and t=60 minutes

[4]Calculated as the difference between fasting insulin and peak insulin concentrations

[5]Calculated from the slope of the line from peak insulin concentration until 60 minutes post-infusion

Table 2.5. Histomorphic quantification of lumen:alveolar ratio, % epithelial proliferation, and % stromal proliferation for control (n=6 for 21 d BEC and n=8 for 21 DIM) and phase shifted (n=6 for 21 d BEC and n=8 for 21 DIM) dairy cows[1]

	21 d BEC				21 DIM				Physiological State			
Item	**CON**	**PS**	**SEM**	***P*-value[2]**	**CON**	**PS**	**SEM**	***P*-value[2]**	**21 d BEC**	**21 DIM**	**SEM**	***P*-value[2]**
Lumen:Alveolar Ratio	0.16	0.086	0.019	0.02	0.24	0.19	0.038	0.36	0.13	0.21	0.024	0.013
% Epithelial Proliferation	12.44	5.22	1.29	<0.01	0.77	0.78	0.23	0.98	8.83	0.78	0.93	<0.01
% Stromal Proliferation	2.89	1.63	0.69	0.23	0.63	0.56	0.12	0.67	2.26	0.59	0.34	<0.01

[1] CON cattle were exposed to consistent cycles of 16 h: 8 h dark. Whereas PS cows were exposed to 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC.
[2]P-value assessed between treatments at each timepoint or physiological state

Table 2.6. Postpartum dry mater intake, milk production, milk component concentration and yield, and melatonin from 1 to 60 DIM for control (n=8) and phase shift (n=8) treated cows[1]

	Treatments			
Item	**CON**	**PS**	**SEM**	***P*-value**
Postpartum Dry Matter Intake [2], kg/day	17.4	16.7	0.70	0.46
Milk Yield[3], kg/day	42.6	40.3	0.80	0.05
Fat, kg/day[4]	1.66	1.52	0.07	0.15
Protein, kg/day[4]	1.20	1.13	0.04	0.24
Lactose, kg/day[4]	2.03	1.86	0.07	0.09
Solids, kg/day[4]	5.30	4.87	0.18	0.10
Fat, %[4]	3.95	3.90	0.13	0.80
Protein, %[4]	2.85	2.90	0.05	0.48
Lactose, %[4]	4.76	4.71	0.05	0.51
Solids, %[4]	12.52	12.43	0.21	0.78
MUN, mg/dL[4]	7.87	8.22	0.29	0.57
Log_{10} SCC[4]	4.93	4.76	0.18	0.50

[1] CON cattle were exposed to consistent cycles of 16 h: 8 h dark. Whereas PS cows were exposed to 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC

[2]Dry matter intake compiled from 1-21 DIM

[3]Milk yield from 1-60 DIM

[4]Milk composition taken from milk samples on 7, 14, 21, 30, and 60 DIM

A)

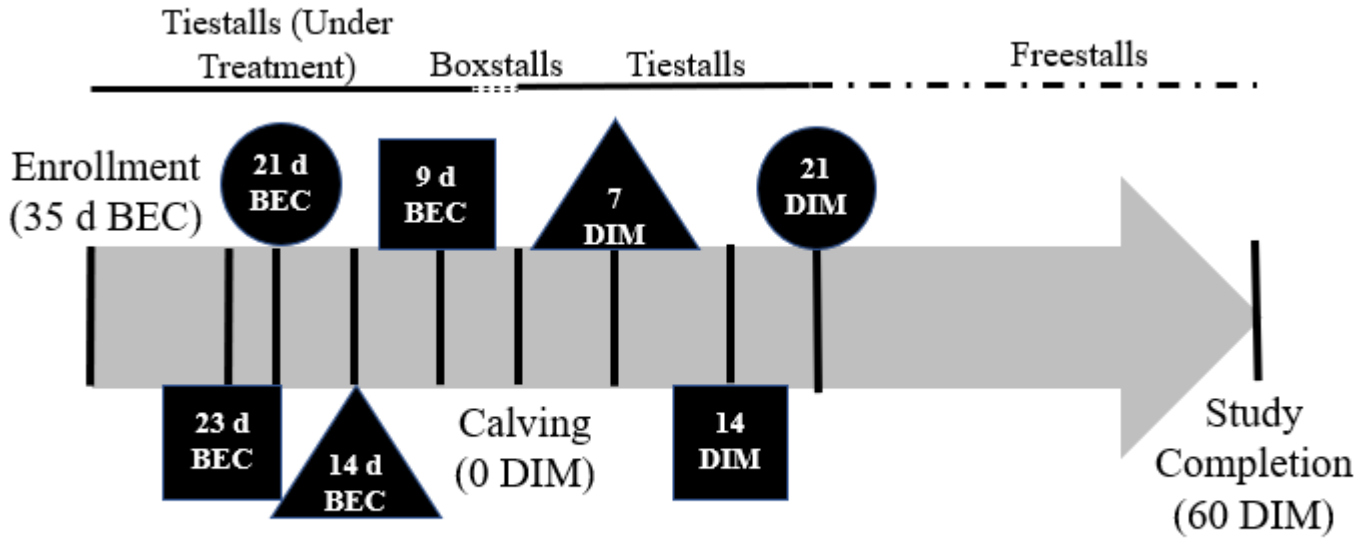
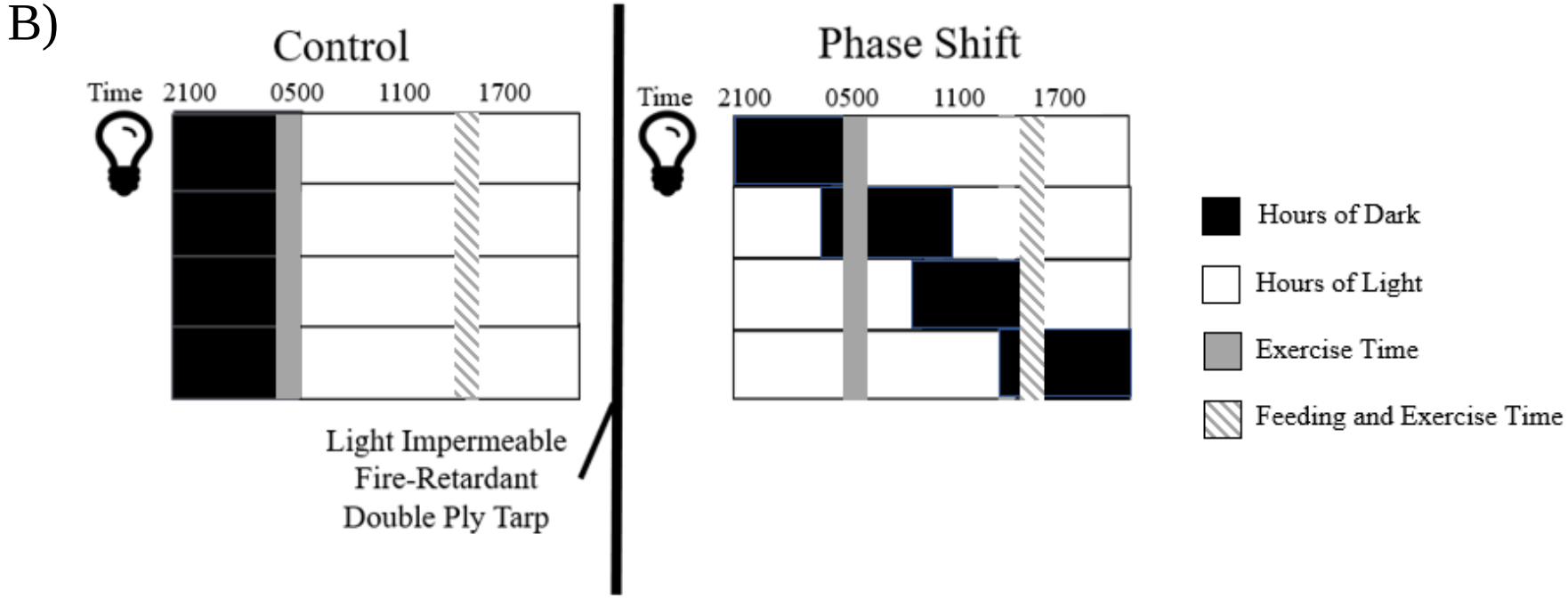


Figure 2.1. Schematic diagram of the study design. Sixteen multiparous Holstein cows were blocked to either a control (CON) or phase shift (PS) treatment at 35 d BEC. A) At study enrollment cows were housed in the tiestalls under lighting treatment as seen in figure 1B. Directly before calving at approximately 3 d BEC cows were moved to boxstalls where they experienced CON lighting. Cows were moved back to the tiestalls after calving through 21 DIM when they were moved to the freestall barn for the remainder of the study. Cows received IVGTT (▲) at 14 d BEC and 7 DIM, mammary biopsies (●) at 21 d BEC and 21 DIM, and temperature collection over 48 h to assess circadian rhythm (■) at 23 d BEC, 9 d BEC, and 14 DIM. B) The treatments were housed in the same barn under treatment while they were separated by a double-ply light impermeable tarp. CON animals received a consistent timing of 16 h of light (0500-2100), 8 h of dark (2100-0500) from 35 d BEC until 3 d BEC. PS cows received a 6 h forward shifting timing of the start of the photophase every three days for 16 h of light (0500-2100; 1100-0300).and 8 h of dark (2100-0500; 0300-1100). Feeding and exercise time was held constant for all animals at 1600 h when animals were under treatment.

Figure 2.1 continued



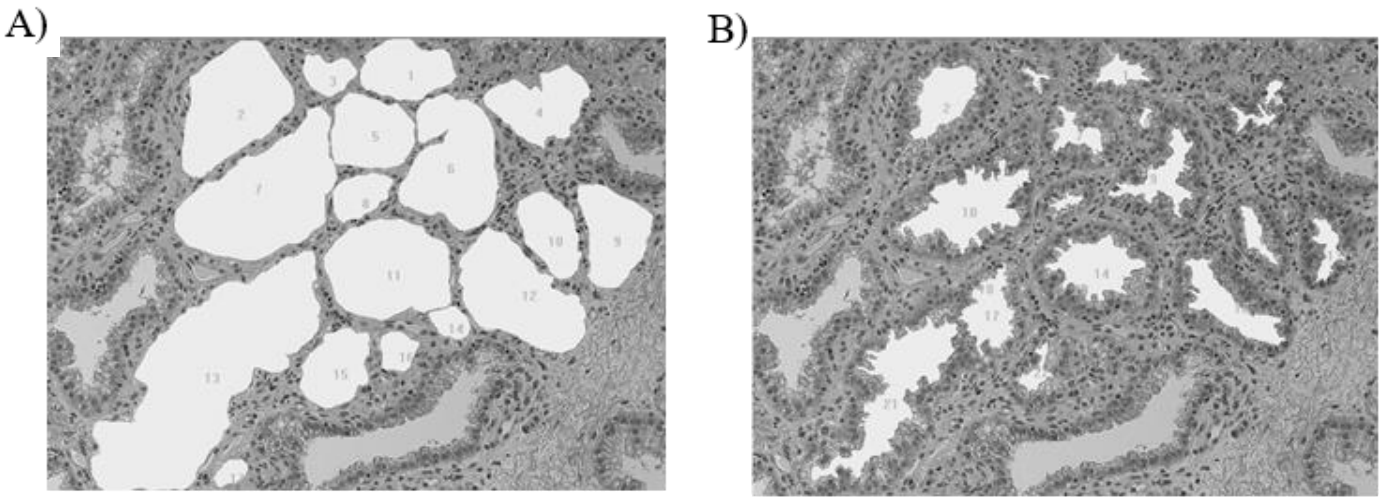


Figure 2.2. Representative images of histological mammary tissue biopsies used to quantify lumen:aleveolar ratio, percent epithelial proliferation, and percent stromal proliferation

A. H&E stained cross section of mammary tissue in the DIM state. The complete alveoli in the image are drawn around to record their collective area.

B. H&E staining cross section of the same mammary tissue sample in the DIM state. The lumen of the complete alveoli in the image are drawn around to record their collective area.

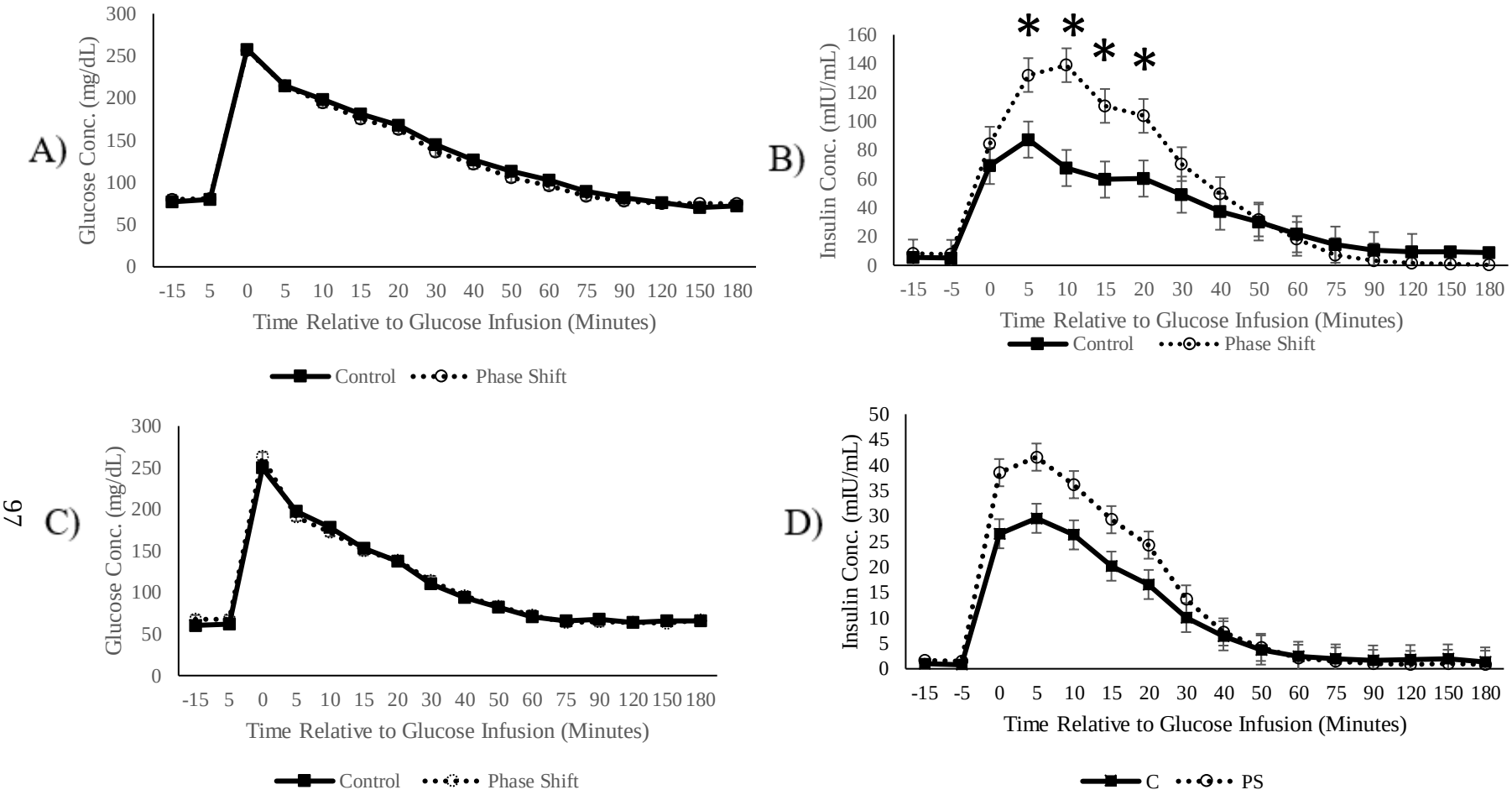


Figure 2.3. Response curves of blood glucose concentration on A) 14 d BEC C) 7 DIM and insulin concentration on B) 14 d BEC and D) 7 DIM during the intravenous glucose tolerance test (IVGTT) in control (solid line) and phase shifted (occluded, dashed line) dairy cows[1]

[1]CON cattle were exposed to consistent cycles of 16 h: 8 h dark. Whereas PS cows were exposed to 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC.

On 14 d BEC there was no difference in glucose (P=0.38) or insulin (P=0.19) levels between treatments, although there was a treatment by time point interaction for insulin levels, with * indicating a difference (P=0.05) in plasma insulin between treatments at the designated timepoint. At 7 DIM there was no difference in glucose (P=0.39) or insulin (P=0.27) levels between treatments or treatment × timepoint interactions. Error bars are the standard error of the mean for the least square means of the treatment x timepoint interaction

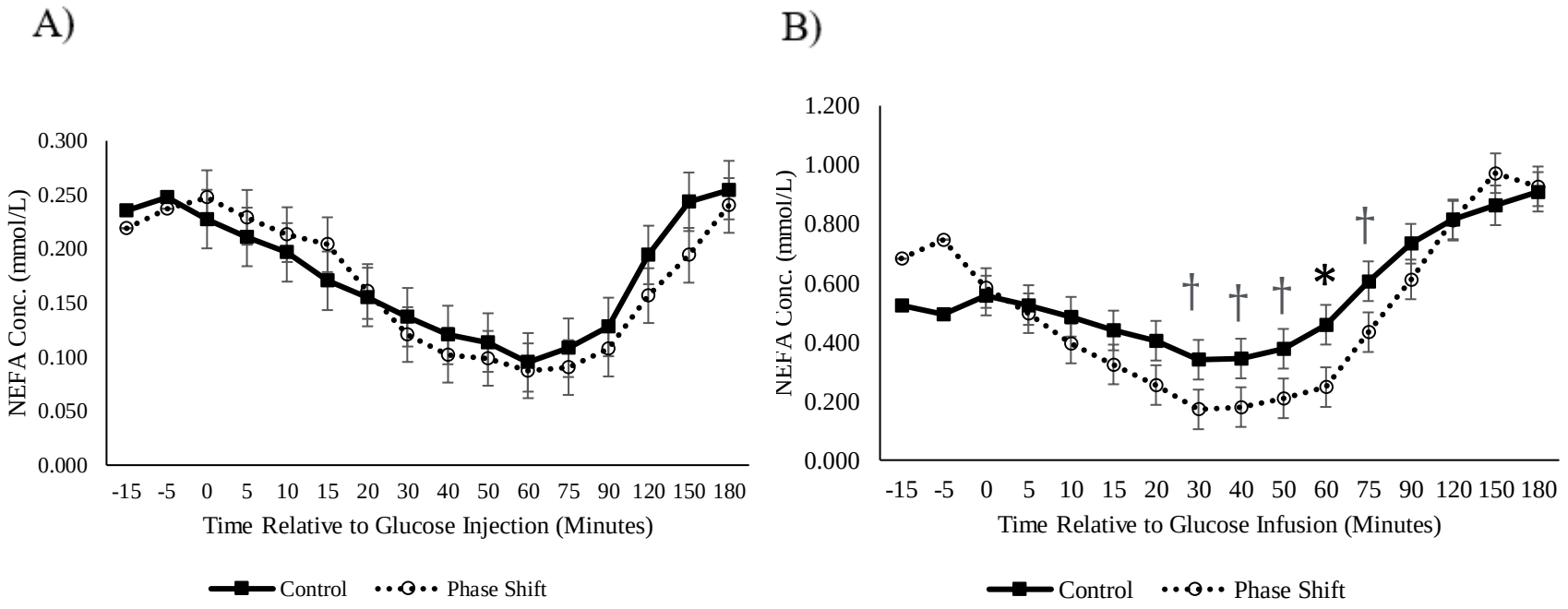
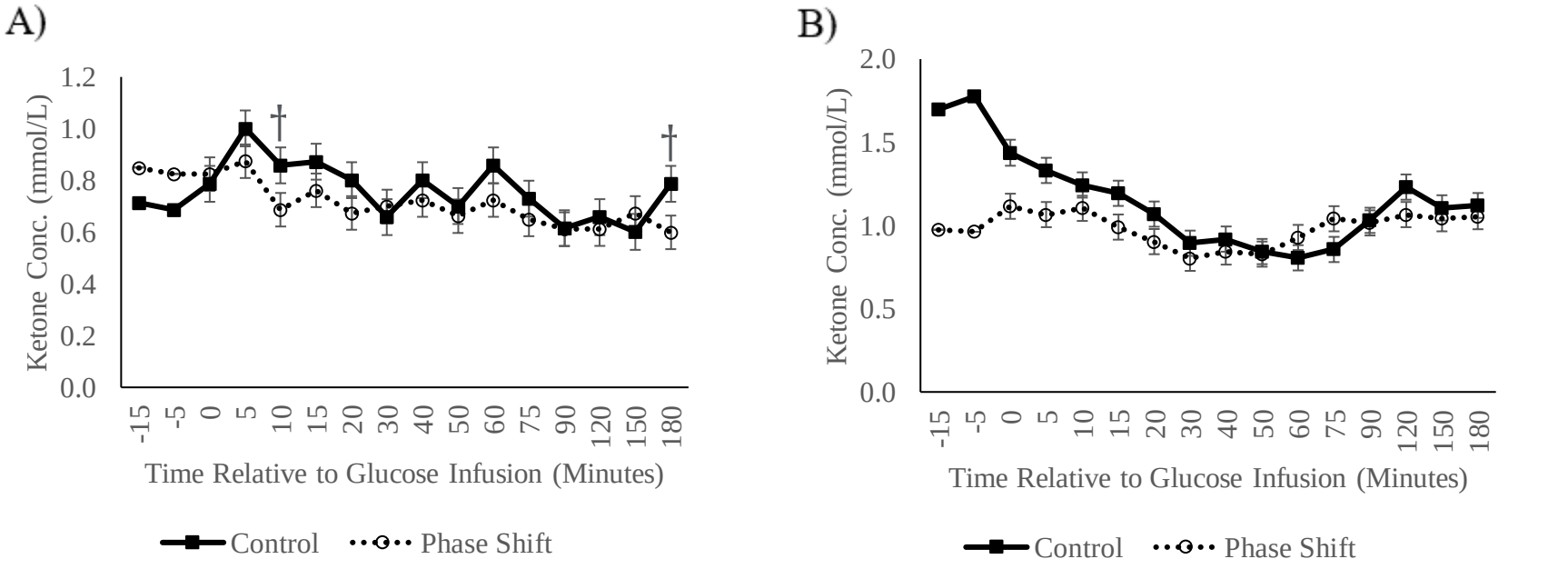


Figure 2.4. Response curves of blood NEFA concentration on A) 14 d BEC and B) 7 DIM during the intravenous glucose tolerance test (IVGTT) in control (solid line) and phase shifted (occluded, dashed line) dairy cows[1]

[1]CON cattle were given a consistent 16 h of light and 8 h of dark each day. Whereas the PS animals were given 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC

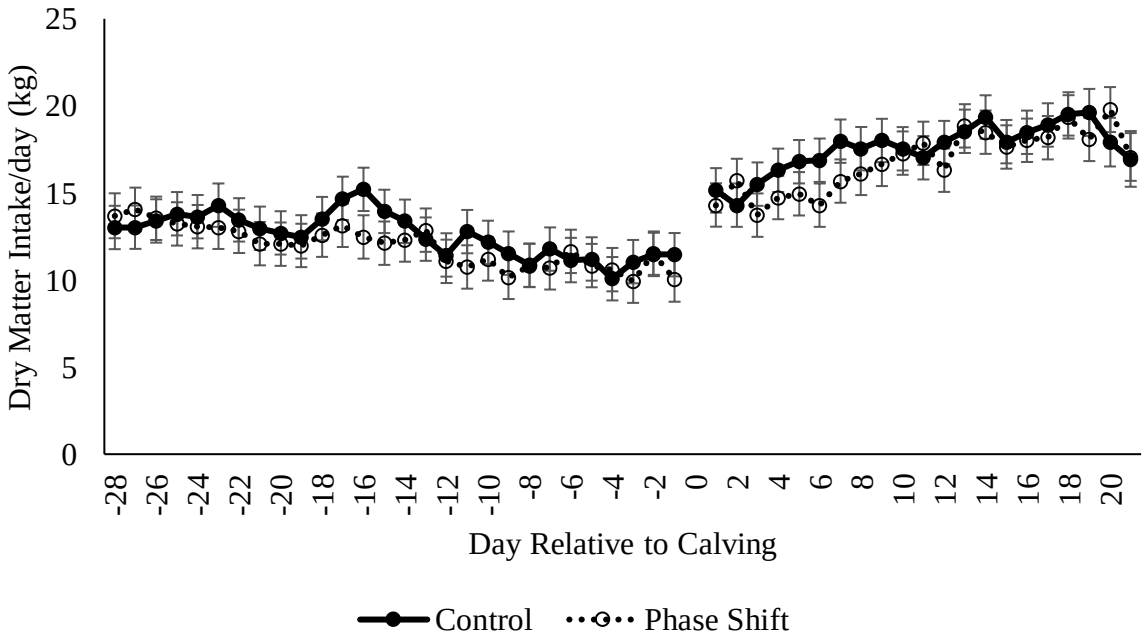
On 14 d BEC there was no difference in NEFA (P=0.76) levels between treatments. At 7 DIM there was no difference in NEFA (P=0.25) but there was a trend for treatment × timepoint interaction (P=0.09), with * indicating a difference (P=0.05) and † indicating a trend (P<0.10, P>0.05) in plasma NEFA at the designated timepoint levels between treatments or treatment × timepoint interactions. Error bars are the standard error of the mean for the least square means of the treatment x timepoint interaction



Supplemental Figure 1. Response curves of blood BHB concentration on A) 14 d BEC and B) 7 DIM during the intravenous glucose tolerance test (IVGTT) in control (solid line) and phase shifted (occluded, dashed line) dairy cows[1]

[1]CON cattle were given a consistent 16 h of light and 8 h of dark each day. Whereas the PS animals were given 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC

On 14 d BEC there was a trend for treatment BHB (P=0.08) with † indicating a trend (P<0.10, P>0.05) in plasma BHB at the designated timepoint levels between treatments. At 7 DIM there was no difference in BHB (P=0.16) between treatments



Supplemental Figure 2. Daily dry matter intake in control (solid line) and phase shifted (occluded, dashed line) dairy cows[1]

[1]CON cattle were given a consistent 16 h of light and 8 h of dark each day. Whereas the PS animals were given 16 h of light and 8 h of dark with a forward shifting photophase of 6 h every three days from 35-3 d BEC

Dry matter intake was not different in the between treatments in the prepartum (P=0.52; 12.6 vs. 12.0 kg/day). However, dry matter intake was greater in the postpartum versus prepartum period (P<0.01; 17.0 vs. 12.5 kg/day)

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CHAPTER 3. LATE GESTATIONAL PROTEIN AND ADIPOSE RESERVES REFLECT THE AMOUNT OF MOBILIZATION OF THESE RESOURCES IN PERIPARTURIENT CATTLE

3.1 Abstract

Due to insufficient dry matter intake and heightened nutrient requirements in early lactation, periparturient dairy cows mobilize adipose and muscle tissues to bridge energy and amino acid gaps. Our objective was to evaluate the relationship between the relative amount of late pregnancy protein reserves and early lactation performance. At 35 d before expected calving (BEC) *longissimus dorsi* muscle depth (LDD) was measured in forty-eight multiparous Holstein cows using ultrasound images. Cattle were assigned to either high muscle (HM; n=27; LDD>4.20 cm) or low muscle (LM; n=21; ≤4.10 cm) groups based on visual separation of two groups with no cattle between 4.10 and 4.20 cm. Tissue mobilization was evaluated via ultrasound images of LDD and backfat depth (BFD) at 21 and 7 d BEC as well as at 0, 10, 30, and 60 days in milk (DIM). Plasma concentrations of 3-methylhistidine (3-MH), creatinine (CRE), nonesterified fatty acids (NEFA), and β-hydroxybutyrate (BHB) were evaluated weekly. Milk yield and milk component data were collected through 60 DIM. *Longissimus dorsi* depth decreased more in HM versus LM from 21 d BEC through 60 DIM (1.41 vs. 1.03 cm) resulting in no difference in LDD between groups at 60 DIM (3.41 vs. 3.09 cm). HM cows also accreted more fat and mobilized more fat over the experimental time period. The general observation was the greater the LDD or BFD at 21 d BEC, the more of that respective tissue cattle mobilized through 30 DIM (R^2=0.47 and 0.82, respectively). There was no difference between groups in milk production (40.6 vs. 42.0 kg/d), however HM cows tended to give birth to heavier calves (44.3 vs. 42.3 kg). Together these findings suggest greater protein reserves in late gestation may affect nutrient partitioning to the

growth of the developing fetus, the amount of protein mobilized through 60 DIM, but does not affect milk production.

3.2 Introduction

In dairy cattle, physiological adaptations from gestation to lactation are marked by coordinated changes in metabolism to partition nutrients to support the developing fetus and the subsequent demands of milk synthesis (Bauman & Currie, 1980). The coordinated changes in metabolism that occur, ensure the cow maintains homeostasis, while responding to physiological demands of late gestation and the onset of lactation. The metabolic adaptations to support the extra energetic and nutrient demands include, increased tissue mobilization, increased liver gluconeogenesis, and reduced peripheral insulin sensitivity (Komaragiri and Erdman 1997; Aschenbach et al. 2010; De Koster and Opsomer 2013).

In early lactation, energy and protein gaps result from elevated nutrient requirements coupled with the cow's inability to consume adequate dry matter. To overcome this deficit, cattle mobilize adipose and muscle tissue reserves to meet glucose and amino acid requirements of fetal growth and lactation. High rates of adipose and protein mobilization in cattle were reported to be initiated as many as ten days prior to parturition (Grummer 1995; van der Drift et al. 2012). Tissue mobilization is subsequently heightened at calving with the spike of glucocorticoids, promoting the mobilization of substrates from tissues (Drackley, 1999). Increased mobilization is associated with a further reduction in insulin sensitivity (De Koster and Opsomer 2013). A mean of 54 kg of body fat and 21 kg of protein were estimated to be mobilized from two weeks prior to parturition until five weeks in lactation (Komaragiri and Erdman, 1997). However significant variation was observed in the amount of tissue mobilized between animals (Komaragiri and Erdman, 1997; Weber et al., 2013). Excessive tissue mobilization contributes to metabolic disease development

during the periparturient period, and thus impacts the welfare of cattle and economic returns to the producer (Leblanc 2010). An improved understanding of the metabolic changes that occur over the periparturient cattle, and the environmental stressors that affect these changes can help in the development of management strategies that mitigate excessive tissue mobilization (Grummer, 2008).

There are various methods that can be used to quantify the amount of muscle and adipose tissue mobilized in early lactation (McCabe and Boerman, 2020). These include tissue depth ultrasonography and analysis of the metabolites 3-methylhistidine (3-MH) and creatinine (CRE) for muscle, and NEFA and BHB for fat mobilization (Roberts et al., 2012; van der Drift et al., 2012; Megahed et al., 2019). Ultrasonography of the *longissimus dorsi* muscle and backfat in the 12^{th} intercostal space of beef cattle is highly correlated with total body protein and adipose (Bruckmaier et al., 1998; Greiner et al., 2003; Schwager-Suter et al., 2020). Ultrasound quantification of *longissimus dorsi* muscle of dairy cattle found cows on average mobilized 19% of their protein reserves and 35% of their adipose reserves from four weeks prior to calving through seven weeks in milk (van der Drift et al., 2012). Moreover, three-methyl histidine (3-MH) is used as a plasma marker of muscle mobilization, as it is a metabolite formed during breakdown of muscle tissue that cannot be resynthesized into skeletal muscle (Houweling et al., 2012). Plasma creatine (CRE) levels reflect total muscle mass. CRE is a nonenzymatic byproduct of phosphocreatine and creatine breakdown in muscle tissue (Wyss and Kaddurah-Daouk, 2000) and is synthesized at a constant rate in all muscle tissues. Therefore, cows with more muscle mass will have higher CRE concentrations. Whereas, plasma concentrations of NEFA and BHB are well characterized indicators of the level of adipose lipolysis (Grummer, 2008).

There are several experiments that demonstrate that cows mobilize adipose tissue in early lactation relative to the amount of adipose reserves they have in late gestation (Kokkonen et al., 2005; Pires et al., 2013). However, there are a limited number of studies that have examined the relationship between the amount of protein reserves in late gestation dairy cows and the amount of muscle and adipose tissue mobilized through the transition period. Here we examined the differences in tissue mobilization of nutrient reserves among cows characterized as having low versus high muscle depending on their *longissimus dorsi* depth (LDD) in late gestation. We hypothesized that cows with greater LDD approximately five weeks prior to calving would mobilize more muscle tissue. Greater protein mobilization was surmised to be associated with increased milk production, and greater milk protein yield through 60 days in milk (DIM). Therefore, our study objectives were to determine if dairy cows with high versus low LDD in late gestation had differences in protein and adipose mobilization, milk production, and dry matter intake through the first two months of lactation.

3.3 Materials & Methods

3.3.1 Animal Management and Experimental Design

Data for this study was collected over two experiments performed at Purdue University Animal Science Research and Education Center (ASREC) dairy unit. All procedures described were reviewed and approved by IACUC protocol #1701001523 prior to the start of experiments. The first experiment took place from January-July 2018, and data were collected from 32 multiparous cows with 2.78 ± 1.56 (mean ± SD) lactations, milk yield of 12,721 ± 2,467 kg in the previous lactation, and body weight of 756 ± 96 kg at experiment enrollment. The second experiment took place from February-June 2019, and data were collected from 16 multiparous

cows with 2.88 ± 0.91 lactations, previous lactation milk yield of 12,277 ± 3,460 kg, and body weight of 752 ± 66 kg at five weeks before expected calving (BEC). In both experiments, cows were blocked by lactation number, previous lactation yield and number of metabolic events (ketosis, mastitis, hypocalcemia, metritis, retained placenta, and displaced abomasum) in the previous lactation into one of two treatments, control (CON) or phase shift (PS) at 35 d BEC. Animals that were enrolled in both experiments, were assigned to the opposite treatment in the second experiment (n=3/treatment). Further details on CON and PS treatments, experimental procedures, and power analysis for this study design, are available in our previous manuscripts (Suarez-Trujillo et al., 2020; McCabe et al., 2021 Accepted).

At the time of experiment enrollment, 35 d BEC, images of LDD and BFD were captured on all animals using an Aloka SSD-500 ultrasound (Wallingford, CT). Ultrasound measurements of BFD and LDD were also taken at 21 and 7 d BEC and 0, 10, 30, and 60 DIM. Ultrasound images were captured on the right side at the 12th intercostal space by a separate trained individual in each experiment in methods adopted from Schäff et al. (2013). Three images were collected at each time point of LDD and BFD measurements. LDD was subsequently measured in cm, while BFD was measured in mm using ImageJ software (NIH, Bethesda, MD). A predetermined five cm length was used to standardize the scale between images. The resulting three measurements of each timepoint were averaged. Measurements across the three replicates with an intra CV greater than 15% were removed as outliers.

When tissue depth measurements were compared between PS and CON at each time point, there was no effect of treatment. Cows, were then post-hoc assigned into low and high LDD groups based on measurements at experiment enrollment (35 d BEC) to evaluate the relationship of prepartum LDD and BFD with calf birth weight, feed intake, early lactation performance, and

metabolic health parameters. Based on the continuum of LDD at 35 d BEC, the cutoff between high and low muscle cows was made between 4.10 and 4.20 cm. Cows that had a LDD > 4.20 cm at experiment enrollment (35 d BEC) were assigned to the high muscle (HM) group (n=27; range 4.22-6.50 cm, 4.96 ± 0.51 cm). Whereas cattle with a LDD <4.10 cm at enrollment were assigned to the low muscle (LM) group (n=21; range 2.04-4.04 cm, 3.36 ± 0.56 cm). The HM and LM groups were similar in lactation number (2.89 ± 0.85 vs. 2.63 ± 1.11), treatment (14 CON, 13 PS vs. 10 CON, 11 PS), and experiment number (20 first experiment; 7 second experiment vs. 12 first experiment, 9 second experiment). There was no difference in previous lactation milk yield between HM and LM cows (12,734 vs. 12,784 kg; $P>0.05$).

3.3.2 Feed Intake

Cows were fed *ad libitum* TMR from 35 d BEC through 60 DIM. Feed intake of individual cows was measured daily from 35 d BEC until approximately 15 DIM, when cows were moved to the free stall barn. Animals were fed *ad libitum* for 10% refusals from 35 d BEC to 15 DIM. In both experiments in the prepartum period, cows were fed at 1600 h each day. In the postpartum period in experiment 1, cows were fed at 0800 h each day and in experiment 2 at 1600 h each day. Feed ingredient samples were collected every two wks and analyzed for nutrient composition. Samples were dried at 60°C for 48 h and ground through a 1 mm mill (Retsch GmbH, Haan, Germany). Feed samples were measured for neutral detergent fiber and acid detergent fiber (Van Soest, Robertson, and Lewis 1991;Ankom, Macedon, NY). Percent ash content was determined by contents remaining after a 24-hour oven cycle at 600ºC. Crude protein was determined on a pure nitrogen basis by analysis (LECO, St. Joseph, MI).

3.3.3 Body Weights and Body Condition Scoring

Body measurements were taken on each animal at -35, 0, 30, and 60 d relative to calving. Cattle were weighed directly following the morning milking and within one hour after calving on the day of parturition. At calving, calf gender was recorded, and calves were weighed. Body condition scoring (BCS) was performed by three trained researchers and the scores were averaged for each timepoint using the five-point scoring system using quarter point increments (Wildman et al. 1982).

3.3.4 Milk Sampling and Analysis

Animals were milked twice daily at 0500 h and 1600 h. In both studies, milk samples from each milking were collected at 7, 14, 21, 30, and 60 DIM. Milk samples were analyzed for protein, fat, milk urea nitrogen, and lactose using Fourier Transform Infrared Spectroscopy (MilkoScan 7 RM, Foss, Hillerrød, Denmark) at Dairy One (Ithaca, NY). Respective daily component yields were calculated by multiplying the component percentage by the milk weight and then summing the product of the morning and afternoon milk weights. Component percentages were determined by dividing the daily component yield by the daily milk yield.

3.3.5 Blood Sampling and Metabolite Analysis

In both experiments, blood samples were collected in the morning between 0430 and 0530 h from the coccygeal tail vein into K_2 EDTA tubes (Becton Dickinson, Franklin Lakes, NJ). Blood was collected at 35, 28, 21,14, and 7 d BEC and 2, 7, 14, and 21 DIM. Within 60 min of blood collection, tubes were centrifuged at 4,000 g for 15 minutes at 4°C, and aliquots of plasma were frozen at -20^{o} C until further analysis. At the same timepoints, blood was collected from the coccygeal tail vein using a sterile syringe (Covidien; Dublin, Ireland) and a 16-gauge needle

(Becton Dickinson, Franklin Lakes, NJ) for a whole blood sample. This sample was then immediately analyzed for glucose and β-hydroxybutyrate (BHB) using the Centrivet meter (ACON Laboratories; San Diego, CA).

Plasma samples were analyzed for CRE and 3-MH at 35 and 21 d BEC as well as 2, 7, 14, and 21 DIM. The metabolites were analyzed via liquid chromatography tandem mass spectrometry (LC-MS/MS). In short, 500 µL of plasma was extracted using acetonitrile (1:4 v/v), vortexed and centrifuged at 4,000 *g* for 10 min. The supernatant was collected, vacuum-dried, and stored at -80ºC until further analysis. Chromatography was performed using an Imtakt Intrada Amino Acid 3 µm 2x150 mm column (Chrom Tech Inc. Apple Valley, MN) as described and modified by Zhao et al. (2016). Plasma CRE concentrations were determined via an internal spiked marker of d4 labeled CRE (Sigma Aldrich; St. Louis, MO) and 3-MH was determined via a standard curve.

Plasma samples from 35 and 21 d BEC and 2, 7, 14, and 21 DIM were analyzed for insulin. Additionally, samples collected at 35, 21, and 7 d BEC and 2, 7, 14, and 21 DIM were analyzed for NEFA. Bovine insulin was measured using an ELISA following the manufacturer's approach (ALPCO; Salem, NH) and NEFA was measured using a commercial kit (WAKO; Mountain View, CA). Intra plate CVs were 3.44% and 5.06% for NEFA and insulin, respectively. Inter plate CVs were 3.52% and 16.94% for NEFA and insulin, respectively.

3.3.6 Statistical Analysis

Post-hoc power analysis was performed using published data from studies evaluating changes in LDD from approximately one week BEC through eight weeks postpartum. Our sample size of 48 cows (n=21 and n=27 per group) was deemed sufficient, with a power of 0.80, based on using 20 animals per group and 0.55 cm difference in LDD between calving and eight wks postpartum, with a standard deviation of 0.60 as reported by van der Drift et al. (2012).

Data were analyzed using the MIXED Procedure of SAS 9.4 (Cary, NC). The model included the fixed effects of group, timepoint, and their interaction, with the random effect of cow nested within group by experiment number. The covariates of previous treatment (CON or PS) and experiment number were also included in the model to rule out the effect of treatment on evaluated variables and the variation between experiments. Data were screened for influential outlying data points where Cooks' D values > 0.3 were removed ($n \leq 1$) for each dataset. Data was considered significant at $P \leq 0.05$ and trends were indicated if $P \leq 0.10$ to > 0.05.

$$Y_{ijkl}=\mu+G_i+ D_j + G \times D_{ij}+T_k+N_l+C(G \times N)_{kl}+e_{ijkl}$$

Where Y_{ijkl} is the dependent variable, μ is the overall mean; G is the fixed effect of group (i=HM or LM), D is the fixed effect of day of lactation (j=35 d BEC to 60 DIM), T is the covariate of treatment (k=CON or PS), N is the covariate of experiment number (l=1 or 2), C(G×N) is the random effect of cow nested within treatment and experiment number; and e_{ijkl} is the random error term. All metabolites and hormone data were separated between the pre and postpartum timepoints to determine the effect of tissue depth during the prepartum on postpartum performance. For BCS, bodyweight, LDD and BFD, a one-way ANOVA test was performed with significant differences determined using Tukey's honestly significant difference (HSD) test. Calf birthweight was evaluated by including the fixed effect of calf gender as well as the fixed effect of group and the interaction of group × gender, and the covariates of experiment number and treatment.

3.4 Results

3.4.1 Feed Intake

In both trials, all cows were fed a similar *ad libitum* prefresh and lactation diet (Table 1). The forage in the prefresh diet were composed of corn silage, rye grass hay, and mixed mostly

legume grass silage. Whereas the lactation diet consisted of mixed mostly legume silage, corn silage, soybean meal, high moisture corn, and a vitamin and mineral premix supplement. The prefresh diet consisted of 51-53% DM, 15.9-16.9% CP, 35.5-37.5% NDF, 20-24% ADF, and 5-8% ash. Whereas the lactation diet consisted of 51-53% DM, 13.8-14.2% CP including rumen protected lysine and methionine, 22-27% NDF, 16-18% ADF, and 5-8% ash. There were no differences between LM and HM groups in daily DMI during the prepartum and postpartum periods ($P>0.05$; Supp. Figure 1).

3.4.2 Tissue Mobilization via Ultrasounds

HM cows had greater LDD than LM cows from 35 d BEC through 30 DIM (Figure 1; $P<0.05$). At 60 DIM, there was no difference in LDD between groups (3.42 cm vs. 3.09 cm; $P>0.05$). Between 35 d BEC and calving, there was no significant change in LDD in HM cows. Whereas the LM group exhibited a significant gain in LDD between 35 d and 21 d BEC (Figure 1), and then a loss between 7 d BEC and parturition. Both the HM and LM groups lost LDD from calving to 60 DIM (Table 2) HM cows began losing LDD between 0-10 DIM whereas LM cows first lost LDD between 7 d BEC and 0 DIM. The initial lowest LDD for both groups was observed at 30 DIM. Although between 30 and 60 DIM there was no significant change in LDD in groups, by 60 DIM there was no difference in LDD between groups.

At 35 d BEC, there was no difference in BFD between groups (3.50 vs. 3.20 mm; Figure 2). However, HM cows gained BFD between 35 d BEC and 21 d BEC, resulting in HM animals having greater BFD then LM group at three weeks prepartum and at calving (Figure 2). Between calving and 60 DIM, HM cows mobilized more BFD than LM cows resulting in a significant group by timepoint interaction (P=0.04). No difference was observed in BFD between groups from 10

DIM onward. The greatest change in BFD for all cows was observed between 0-30 DIM, relative to differences between 35 d BEC-0 DIM and 30-60 DIM.

3.4.3 Body Condition Analysis

At 35 d BEC, HM cows weighed more than LM cows (Table 2). There was no difference in bodyweight or change in bodyweight between groups at 0, 30, and 60 DIM. HM and LM cows lost significant bodyweight from 35 d BEC through 60 DIM ($P<0.05$). HM cows also had greater BCS at 35 d BEC ($P<0.05$) and lost more BCS from 35 d BEC through 60 DIM ($P=0.04$). The largest BCS losses were observed from 0-30 DIM compared to 35 d BEC-0 DIM and 30-60 DIM, respectively ($P<0.05$).

3.4.4 Energy Related Metabolites and Hormones

The postpartum and prepartum period were analyzed separately due to differences in nutrient requirements between late gestation and early lactation (Bell, 1995). There were no differences in any of the metabolites or insulin levels between groups during the prepartum time points measured (Supp. Table 1). In the postpartum, there was no difference between the groups in insulin, BHB, or glucose concentrations ($P>0.05$; Table 4), but HM cows had greater NEFA concentrations (0.70 vs. 0.53 mmol/L; $P=0.02$). There was no difference between groups in CRE or 3-MH concentrations ($P>0.05$). However, the ratio of 3-MH:CRE tended to be greater in HM cows (0.153 vs. 0.134; $P=0.06$).

3.4.5 Tissue Mobilization Relationship

Linear regression analysis was used to determine if there was a relationship between amount of muscle or adipose tissue reserves and amount mobilized during the transition period.

Muscle and adipose tissue accretion occurred between 35 d BEC and 21 d BEC to result in maximal depth for both groups at 21 d BEC, whereas the initial lowest tissue depth was first realized at 30 DIM for both groups. Thus, the relationship between tissue depth at 21 d BEC and change in tissue depth between 21 d BEC and 30 DIM was used for regression analysis as they represented the peak and nadir of LDD and BFD. A positive relationship was found between LDD at 21 d BEC and the amount of muscle mobilized through 30 DIM (R^2=0.47; Figure 3). While the percentage of tissue mobilized varied between cows (Range: 51% loss to 67% LDD gain), on average, cows mobilized 1.02 cm or 20.4% of their LDD present from three wks prepartum through 30 DIM. There was also a positive relationship between BFD at 21 d BEC, and the amount of BFD mobilized from 21 d BEC through 30 DIM (R^2=0.82; Figure 3.4). There was a large range in BFD mobilization (79% loss to 120% gain), but on average, cows mobilized 1.03 mm or 11.0% percent of their BFD at 21 d BEC through 30 DIM.

3.4.6 Production Outcomes

All cows gave birth to a single calf. There was a trend for HM cows (16 F; 11 M) to give birth to heavier calves compared to LM cows (9 F; 12 M) (44.3 vs. 42.3 kg; *P*=0.06; Table 4). There was no difference between HM and LM cows in milk production per day from 1-60 d DIM (40.6 vs. 42.0 kg/d; *P*>0.05). LM cows tended to have greater milk protein concentration across the five timepoints sampled (2.91 vs. 2.83%; *P*=0.09). However, there were no differences in milk fat or lactose concentrations on the days that milk composition was measured (*P*>0.05). Milk urea nitrogen concentrations were also not different (*P*>0.05).

3.5 Discussion

Across all cows LDD decreased from three weeks BEC to a study nadir at 30 DIM, with a linear relationship between the depth of muscle and adipose in late gestation and the amount of protein and fat cows mobilized through 30 DIM. However, the profiles of muscle and fat accretion and mobilization were distinct between cattle assigned to high versus low muscle depth groups. Between five and three weeks BEC the high muscle group had no change in muscle but accreted a significant amount of fat, whereas the low muscle group accreted muscle but had no change in BFD. These distinct patterns resulted in the animals in the high muscle group with greater muscle and fat reserves at three weeks BEC. Moreover, although the start of mobilization of *longissimus dorsi* muscle reserves was earlier in animals in the LM group, the HM group mobilized more muscle and more fat by 30 days postpartum. Consistent with greater loss in LDD and BFD, HM cows also tended to have a greater 3-MH:CRE ratio and plasma NEFA levels in the postpartum period, supporting they mobilized more protein per unit of total body protein and had greater lipolysis rates in the postpartum period. Together these findings indicate that animals with high versus low muscle reserves used different sources of nutrients to meet the energetic and amino acid demands of the transition period.

3.5.1 Longissimus Dorsi Depth (LDD) Impact on Protein Mobilization

Our hypothesis that cows with greater LDD at experiment enrollment would have a greater rate of protein mobilization across the experimental period was supported. Animals with low LDD at 35 d BEC, gained tissue depth for the first few weeks of the experiment and then reserves began to decrease from one wk BEC unto their initial nadir at 30 DIM. The accretion of muscle suggests the potential for a metabolic target level of protein reserves in cows that is required to support fetal growth and lactation. Furthermore, despite no statistical difference between 30 and 60 DIM, cows

may have mobilized additional LDD between these timepoints and then reaccreted that tissue by 60 DIM. Whereas cattle with greater muscle reserves, accreted fat between 35 d and 21 d BEC, and significant mobilization of muscle only became evident between calving (0 DIM) and 10 DIM. This suggests that LM cows needed to mobilize more protein to support energetic and amino acid requirements of late gestation than HM cows. Both groups on average mobilized approximately 20.4% of their 21 d BEC LDD through 30 DIM, which is consistent with the timing and extent of protein mobilization in the literature (Komaragiri and Erdman, 1997; Kokkonen et al., 2005; van der Drift et al., 2012).

The degree of muscle mobilization in both groups was best reflected in a 3-MH:CRE ratio. A previous study documented a moderate positive correlation between LDD and CRE (Megahed et al., 2019), however plasma CRE levels at enrollment did not reflect differences in LDD between groups in our study. Although, as LDD decreased through early lactation, CRE concentrations accordingly did as well, which is consistent with the findings of others (Kokkonen et al., 2005). Furthermore, LDD mobilization was not related with 3-MH concentrations. This is in contrast to previous reports of a strong relationship between protein mobilization and 3-MH levels (Doepel et al., 2010; van der Drift et al. 2012), however, when protein mobilization was standardized via the ratio of 3-MH:CRE (Pires et al., 2013), the relationship between metabolites and the degree of LDD mobilization was reflected.

3.5.2 LDD Impact on Adipose Mobilization

The changes in adipose tissue reserves across the study were distinct between cattle with HM and LM at 5 wk BEC. At the beginning of the experiments, HM cattle had a greater BCS and BW compared to LM cattle. HM cattle gained BFD during the late dry period to have greater BFD than LM cows at 21 d BEC and calving. Subsequently HM cows mobilized more BFD over the

experiment than LM cows so that there was no difference in BFD, BW, or BCS at 60 DIM. This mobilization resulted in greater postpartum plasma NEFA concentrations in HM compared to LM cows. Where HM cows exhibited a trend for greater NEFA concentrations at 21 DIM, which corresponded with the decrease in BFD observed in HM cows from 10-30 DIM. This timing of adipose mobilization is similar to the values reported in the literature (Komaragiri and Erdman 1997; Reynolds et al. 2004; Bünemann et al. 2019). Overall, approximately 11.0% of BFD was lost from 21 d BEC through 30 DIM. Initial, BFD in our study was lower than those observed in cattle with similar body conditioned periparturient Holstein-Friesian and Holstein-Friesian crosses (van der Drift et al., 2012). Differences between studies may be due to the area ultrasound measurements were taken. Use of the thoracic vertebrae ultrasound location to evaluate tissue depth tends to be a smaller tissue reserve than other locations such as between the *tuber coxae* and *tuber ischiadicum* or over the transverse process of the lumbar vertebrae (Kokkonen et al., 2005). This location was chosen because it is thought to be the best representation of whole-body tissue reserves. Moreover, greater changes in BFD measurement may be observed in breeds of cattle that have greater levels of subcutaneous fat (Greiner et al., 2003).

The amount of LDD or BFD cattle mobilized was related to the amount of reserves they had available at experiment enrollment, which is consistent with previous findings (van der Drift et al., 2012; Pires et al., 2013). Cows that had the lowest LDD at study enrollment, mobilized the least amount of tissue or potentially accreted tissue from study enrollment through termination. Therefore, there may be a lower limit of tissue reserves at which cows do not mobilize.

3.5.3 Tissue Reserves Did Not Impact Production or Intake

While there were relative differences in milk production between LM and HM cows from 1-60 DIM (42.0 vs. 40.6 kg/d), there were no statistical differences in milk production, milk

components, or DMI. Previous research found cattle that were high adipose tissue mobilizers experienced prolonged negative energy balance, reduced intake, had elevated milk fat concentrations and produced less milk compared to their herd mates with low or medium levels of mobilization (Tamminga et al., 1997; Roche et al., 2013; Weber et al., 2013). While we did not observe differences in milk fat in our study, we hypothesized that cows that mobilize more protein would have elevated milk protein concentrations. Contrary to what we observed, previous research on the impact of increased amino acid supply in early lactation through abomasal infusions of casein or amino acid profiles of casein, found higher amino acid supplies increased milk protein and milk lactose output (Larsen et al., 2014; Larsen et al. 2015). Thereby suggesting cows divert surplus amino acids towards milk protein synthesis and gluconeogenesis to form lactose. Thus, the higher mobilization of muscle in HM cows may have not resulted in higher supply of amino acids to the mammary gland.

In late gestation, instead of storing amino acids in skeletal muscle, HM cows may have spared them for the developing neonate. Amino acids and glucose flow to the fetus through the process of facilitated diffusion, which drives skeletal muscle synthesis and therefore calf size (Bell et al., 1995). The greatest gains in fetal size and amino acid requirements are observed in the third trimester (Bauman and Bruce Currie, 1980). Previous work in beef cattle has shown that dams supplemented with protein in the late gestational period gave birth to heavier calves (Stalker et al., 2007). Whereas dairy cows that were supplemented with methionine in the late gestational period produced larger male calves, and female calves performed better than a control through weaning (Batistel et al., 2019). However, these supplemented dams also experienced greater dry matter intake, which may influence the amount of nutrients available to the developing fetus.

Although the LDD group categorization was not associated with significant differences in milk yield, milk components, or dry matter intake, there was a was a trend for HM cows to give birth to heavier calves. Although not directly measured, heavier calves combined with higher NEFA and numerically lower milk yields with higher fat content suggest that animals with greater protein reserves in late gestation may suggest a greater degree of insulin resistance in the transition period. Previous studies support this postulate, as cows that mobilized more muscle and fat tissue in early lactation were hypoinsulinemic relative to cows that mobilized less tissue and developed greater insulin resistance to facilitate greater rates of tissue mobilization (Kokkonen et al., 2005; Weber et al., 2013; Zachut et al., 2013). However, this postulate was not supported by basal values of non-fasting insulin and glucose levels in cattle used in our study, as HM and LM groups were isoglycemic and isoinsulinemic throughout the study.

Future work will need to address relationships between LDD and BFD changes over the dry and early lactation period. Previous work has documented a large amount of variability in the type and amount of tissue mobilized over the transition period between cows (Komaragiri et al., 1998). While diet and management strategies have been documented to alter tissue reserves through the periparturient period (Doepel et al., 2002; Douglas et al., 2006; Jaurena and Moorby, 2017). Future work on the appropriate level of tissue reserves at dry off through late gestation could affect health and lactation performance.

3.6 Conclusion

The profiles of muscle and fat accretion and mobilization were distinct between multiparous cattle with high versus low muscle depth at five weeks before expected calving. Cows with greater muscle reserves at five wks BEC mobilized more protein and adipose through 30 DIM. High muscle cows gained more adipose tissue during the dry period whereas LM cows gained

additional muscle reserves. These distinct profiles suggest that metabolic adaptations to the energetic demands of the periparturient period were different and affected by the amount of initial muscle reserves. High muscle cows may have partitioned more nutrients towards the developing fetus thereby leading to larger calves. After calving, high muscle cows mobilized more protein and adipose tissue through early lactation, but higher mobilization of tissue did not result in greater milk yield or milk nitrogen fractions. Future research is to understand how cattle tissue reserves are altered throughout the late gestation and early lactation period to maximize cow's milk production, health, and welfare.

3.7 Tables & Figures

Table 3.1 Nutrient composition for the two rations fed during the two experiments. The prefresh diet was fed from 35 d before expected calving (BEC)-0 days in milk (DIM) and the lactating diet was fed 0-60 DIM

Ingredients (g/100 g diet DM)	First Experiment		Second Experiment	
	Prefresh	**Lactating**	**Prefresh**	**Lactating**
Corn silage	32.7	24.6	32.2	29.8
Mixed mostly legume silage	5.4	19.1	5.3	8.4
Rye hay	16.2	7.8	-	-
Wheat straw	-	-	15.3	6.9
Alfalfa hay	-	-	-	9.3
Whole fuzzy cottonseed	-	6.0	-	-
Dry ground corn	14.4	4.8	10.5	9.6
High moisture corn		17.4	-	14.8
Cottonseed hulls	6.6		7.2	-
Soybean meal	6.4	5.5	6.6	5.8
Soyplus[1]	3.6	4.8	-	-
Blood meal	-	-	8.0	4.5
Distillers grain with solubles	0.7	-	-	-
LysAAMet[2]	0.6	0.8	1.5	0.8
Calcium carbonate	1.2	1.1	1.2	1.0
Biochlor[3]	7.0	-	7.1	-
Vitamin and mineral mix	2.9[4]	3.1[5]	3.0[4]	2.7[5]
QLF 63/43[6]	2.3	3.7	2.2	4.1
Palmit 80[7]	-	1.3	-	2.2
Nutrient Composition	-	-	-	-
Dry matter	51.6%	53.2%	52.9%	51.8%
Crude protein	16.9%	14.2%	15.9%	13.8%
NDF	35.5%	22.3%	37.5%	27.2%
ADF	20.5%	15.8%	24.4%	18.4%
Ash	7.8%	7.9%	5.7%	5.1%

Table 3.1 continued

[1]Landus Cooperative, Ames, IA
[2]40.6% Smartamine (Adisseo, Alpharetta, GA), 39.9% Biocycle Plus (Agrarian Solutions, Middlebury, IN), and 19.6% AjiPro-L (Ajinomoto Animal Nutrition, Chicago, IL)
[3]Arm and Hammer Animal Nutrition, Ewing Township, NJ
[4]Prefresh vitamin and mineral mix contained 28.55% MegalacR (Church and Dwight Co., Princeton, NJ), 13.25% Diamond V XP (Diamond V, Cedar Rapids, IA), 13.96% magnesium oxide, 9.11% monocalcium phosphate, 8.54% magnesium sulfate, 7.32% salt, 6.68% vitamin E 20,000 IU, 6.07% calcium sulfate, 5.94% mineral premix, 0.43% Rumensin 90 g/lb (40.8 g/kg; Elanco Animal Health, Greenfield, IN) and 0.15% vitamin A.
[5]Lactating vitamin and mineral mix contained 27.07% sodium bicarbonate, 13.60% salt, 10.89% monocalcium phosphate, 10.72% DCAD Plus (Arm and Hammer), 6.68% Omnigen AF (Phibro Animal Health Corporation, Teaneck, NJ), 6.40% Diamond V XP (Diamond V), 6.59% magnesium oxide, 5.99% calcium sulfate, 5.90% fat yellow grease, 3.38% mineral premix, 1.80% ground corn, 0.78% vitamin E, and 0.21% Rumensin 90 g/lb (40.8 g/kg; Elanco Animal Health).
[6]Quality Liquid Feeds, Dodgeville, WI
[7]ADM Animal Nutrition, Quincy, Il

Table 3.2 Effect of high muscle (HM; n-27) and low muscle (LM; n=21) at 35 d before expected calving (BEC) on body condition score (BCS), bodyweight, and respective changes in tissue depth from 35 d BEC through 60 DIM

	35 BEC		0 DIM		30 DIM		60 DIM			*P*-values		
	HM	LM	HM	LM	HM	LM	HM	LM	SEM[2]	Group[1]	Day	Group × Day
BCS	3.54[a]	3.24[b]	3.42[ab]	3.19[bc]	2.94[cd]	2.76[d]	2.75[d]	2.68[d]	0.08	<0.01	<0.01	0.10
BCS Change per Month	-	-	-0.10[a]	-0.03[a]	-0.48[b]	-0.45[b]	-0.15[a]	-0.02[a]	0.05	0.04	<0.01	0.69
Bodyweight (kg)	783[a]	720[bc]	756[b]	700[bc]	674[cd]	629[d]	672[cd]	630[d]	15.7	<0.01	<0.01	0.57
Bodyweight Change (kg)	-	-	-26.2	-19.2	-80.3	-72.3	-2.6	1.5	7.8	0.26	<0.01	0.97
Longissimus dorsi depth (LDD) per Month Δ (cm)	-	-	-0.31[b]	0.40[a]	-0.98[c]	-0.55[bc]	-0.33[bc]	-0.16[b]	0.15	<0.01	<0.01	0.13
Prepartum/Postpartum LDD Δ (cm)	-	-	-0.33[b]	0.39[a]	-	-	-1.31[c]	-0.70[b]	0.17	<0.01	<0.01	0.79
Overall LDD Δ (cm)	-	-	-	-	-	-	-1.64	-0.30	0.15	<0.01	-	-
Backfat depth (BFD) Δ per Month (mm)	-	-	0.70[a]	0.00[ab]	-1.20[c]	-0.60[bc]	-0.40[bc]	-0.10[ab]	0.30	0.66	<0.01	0.04
Prepartum/Postpartum BFD Δ (mm)	-	-	0.68[a]	0.00[ab]	-	-	-1.67[c]	-0.70[b]	0.28	0.43	<0.01	0.03
Overall BFD Δ (mm)	-	-	-	-	-	-	-1.64	-0.30	0.15	<0.01	-	-

[a,b]Differences in letters indicate a significant difference ($P<0.05$) between group or timepoint least square means

[1]SEM determined by largest group × day standard error

[2]Cows were assigned to group based on longissimus dorsi depth (LDD) at 35 d BEC. HM LDD was >4.20 cm and LM LDD was ≤ 4.10 cm at 35 d BEC

Table 3.3 Effect of high muscle (HM; n=27) and low muscle (LM; n=21) at 35 d before expected calving (BEC) on postpartum energy related metabolites and hormone of insulin, glucose, $\beta - hydroxybutyrate$ (BHB), non-esterified fatty acids (NEFA), creatinine (CRE), and 3-methyl histidine (3-MH)

	2 DIM		**7 DIM**		**14 DIM**		**21 DIM**			***P*-Values**		
Item	**HM**	**LM**	**HM**	**LM**	**HM**	**LM**	**HM**	**LM**	**SEM[1]**	**Group[2]**	**Day**	**Group × Day**
Insulin (ng/mL)[3]	0.26	0.28	0.22	0.33	0.17	0.27	0.31	0.22	0.09	0.72	0.74	0.37
Glucose (mg/dL)[3]	67.9	68.8	68.2	65.3	66.3	64.5	70.0	68.1	2.22	0.35	0.15	0.69
BHB (mmol/L)[3]	1.08	0.94	1.19	1.09	1.11	0.90	1.03	0.83	0.16	0.15	0.43	0.97
NEFA (mmol/L)[3]	0.64^{xy}	0.50^{xy}	0.74^{x}	0.59^{xy}	0.66^{xy}	0.59^{xy}	0.75^{x}	0.44^{y}	0.08	0.02	0.46	0.29
CRE (ng/mL)[3]	3,524	3,579	3,339	3,485	3,181	3,233	3,099	3,099	103	0.56	<0.01	0.79
3-MH (ng/mL)[3]	473	445	491	440	549	495	489	443	31.2	0.22	<0.01	0.90
3-MH:CRE[3]	0.131	0.121	0.148	0.124	0.174	0.149	0.160	0.141	0.010	0.06	<0.01	0.71

[x,y]Differences in letters indicate a trend (P≤0.10, >0.05) between group × timepoint least square means

[1]SEM determined by largest group × day standard error

[2] Cows were assigned to group based on longissimus dorsi depth (LDD) at 35 d BEC. HM LDD was >4.20 cm and LM LDD was ≤ 4.10 cm at 35 d BEC

[3]Samples collected at 2, 7, 14, and 21 DIM

Table 3.4 Relation between high muscle (HM; n=27) and low muscle (LM; n=21) at 35 d before expected calving (BEC) on milk production and milk components through 60 days in milk (DIM)

Item	HM	LM	SEM	Group *P*-value[1]
Calf birthweight (kg)	44.3	42.3	0.76	0.06
Milk yield (kg/d)[2]	40.6	42.0	0.78	0.19
Fat %[3]	4.24	4.01	0.11	0.12
Protein %[3]	2.83	2.91	0.03	0.09
Lactose %[3]	4.66	4.75	0.05	0.24
Fat yield (g)[3]	1701	1614	61.4	0.30
Protein yield (g)[3]	1136	1157	35.6	0.65
Lactose yield (g)[3]	1883	1909	65.2	0.77
Milk urea nitrogen (mg/dL)	7.59	7.87	0.48	0.45

[1]Cows were assigned to group based on longissimus dorsi depth (LDD) at 35 d BEC. HM LDD was >4.20 cm and LM LDD was ≤ 4.10 cm at 35 d BEC

[2]Calculated from production records from 1-60 DIM

[3]Compiled from milk samples collected at 7, 14, 21, 30, and 60 DIM

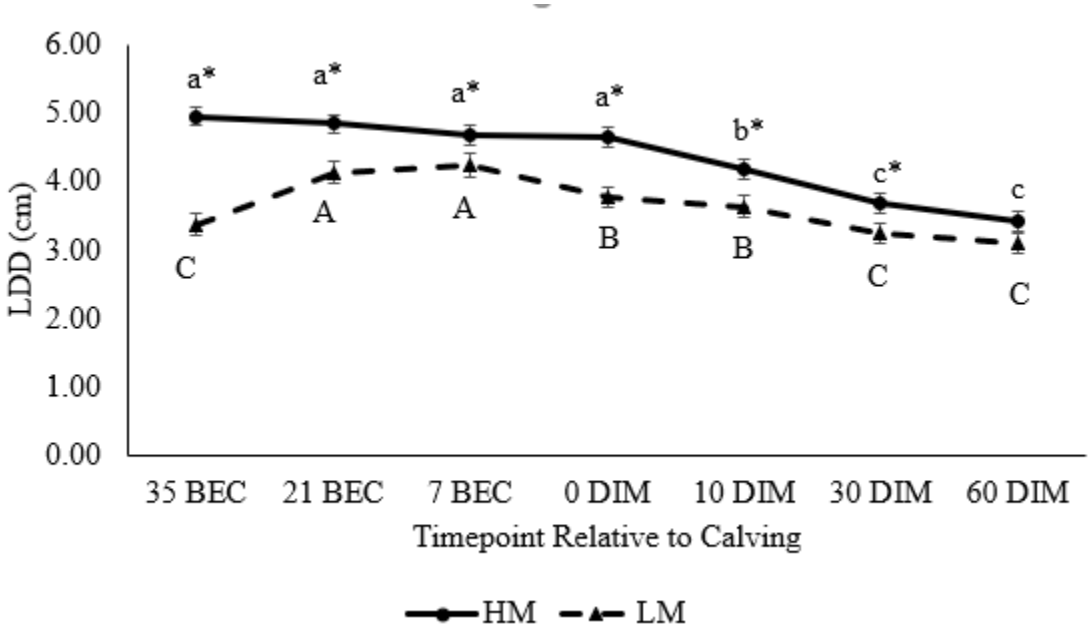


Figure 3.1 LDD between high muscle (HM; n=27) and low muscle (LM; n=21) cows at different timepoints from 35 days before expected calving (BEC) to 60 days in milk (DIM)[1]

[A,B; a,b] Differences in lowercase letters indicate a difference between timepoints within HM, uppercase letters indicate a difference between days within LM, and an asterisk indicates a difference between groups within a day at $P<0.05$

[1]Cows were assigned to group based on longissimus dorsi depth (LDD) at 35 d BEC. HM LDD was >4.20 cm and LM LDD was ≤ 4.10 cm at 35 d BEC

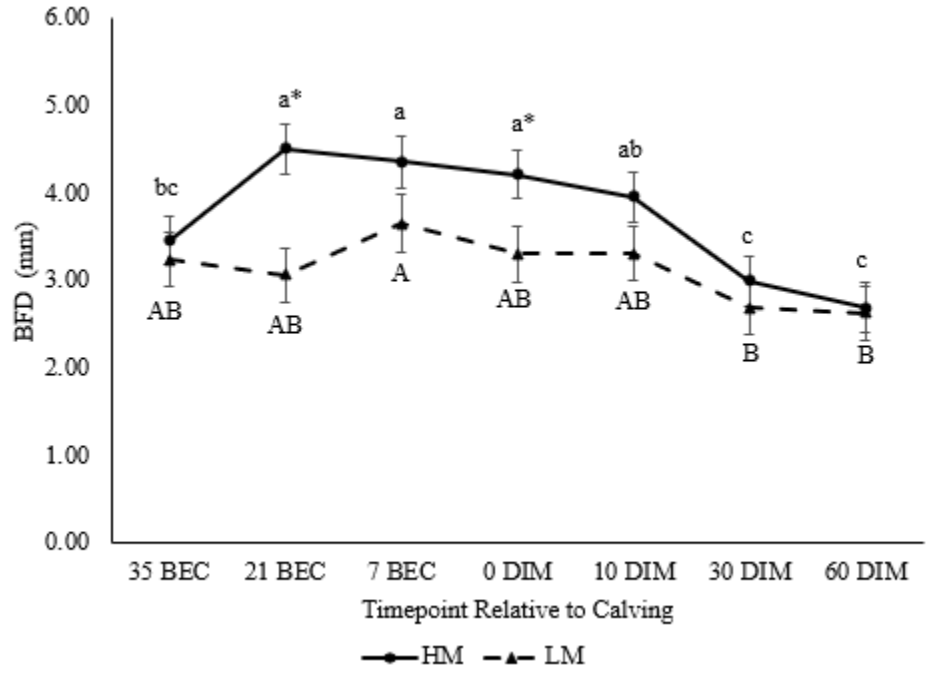


Figure 3.2 BFD between high muscle (HM; n=27) and low muscle (LM; n=21) cows at differen timepoints from 35 days before expected calving (BEC) to 60 days in milk (DIM)1

[1]Cows were assigned to group based on longissimus dorsi depth (LDD) at 35 d BEC. HM LDD was >4.20 cm and LM LDD was ≤ 4.10 cm at 35 d BEC

[A,B; a,b] Differences in lowercase letters indicate a difference between timepoints within HM, uppercase letters indicate a difference between days within LM, and an asterisk indicates a difference between groups within a day at $P<0.05$

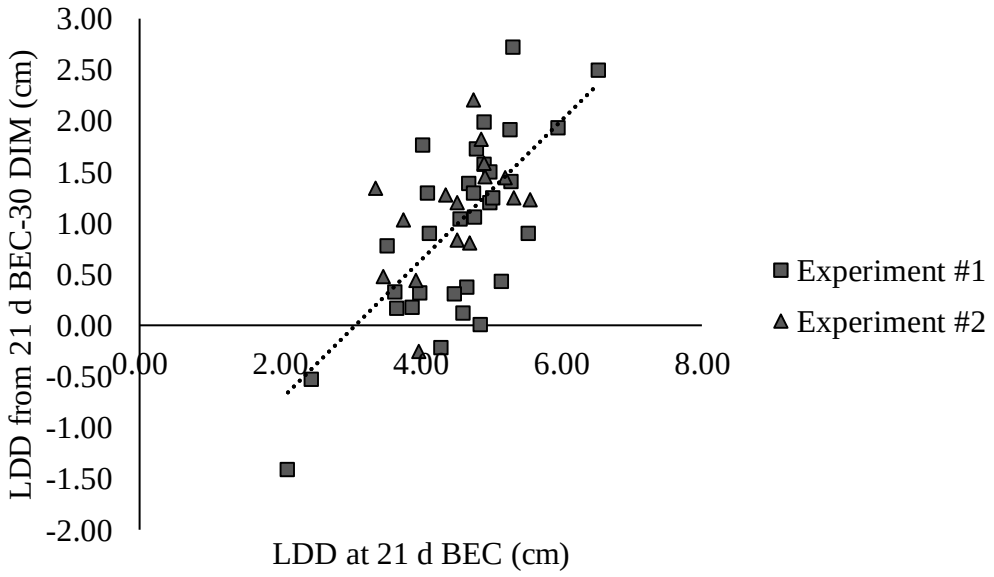


Figure 3.3 The relationship between longissimus dorsi depth (LDD) at 21 days before expected calving (BEC) and the amount of LDD mobilized from 21 d BEC to 30 days in milk (DIM) from the first experiment (■ ; n=32) and the second experiment (▲; n=16). The greater the LDD cows have at 21 d BEC, the more LDD they mobilize from three weeks prepartum through 30 DIM (R^2=0.47). Negative LDD mobilized is equivalent to muscle accretion over the timepoints measured.

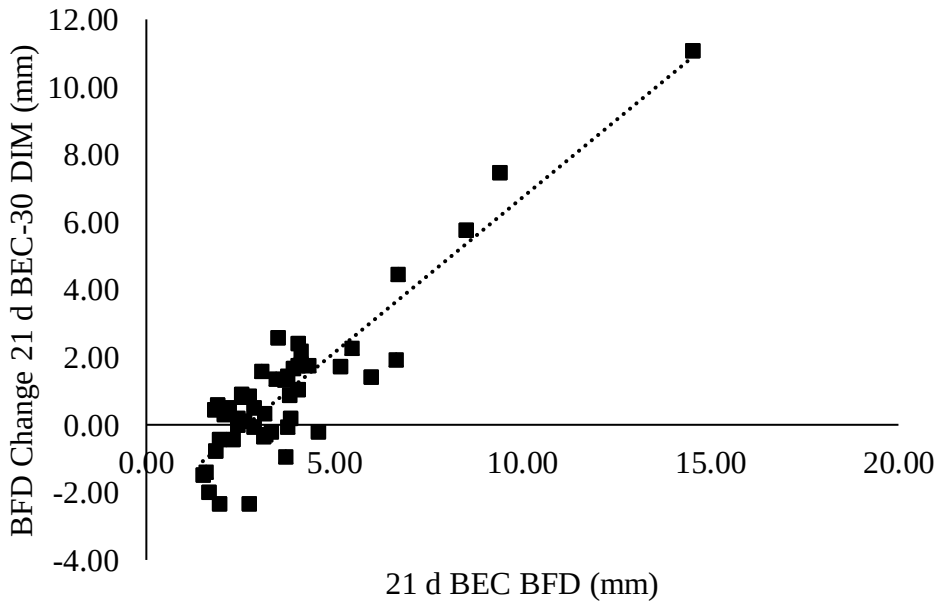
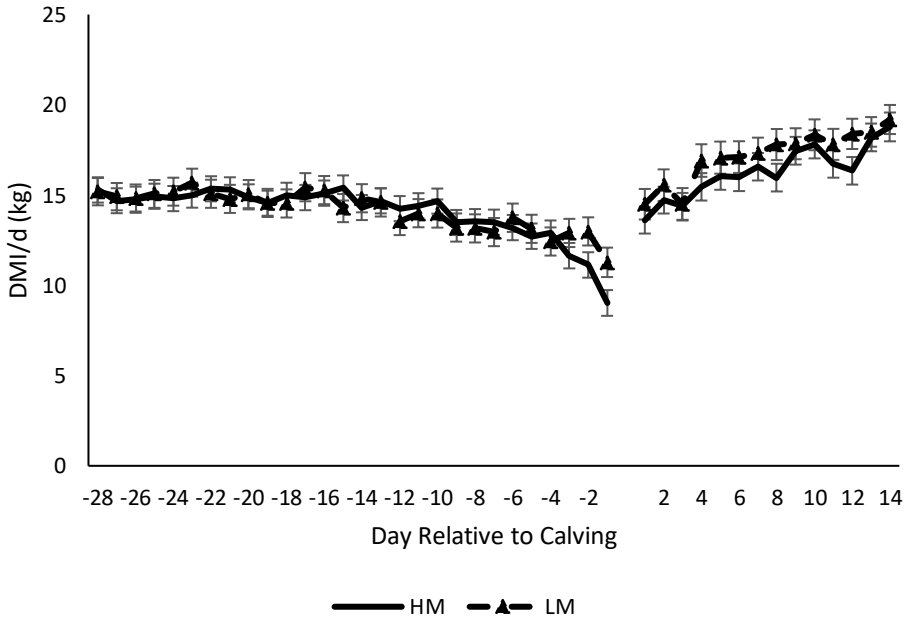


Figure 3.4 The relationship between backfat depth (BFD) at 21 d before expected calving (BEC) and change in BFD from 21 d BEC to 30 days in milk (DIM) from the first experiment (■ ; n=32) and second experiment (▲; n=16). The greater amount of BFD cows have at 21 d BEC the more BFD they mobilize from three weeks prepartum through 30 DIM (R^2=0.82). Negative BFD mobilized is equivalent to adipose accretion over the timepoints measured.

Supplemental Table 1. Effect of high muscle (HM; n=27) and low muscle (LM; n=21) at 35 d before expected calving (BEC) on prepartum energy related metabolites and hormone of insulin, glucose, $\beta - hydroxybutyrate$ (BHB), non-esterified fatty acids (NEFA), creatinine (CRE), and 3-methyl histidine (3-MH)

	Group[1]			*P*-Value		
Item	**HM**	**LM**	**SEM**	**Group**	**Day**	**Group × Day**
Insulin (ng/mL)[2]	0.65	0.75	0.09	0.40	0.42	0.69
Glucose (mg/dL)[3]	77.8	79.1	0.87	0.16	0.25	0.35
BHB (mmol/L)[3]	0.53	0.53	0.04	1.00	0.12	0.30
NEFA (mmol/L)[4]	0.18	0.17	0.03	0.78	<0.01	0.63
CRE (ng/mL)[2]	3,289	3,128	81.7	0.16	0.10	0.79
3-MH (ng/mL)[2]	435	401	25.2	0.33	0.18	0.26
3-MH:CRE[2]	0.128	0.125	0.007	0.75	0.75	0.20

[1]Cows were assigned to group based on longissimus dorsi depth (LDD) at 35 d BEC. HM LDD was >4.20 cm and LM LDD was ≤ 4.10 cm at 35 d BEC
[2]Metabolite and hormone samples recorded at 35 and 21 d BEC
[3]Metabolite samples recorded at 35, 28, 21, 14, and 7 d BEC
[4]Metabolite samples recorded at 35, 21, and 7 d BEC



Supplemental Figure 1. Dry matter intake between high muscle (HM; n=27) and low muscle (LM; n=21) cows from 28 d before expected calving (BEC) to 15 days in milk (DIM)[1] Group *P*-value: 0.33 Group × Day *P*-value: 0.25

[1]Cows were assigned to group based on longissimus dorsi depth (LDD) at 35 d BEC. HM LDD was >4.20 cm and LM LDD was ≤ 4.10 cm at 35 d BEC

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CHAPTER 4. CONCLUSION

There are several factors that can impact a dairy cow's lactation performance. Events that occur during the late gestation period have been found to have carryover effects impacting early lactation milk production. One of the areas that can be impacted is the animal's circadian rhythm, which prepares the body to anticipate the timing of daily events by relying on environmental cues. Chronic disruptions in the timing of events can influence the circadian rhythm and lead to poor animal health and productivity. Additionally, one of the main markers of lactational success is the cow's ability to transition from supporting the nutrient requirements of the fetus in late gestation to partition nutrients to allow for high levels of milk production in early lactation. One of the greatest metabolic challenges cows experience is insufficient dry matter intake. To overcome a nutrient gap, cows mobilize tissue substrates of adipose and protein from their reserves to meet the elevated nutrient requirements of early lactation. A miscoordination of tissue mobilization can result in costly metabolic diseases that negatively impact health and lactation performance.

In the first study, PS cattle were confirmed to have disrupted circadian rhythm compared to CON animals as highlighted by attenuated internal temperature rhythm over a 48 h period. Cattle exposed to PS timing of light in the late gestation period developed insulin resistance at 14 d BEC and at 7 DIM, even after the treatment was eliminated. Furthermore, PS cows exhibited lower mammary development at 21 d BEC, which was also associated with lower milk yield through 60 DIM. Despite no difference in dry matter intake, this study demonstrated that circadian disruptions had a carryover effect from late gestation into early lactation. Therefore, emphasizing the need to minimize disruptions for cows during the dry period to maximize mammary development and milk production in the subsequent lactation.

Our second study demonstrated that cows mobilize tissue in early lactation based on the amount of reserves they have available. This resulted in HM cows mobilizing more LDD and BFD than LM cows through the first 30 d PP. While at 60 d PP, there was no difference in LDD or BFD. However, during the last five weeks of the dry period, HM cows gained additional adipose tissue whereas LM cows gained additional muscle tissue. Since HM cows did not use amino acids to synthesize tissue in late gestation, the nutrients were partitioned towards the developing fetus, which resulted in HM cows tending giving birth to larger calves. While in the early lactation period there was no effect of group on milk production, milk components or feed intake, HM cows mobilize more muscle and adipose tissue as noted by a greater 3-MH:CRE ratio and NEFA plasma concentrations in early lactation. Although, tissue reserves in late gestation did not impact milk performance metrics, they influence nutrient partitioning from late gestation through early lactation.

The dry period is of great importance since it prepares the cow for her upcoming lactation and can be influenced by external disruptors. Future studies will need to identify how circadian rhythm disruptions are affected by the timing and magnitude of disruptions on health and production outcomes in the subsequent lactation. While this research highlights the importance of keeping a consistent timing of environmental events, the ideal level of adipose and protein reserves in late gestation is still unknown. Minimizing the effects of circadian disruptions and ensuring cows have the correct quantity of tissue reserves in late gestation could position them for a successful transition to lactation by also minimizing negative health outcomes.

PUBLICATIONS

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