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**THE INFLUENCE OF THE TIMING OF FEED INTAKE ON RUMINAL
FERMENTATION AND MILK SYNTHESIS**

A Thesis in

Animal Science

by

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THESIS ABSTRACT

The rate of feed intake has been shown to vary over the day and is expected to create a daily rhythm of nutrient absorption. With daily fluctuations in available nutrients, milk synthesis may also vary over the day. Additionally, milk synthesis has been shown to fluctuate independently of nutrient availability, which would create times over the day that the nutrient profile does not match the nutritional needs of the mammary gland. Two studies were conducted to examine the circadian patterns of the dairy cow and to understand the relationship between timing of feed intake and milk synthesis.

The object of the first experiment was to characterize the effect of timing of feed intake on the pattern of milk synthesis. The treatments for this experiment were cows fed once a day (1x fed) or every six hours in four equal meals (4x fed). The cows were milked twice a day for the first 14 d and then four times a day for the last 7 d of each period. There was a circadian pattern to milk and milk component synthesis over the day. Milk yield was high during the first milking interval (MI), decreased over the next two milking intervals, and for the 1x fed increased during the last milking interval but remained lower for 4x fed. Milk protein fluctuated over the day as well. Milk protein percent for both treatments peaked during the third milking interval with 4x fed peaking higher than 1x fed. The largest range was observed for milk fat over the day. The second and third milking interval exhibited the highest milk fat concentration and yield. The 4x fed was consistently higher in fat percent at each timepoint, but milk fat yield was only higher during the first two milking intervals. Non-esterified fatty acids were the only plasma metabolite with an effect of time, but no effect of treatment. The experiment demonstrated a clear pattern to milk yield and milk component synthesis that is affected by the timing of feed intake.

The object of the second experiment was to identify a feeding regimen that complements the natural pattern of feed intake and milk synthesis. Cows were fed either a control diet (30.1%

NDF) or two regimens that consisted of a combination of a high fiber diet (31.8% NDF) and a low fiber diet (26.9% NDF). The treatments for the experiments were the control diet fed at 0800 h (Con), the high fiber diet fed at 0800 h and the low fiber diet at 2200 h (HL), and the low fiber diet fed at 0800 h and high fiber diet at 1300 h (LH). The high and low fiber diets were fed at a ratio to provide the same nutrients as the control diet. Feeding times were selected to provide the rumen with a low fiber diet either during the high or low intake period of the day. There was a tendency for lower daily milk fat yield ($P < 0.07$) and fat corrected milk (FCM; $P < 0.07$) for HL compared to LH. There was a treatment effect for DMI ($P = 0.01$) with Con consuming 1.9 kg more than HL, but there was no difference between HL and LH. There was no effect of treatment on empty body weight. There was also no treatment effect to plasma metabolites, but as reported in the first experiment, there was an effect of time ($P < 0.001$ for all treatments). There was no treatment effect on VFA concentrations, but there was a tendency for a treatment by time interaction for valerate ($P = 0.07$). All VFA concentrations peaked at approximately 1800 h and the acetate to propionate ratio peaked at 1200 h. Splitting the TMR ration into two separate rations that differ in their fiber level and feeding these at specific times over the day, decreases DMI with minimal effects on milk production. Lastly, there was a tendency for feed efficiency to be higher for LH compared to HL ($P = 0.096$).

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Chapter 1

Introduction

Milk production and intakes are traditionally discussed and reported as daily sums. For example, the Dairy Herd Improvement Association (DHIA) combines morning and evening milking in their analyses, and the scientific literature almost exclusively reports daily values for milk and milk components. It is commonly recognized that intake, milk yield, and milk composition changes over the day, but this pattern is not well described nor commonly considered in dairy management (Quist et al., 2008). A better understanding of the daily pattern of intake and milk synthesis may provide insight into mammary gland function. Recent reports have demonstrated that many daily patterns of physiological processes are influenced by an internal clock with roughly a 24-hour period (Dibner et al., 2010, Reppert and Weaver, 2002). Rhythms with a 24 h cycle are referred to as circadian rhythms and it would be highly likely that these circadian rhythms influence milk and milk component synthesis.

The circadian system is responsible for the sleep/wake cycle, also known as activity/rest cycles, among other physiological and behavioral patterns (Panda et al., 2002b, Reppert and Weaver, 2002). Through these physiological and behavioral patterns, the circadian system can influence intake as well as metabolic efficiency in rodents (Froy, 2007, Froy, 2011). In the cow, naturally occurring higher intake periods of the day may alter timing of ruminal volatile fatty acid (VFA; for a complete list of abbreviations refer, to Appendix A) production and nutrient passage from the rumen, ultimately leading to a change in nutrient absorption over the day. Animal metabolism and milk synthesis are both expected to be influenced by the timing of nutrient

availability and potential by internal timekeeping mechanisms, like a clock, in the mammary gland.

The objectives of this research are to determine the circadian pattern of milk synthesis and the influence of the timing of nutrient availability on milk synthesis. First, the influence of the timing of nutrient intake on the circadian pattern of milk synthesis is described in Chapter 3. There was a clear pattern to milk and fat synthesis either due to nutrient availability or a functional internal clock in the mammary gland. Secondly, Chapter 4 investigates a feeding strategy designed to stabilize rumen fermentation and nutrient absorption by feeding multiple rations over the day formulated to equalize temporal intake of fermentable organic matter. There was no change in milk production or body weight gain among the treatments, but daily intake was decreased. Additionally, there were clear changes over the day in blood metabolites and volatile fatty acids suggesting a circadian rhythm also regulates these variables.

Chapter 2

Literature Review

2.0 Background

In 2002, total mixed rations (TMR) were fed on 90% of large dairy farms (> 500 cows) and 78% of the time on medium dairy farms [100-499 cows; (USDA, 2002)]. The TMR was developed to reduce the selective consumption of highly fermentable feedstuffs and promote a stable fermentation pattern in the rumen (Coppock et al., 1981). The principle aim is to provide a continuous and consistent amount of fermentation occurring in the rumen, a direct product of the rates of intake, digestion, and passage. The dairy cow has a natural pattern of feeding behavior that results in varying rates of intake over the day (DeVries et al., 2003, Shabi et al., 1998). Temporal differences in the amount (flux) of digestion and VFA production are expected as a result of the variable rates of intake over the day that cause changes in the rumen nutrient pool size, digesta digestibility, and microbial digestion capacity. Differences may also exist in the rate of passage, although this is difficult to experimentally observe.

The natural feeding pattern of the dairy cow has a major impact on the ability of a TMR to stabilize rumen fermentation. The rate of intake over the day has not been directly investigated, but other research into feeding frequency and sorting provide insight. For example, the pattern of blood metabolites is profoundly changed when the frequency of concentrate feeding is increased from 2 to 6 times per day (Sutton et al., 1985). Additionally, plasma VFA profiles provide some evidence that increased feeding frequency changes the rumen environment and the profile of available nutrients (Sutton et al., 1986). French and Kennelly (1990) provide further support that increasing feeding frequency changes the rumen environment by showing that there

was greater variation in rumen pH of cows fed twice a day versus twelve times a day.

Interestingly, in cows fed twice a day, the rumen pH decreased below 5.5 twice during the day whereas the pH in cows fed twelve times a day never dropped below 5.5. In cows fed complete TMR rations that include both small and long particles, sorting provides an opportunity for the cows to decide what and when to consume. It is well-described that cows sort against long particles, which are high in neutral detergent fiber (NDF) and are less digestible, and sort in favor of the smaller particles, which are lower in NDF and more digestible (Leonardi and Armentano, 2003). However, the ability to sort is limited in cows fed individually to low or moderate refusals (5 to 10% of DMI). Selection with limited feed available is expected to result in a progressive increase in NDF concentration as the day proceeds, but by the end of the day the cow will have eaten a ration similar to that fed regardless of sorting.

Activity and rest cycles are regulated through a system in the brain comprised of oscillators that track the 24 hour light/dark cycle of the earth. These 24 h cycles are known as the circadian system (from the Latin words “circa” meaning “around” and “dies” meaning “day”). The circadian cycles include feeding behavior and have been most thoroughly demonstrated in rodent (Stephan, 2002). The circadian system is also connected to cellular energy metabolism (Asher and Schibler, 2011), which has implications for both feeding behavior and energy homeostasis. Since the dairy cow’s ability to eat is a balance between rumen fill and energy needs (Baile and Della-Fera, 1981), it is logical to expect that the circadian system connects metabolic demands and feeding behavior. Combining an understanding of the circadian system with an understanding of ruminant nutrition and physiology may help the dairy industry develop feeding strategies that maximize milk yield and efficiency.

2.1 Circadian System

All organisms from cyanobacteria to plants and mammals have evolved the ability to anticipate the Earth's rotation in order to synchronize the gathering of food with predator abundance and other environmental dangers (Dunlap, 1999, Panda et al., 2002a). An organism's ability to track the light/dark cycle is predominantly controlled by a central circadian pacemaker (Ralph et al., 1990), and it has been shown to influence sleep/wake activities as well as hormone and neural patterns (Froy, 2011, Rusak, 1989, Wijnen and Young, 2006).

The central circadian pacemaker regulates a coordinated cascade of messages to the body on the phasing of the light/dark cycle. It is considered the master clock of the entire body and is referred to as the suprachiasmatic nucleus (SCN; See Appendix B for a list of definitions). In the SCN, neurons are the autonomous units that oscillate with roughly a twenty-four hour cycle independent of any external stimulus [For a more complete description of the circadian system, please refer to the review by Welsh et al. (2010)]. The neurons receive direct input from the retinohypothalamic tract which enables them to perceive stimuli from the environment, such as changes in light intensity across the day (Gooley et al., 2001, Welsh et al., 2010). Lastly, these neurons are a single functional unit that can synchronize other clocks throughout the body with their own output (Reppert and Weaver, 2002).

There are also subordinate clocks located in most other tissues that are referred to as "peripheral clocks". Particularly important, these peripheral clocks have been found in many tissues performing key metabolic functions, some of which include adipose, hepatic, lungs, skeletal muscle, and pancreatic tissues (Dibner et al., 2010, Reppert and Weaver, 2002, Sadacca et al., 2011, Yamazaki et al., 2000). Whereas peripheral clocks typically do not influence the phase of the SCN, the SCN plays a crucial role in regulating peripheral clocks. Under normal conditions, the SCN couples the peripheral clocks to itself so all rhythms in the organism are

operating in the same phase. This synchronization, or coupling, occurs so that multiple organs can coordinate functions (Yamazaki et al., 2000).

The light-dark cycle is the most powerful zeitgeber (“time-giver”) for entrainment of the SCN, which in turns entrains the peripheral clocks. The second most powerful zeitgeber is the timing of feeding. In complete darkness, meal time has been shown to strongly entrain peripheral clocks (Mistlberger, 2009, Stokkan et al., 2001), but entrainment of the SCN remains highly debated (Froy et al., 2009a, b, Hara et al., 2001, Honma et al., 1983b).

Lastly, core body temperature has also been proposed as a zeitgeber with the ability to entrain peripheral circadian clocks. A robust body temperature rhythm has been characterized in many species regardless of environment [see recent review by Refinetti (2010b)]. Many studies have demonstrated that both consuming feed and the onset of activity can change the amplitude and shape of the rhythm, and that feeding time and activity have nothing to do with creating it (Refinetti, 2010a). For example, the rhythm in body temperature persists during total feed deprivation (Sakurada et al., 2000) and in patients confined to bed rest (Golja, 2002). Daily fluctuations in body temperature due to activity and/or feeding have also been shown to synchronize the peripheral clocks (Brown et al., 2002).

The ability of circadian clocks to respond to multiple zeitgebers provides the opportunity for a disconnection of the multiple timekeepers in an organism. This disconnect has been implicated in metabolic issues and disease in humans (Froy, 2010, Hastings et al., 2003). The most common example of this disconnection occurs in shift workers where the negative effects include an increased waist circumference and insulin resistance [summarized by Antunes (2010)]. To mimic shift work, a “forced desynchrony protocol” is used to create the daily schedule of shift work without requiring the use of shift working subjects. This protocol also allows scientists to control for social demographics and other factors. Many negative health impacts have been demonstrated to be caused by experimental desynchronization. There was higher post prandial

concentration of glucose and insulin in the misaligned group, which could indicate a greater chance of developing Type II diabetes mellitus (T2DM) or other associated diseases (Scheer et al., 2009). In epidemiologic studies, rotational shift workers had elevated waist to hip ratios, blood pressure, and insulin and triglyceride levels compared to dayshift workers. These characteristics are biomarkers for metabolic syndrome and indicate an increased risk for T2DM, obesity, and cardiovascular disease (Sookoian et al., 2007). Desynchrony of central and peripheral clocks have not been specifically investigated in the cow, but may similarly contribute to metabolic diseases and reduced efficiency.

2.1.1 Molecular Mechanism

The molecular mechanism of the circadian system has been extensively reviewed elsewhere (Bell-Pedersen et al., 2005, Doherty and Kay, 2010, Jay C, 1999) and will be briefly summarized here. The clock consists of a series of transcriptional/translational negative feedback loops with both positive and negative elements. It completes one cycle in approximately 24 h. The positive regulatory elements consist of circadian locomotor output cycles kaput (Clock) with its paralogue neuronal PAS domain-containing protein 2 (Npas2) and the aryl hydrocarbon receptor nuclear translocator-like protein Arntl or Bmal1. The negative elements consist primarily of the Periods (Per1, Per2, and Per3) and the Cryptochromes (Cry1 and Cry2). Clock and Bmal are transcription factors that heterodimerize when translated and induce transcription of per and cry. Upon translation, Per and Cry move to the cytoplasm where they heterodimerize and then return to the nucleus to inhibit the Clock:Bmal complex. They therefore inhibit their own transcription and create a regulation loop [See Figure 2.1a (Dibner et al., 2010, Doherty and Kay, 2010, Panda et al., 2002a)].

Another critical aspect to this rhythmicity is the post-translational phosphorylation of the Per and Cry proteins. Kinases progressively phosphorylate Per and Cry resulting in degradation [Figure 2.1a (Takahashi et al., 2008b)]. Mutations in these kinases have been shown to cause changes in the period of the rhythm. Both longer and shorter periods have been discovered depending on either the mutation within the kinase or among different kinases (Tataroglu and Schafmeier, 2010). For example, the famous tau mutant hamster, known for having a 20 hour period, is a result of a single point mutation in casein kinase I epsilon (CKI ϵ), the most studied kinase in the primary feedback loop (Leloup and Goldbeter, 2011).

There are secondary feedback loops that are proposed to be responsible for the robustness of the overall rhythm. The most crucial secondary feedback loop consists of the orphan nuclear receptors retinoic acid-related orphan nuclear receptor α (Ror α) and reverse-erb alpha (Rev-erb α) [See Figure 2.1b (Takahashi et al., 2008b)]. Ror α competes with Rev-erb α on the Bmal promoter site through the Ror regulatory elements (Sato et al., 2003) so that Ror α up-regulates Bmal expression and Rev-erb α down-regulates Bmal expression. More specifically, rev-erb α may act as another link between the positive loop (Bmal and Clock) and the negative loop (Per and Cry) by being up-regulated by the positive limb and down regulated by the negative limb of the loop [Figure 1a and 1b (Preitner et al., 2002)].

Genes regulated by both the primary and secondary loops are known as clock controlled genes (CCGs) and are expressed in a tissue specific manner. These CCGs comprise the second tier of the hierarchy of the circadian system and are the downstream targets predominantly responsible for the outward rhythms that impact the activity of metabolic pathways and other physiological processes. The products of the CCGs and their enzymatic activity are often monitored to demonstrate the rhythms of the core clock (Bozek et al., 2009). The impact of the diverse CCGs can be seen on neuronal activity, blood metabolite concentrations, blood pressure,

or protein abundance in specific tissues and will be addressed further in a later section (Teboul et al., 2008).

2.1.2 Metabolism on Molecular Level

Many of the genes involved in secondary loops have important roles in metabolism (Asher and Schibler, 2011, Froy, 2007, Froy, 2011, Wijnen and Young, 2006). For example, 3-20% of hepatic genes are cyclic in nature (Panda et al., 2002a). Specifically, Rev-erb α regulates metabolism in metabolically active tissues like the liver, adipose tissue, skeletal muscle, and pancreas (Duez and Staels, 2008, Panda et al., 2002a, Torra et al., 2000). Briefly, Rev-erb α is directly involved in lipid and glucose metabolism by down regulating apolipoprotein C-III (ApoC-III) and phosphoenolpyruvate carboxykinase [Pepck; See review by Duez and Staels (2009)].

Another CCG that is linked to metabolism is silent mating type information regulation 2 homolog (Sirt1), which monitors the redox status of the cell and influences the circadian system by affecting the ability of the Clock:Bmal complex to bind DNA (Asher et al., 2008). When NAD⁺, an indicator of cellular energy balance, increases in concentration, it is bound by Sirt1 to activate its deacetylase activity. Deacetylating DNA causes it to slightly unwind, which allows the Clock:Bmal complex to bind to DNA and regulate transcription (Wijnen, 2009). Since NAD⁺ acts as the indicator of the cell's energy status, it connects Sirt1 to the metabolic state of the cell and the circadian system. This is supported by increased Sirt1 protein synthesis during starvation. Sirt1 proteins also have an effect on prompting the cell to start mobilizing energy sources through increased gluconeogenesis and lipolysis (Duez and Staels, 2009). Nicotinamide phosphoribosyltransferase (Nampt) is the rate limiting enzyme in NAD⁺ synthesis and another

indicator of a cell's energy balance. Clock and Bmal have also been shown to directly regulate Nampt production aside from cellular metabolism (Nakahata et al., 2009)

Lipid metabolism is an additional function highly regulated by the circadian system. Peroxisome proliferator-activated receptor alpha (PPAR α), a regulator of hepatic lipid metabolism, is activated by Clock, and Clock knockout mice have decreased mRNA expression of PPAR α (Oishi et al., 2005). The reverse has also been shown in that PPAR α changes the amplitude, but not the phase, of Bmal expression in the liver. Furthermore, the amplitude of Bmal expression was found to be dampened in PPAR α knock-out mice (Canaple et al., 2006), which demonstrates that it is part of a feedback loop. As yet another link between metabolism and the circadian system, PPAR α also increases Rev-erba expression (Duez and Staels, 2009). Lastly, Cry1/Cry2 knock-out mice have a decreased ability to synthesize cholesterol and fatty acids, presumably due to a decreased ability to activate the sterol response element binding protein (SREBP) transcription factors (Gachon and Bonnefont, 2010).

Finally, many metabolic hormones have been shown to have a circadian pattern due to circadian regulation of gene expression and hormone secretion (Froy, 2011, Froy and Miskin, 2010). For example, the satiety/hunger hormones leptin, ghrelin, and neuropeptide Y (NPY) all show robust circadian rhythms that are responsive to inversion of feeding and sleep patterns (Bodosi et al., 2004, Bray and Young, 2007, Kalra et al., 2003, Laposky et al., 2008a, Laposky et al., 2008b). Many other hormones have a distinct circadian pattern including glucagon, norepinephrine and epinephrine, and insulin (Allaman-Pillet et al., 2004, De Boer and Van Der Gugten, 1987, Ruiter et al., 2003).

2.1.3 Circadian Regulation of Behavior

Behavior, especially feeding behavior, is influenced by the circadian system because it normally occurs during the wake phase of the sleep/wake cycle. Normally, the sleep/wake cycle is set by the light/dark cycle, but it can also be set by feeding time (Honma et al., 1983a). This fact demonstrates the importance of feeding as a stimulus. In some severe cases, feeding time can override the central rhythm set by the SCN. For example, restricting feeding to only the light phase in mice changes their activity patterns and uncouples multiple oscillators (Damiola et al., 2000, Stokkan et al., 2001). Uncoupling occurs when different tissues, including the SCN, are functioning in different phases relative to each other. Restricted-fed rodents have uncoupled oscillators due to a food-entrainable oscillator (FEO) that responds to feeding time (Mistlberger, 1994, Mistlberger, 2009, Stephan, 2002), but feeding does not entrain the SCN (Froy et al., 2009b). The consequence is that when food availability returns to normal, synchrony is re-established in the SCN with the other clocks in the body (Honma et al., 1983a). The anatomical location of the FEO is not known.

Serious repercussions occur when multiple oscillators are operating in different phases, as previously discussed with regards to metabolic syndrome in shift workers. Restricted-fed rodents, defined as limiting the time for which feed is available, calorie intake, or both, is an experimental scenario in which the FEO and SCN are forced to oscillate in different phases (Damiola et al., 2000). Other mouse models, such as circadian mutants, have been used to understand the circadian influence on obesity and other associated health concerns. Additionally, it has been shown that genetic mutation of circadian control genes increases obesity, hyperlipidemia, hepatic steatosis, and hyperglycemia (Laposky and Turek, 2009). CLOCK mutant mice also have decreased locomotor activity and increased voluntary caloric intake which illustrate that their behavior is different than their wild-type counterparts (Turek et al., 2005).

2.1.4 Mammary Gland

There recently has been interest in the role of the circadian system in the mammary gland. The mRNA expression of the circadian system's core elements changes during mammary gland development (Casey et al., 2009, Metz et al., 2006). Metz et al. (2006) also investigated the mRNA expression of key circadian genes over the day and found differences in expression levels between virgin and lactating mammary tissue (Metz et al., 2006). There were clear patterns of many circadian genes including *Cry*, *Per*, and *Clock* in mRNA extracted from human milk fat (Maningat et al., 2009).

In lactating rats, lipogenesis in the mammary gland has a clear trough around 1600 h, which coincides with the lowest energy intake period of the day (Munday and Williamson, 1983). Lactose synthesis also follows a daily pattern in mammary tissue with marked decreases in rates of synthesis around 1700 h (Carrick and Kuhn, 1978). Lastly, whole-animal *CLOCK* mutant mice are shown to have a disrupted nursing pattern compared to wild-type mice. *Clock* null mice had decreased pup weight gain, a distinct change in nursing behavior, and decreased prolactin concentrations six days post-partum (Hoshino et al., 2006). Cause and effect is difficult to interpret with whole animal disruption of *CLOCK* because changes may be due to central regulation of behavior or mammary physiology.

2.2 Feeding Behavior and Physiology

2.2.1 Introduction

The circadian system is highly conserved (Panda et al., 2002b) allowing many extrapolations across species (Jay C, 1999). Wheel running is commonly used in rodents to

observe behavior in relation to mutations in the clock framework, changes in feeding frequency, or caloric restrictions (Challet et al., 1998). Using this approach, food-anticipatory activity (FAA) was discovered when feed is both restricted calorically and/or temporally altered (Mistlberger, 2009).

Wheel running is not amenable to cattle, but technology has been developed to monitor feeding behavior over the day. This directly determines meal size and timing and provides mechanistic insight into intake regulation. Contrary to popular belief, cattle do not graze continuously or even sporadically throughout the day, but have a distinct pattern of feed intake (Albright, 1993). As in rodents, changes to the environment, feed, and daily schedule has been shown to alter the feeding behavior of cattle (DeVries and von Keyserlingk, 2005, Oba and Allen, 2000). This may have implications in dairy management to structuring the day, especially the timing of milking and feeding.

The timing of feed intake impacts the timing of nutrient digestion and absorption. In theory, the timing of feed intake dictates the abundance of nutrients available for milk synthesis and metabolic processes. Little is known about the relationship between the timing of feed intake and milk synthesis, but specific feeding strategies provide an opportunity to change the timing of nutrient absorption. Milking and feeding at different frequencies and times would offer a better understanding of these interactions. Feeding behavior links nutrition and physiology to the behavioral sciences, which one could argue is primarily driven by the circadian system. Thus, the circadian system could give insight on how to manipulate behavior to increase production or efficiency in dairy cows.

2.2.2 Feeding Behavior

Intake is one of the most important factors to animal production and feeding behavior is the study of how and when an animal consumes feed. From a physiological view cows begin a meal when they are hungry and eat until they reach satiety (van der Veen et al., 2006). In the cow satiety may be stimulated by physical fill of the rumen or through sensing that the energy needs have been met (Baile and Della-Fera, 1981). For the purpose of discussion we will assume that rumen fill is not limiting. To meet the daily energy demand, a certain quantity of feed is consumed. This 'daily quantity' is met through a modification of the number and size of meals (Brobeck, 1955). Changes in intake cannot be made without affecting those parameters.

There are several approaches that allow investigation of feeding behavior in the dairy cow. Weighing feed at set intervals throughout the day allows determination of feed disappearance and can be done manually or with an automatic system. A common automated system uses feeding buckets mounted on load cells integrated with a control system, which records the weight at a specified rate (Dado and Allen, 1993). These systems have been built in research laboratories and the systems are also commercially available. Automated systems provide high resolution of feed disappearance, allow determination of meal bouts and sizes, and interfere minimally with normal behavior. Alternatively, manually weighing the feed at specific intervals over the day can be done, but the interval between observations tends to be much longer (h vs. sec) limiting the ability to observe meal bouts and may interfere or alter normal behavior.

Automated feed observation systems collect a large amount of data that must be processed to determine timing and size of meals. Specifically, parameters must be chosen for the minimum meal size and inter-meal interval. An inter-meal interval determines when two feeding bouts, with an interceding rest period, is considered the same or different meals. Currently there is a lack of consensus on the appropriate inter-meal interval. Tolkamp (2011) has established a

distribution analysis method to determine the minimum inter-meal interval, although the method may not be unbiased. Another approach to intake analysis relies on deconvoluting daily intake into multiple feeding distributions without determination of individual meals. Shabi et al. (2005) first applied this method to dairy cows and observed two higher distributions of feeding behavior during the day which occurred around dawn and towards dusk (2005). This counters the idea that the TMR is consumed in a relatively constant rate over the day but is supported in a review by Albright (1993) which describes a crepuscular pattern of grazing in cattle. Lastly, Boston (2008) used a similar deconvolution model to describe human food intake and there is optimism that this approach could be applied to dairy cows to provide a less biased approach to analysis of feeding behavior over the course.

Presence at the bunk has also been used to determine feeding behavior in group housed dairy cows. The two main ways of monitoring bunk attendance, or more generally feeder attendance, is through visually recording or radio tags. Visually determining behavior typically is accomplished either by observing animal behavior every 5 minutes for 24 h in real time or using a video camera to record the feed bunk with behavior determined by time-lapse viewing at a later time (Vasilatos and Wangsness, 1980). These methods are time consuming, sometimes require interpretation of behavior, and are prone to recorder mis-identification of cows, but can be used to observe non-feeding behaviors such as aggressive interactions (Huzzey et al., 2006). Radio tag systems have been developed that record the presence of a cow at the feed bunk through a receiver placed below the feed (DeVries et al., 2003, Schwartzkopf-Genswein et al., 1999). Radio tags provide an automated record of the attendance at the bunk, which is well correlated to feeding, but does not provide amount of feed consumed.

The timing of intake is important to ruminal fermentation and may be important for efficient use of nutrients. Technology makes it easier to monitor when and how cows consume feed. In research settings this will allow development of feeding strategies that optimize the

timing of feed intake. In the commercial setting, these systems could provide early detection of sick animals as well as insight into diet acceptability. Important to our research objectives, feed observation systems allow calculation of the timing of nutrient intake to demonstrate the limitations of a single TMR fed once per day.

2.2.3 Timing of Nutrient Intake

The pattern of intake has been well documented although circadian analysis procedures have not been commonly applied. The rumen allows the dairy cow to consume a large amount of feed at once and utilize low quality feeds, but makes determination of the timing of nutrient absorption difficult. To further convolute the issue, selective breeding has made it difficult for dairy cow to sustain such high levels of production while consuming a diet consisting primarily of low quality feedstuff. Highly digestible concentrate feeds have been introduced to increase diet energy density, but increases the risk of ruminal acidosis and poor animal health (Owens et al., 1998). The TMR was developed to dampen the effects of concentrate feeds and optimize the ruminal environment. Many think that TMRs offers a constant amount of nutrients to the rumen (Coppock et al., 1981), but it is apparent based on the circadian pattern of feed intake that the amount of nutrients entering the rumen is not constant. Although diet composition is constant, the rate of nutrients entering the rumen is not and is expected to change rumen nutrient pool sizes over the day.

Management, feed characteristics, and ration properties affect feeding behavior. Offering fresh feed stimulates intake and may increase DMI (Beauchemin and Yang, 2005, Hosseinkhani et al., 2008, Kononoff and Heinrichs, 2003, Shabi et al., 2005, Yang and Beauchemin, 2006). However, pushing up feed has little effect on stimulating intake in several experiments (DeVries and von Keyserlingk, 2005, DeVries et al., 2003, 2005, Nocek and Braund, 1985), even despite

the fact that cows will get up and go to the bunk when feed is pushed up. However, pushing-up feed is very important when feed may not be within reach.

Stocking density also alters feeding behavior. At higher stocking densities cows lower in the social order are displaced by the dominant cows in the herd which result in more time per dominant cow at the bunk and less time per submissive cow at the bunk (DeVries et al., 2004, Huzzey et al., 2006). Less dominant cows will return to the feed bunk at off peak hours and tend to eat fewer but larger meals per day.

Feed characteristics also affect the feeding behavior of dairy cows. One of the most important feed characteristics is particle size, which changes the pattern of intake over the day (Beauchemin and Yang, 2005, Kononoff et al., 2003). There are also examples of feed processing, feed digestibility, and dietary macronutrient concentration that alters the number or size of meals (Mooney and Allen, 1997, Oba and Allen, 2000, Stephan, 2002, Taylor and Allen, 2005).

Sorting or selection of feeds by the cow also affects the amount of each nutrient entering the rumen. During the early part of the day cow preferentially sort for smaller particles which tend to be the more fermentable (Leonardi and Armentano, 2003). This also coincides with the higher intake part of the day and exacerbates the high rate of fermentable nutrients entering the rumen. Since cows are fed to limited refusals this leaves less readily fermentable feeds for the latter part of the day (Maulfair et al., 2010) and limits other sorting. Importantly, the rate of refusals has a large impact on the cow's ability to select the daily diet. Developing new strategies in feeding could lessen the effects of sorting by formulating rations that take into account the circadian pattern of feed intake.

The cows natural circadian pattern of feeding behavior and tendency to select more fermentable nutrients results in changes in the amount of nutrients entering the rumen and body over the day (Shabi et al., 2005). The higher intake and concentrations of readily fermented

feedstuffs in the period following the higher intake periods of the day could cause acidotic conditions in the rumen and creates non-steady state concentrations of nutrients available to cow for processes like milk synthesis.

2.2.4 Digestion and Passage Rates

The complexity of the rumen including the interaction of feedstuffs and rumen microbes make prediction of rates of digestion and passage difficult. Many models have been made to simulate digestion, absorption, and passage, but all have inherent flaws (Dhanoa et al., 1995, Firkins et al., 1998, Mertens, 1987, Mertens and Ely, 1979). Experimental procedures used to determine passage rates are difficult, have inherent flaws, and normally assume steady state conditions as summarized by Huhtanen and Kukkonen (1995).

The major flaw in most models is the assumption of steady state conditions and the application of first order kinetics for determination of digestion and passage (Firkins et al., 1998). Physical properties of the feedstuffs such as particle size and specific gravity affect passage rate. Smaller particles and particles with a higher specific gravity tend to pass from the rumen faster. There is also a lag phase in the breakdown of feed due to the time it takes the microbes to attach to the feed particle. Once the microbes digest the readily available portions of the feedstuff there is a decrease in the rate. Lastly, passage rates are influenced by dry matter intake with an increase in passage rate with increased intake (Cochran et al., 1986). It is expected that the rate of digestion and passage of nutrients varies over the day because of increased rumen fill during the high intake period of the day and changes in microbial populations (Fickett).

Digestion and ruminal passage also dictate when and where nutrients are digested and absorbed. Ruminal fermentation results in volatile fatty acids (VFA) that are the dairy cow's main source of energy, but are also linked to acute and subacute ruminal acidosis. As the

production of VFAs increase, rumen pH and rumen fiber digestion decrease (Allen, 1997). Since pH appears to follow a daily pattern one can assume that the fermentation capacity of the rumen also changes which results in fluctuations in VFA production (French et al., 1990, Gustafsson and Palmquist, 1993, Shabi et al., 1998).

2.2.5 Milk Synthesis

The timing of milk and milk component synthesis over the day has not been well investigated in the cow. The scientific literature reports milk yield and components as means over the day, which gives little insight into daily patterns. A difference in milk yield and composition between milkings is commonly appreciated, but uneven milking interval can confound interpretation. The longer the milking interval the more time the mammary gland has to accumulate milk and rate of milk synthesis declines (Erdman and Varner, 1995). The increased pressure may have different impacts on synthesis of individual milk components. Differences in milk and milk component yields between milkings is normally attributed to unequal milking intervals (Erdman and Varner, 1995, Everett and Wadell, 1970b, Hargrove, 1994), while others detected differences between milkings with equal milking interval (Everett and Wadell, 1970a, Gilbert et al., 1973). These experiments consistently reported higher milk yields in the AM milking, higher milk fat concentrations during the PM milking, and either equal or higher protein concentrations during the PM milking. Twice a day milking strategies provides support for a difference in milk and milk component synthesis over the day, but provides limited mechanistic insight.

Very few published experiments report milk yield over the day with a greater resolution than twice daily milking. Van Der Iest and Hillerton (1989) milked cows six times a day for two days to see how quickly the mammary gland can adapt to the change in milking frequency. There

are no statistical analysis applied, but a pattern of milk synthesis can be seen with lowest yields at the fourth and eighth milkings. A cyclic pattern in fat concentration can also be seen over the twelve milkings (Van Der Iest and Hillerton, 1980). Recently Quist et al (2008) examined production data in sixteen commercial dairy herds milked twice or three times a day. There is a clear pattern of milk synthesis in the herds milked twice daily with the lowest milk yields during the evening milking. A pattern also emerges in milk fat concentration with the lowest concentrations reported during the morning milking. For the thrice daily milked herds, milk yield and milk fat and protein concentration have noticeable patterns with milk yield and fat concentration lowest during the morning milking, while milk protein percent appears to be lowest during the evening milking (Quist et al., 2008).

Lastly, photoperiod has a well-known effect on dairy cows during lactation, puberty, and the dry period [See Review by Dahl (2000)]. A long day photoperiod during lactation increases milk production that persists throughout lactation. Furthermore, a short day photoperiod is optimal for mammary development during the dry period and results in increased milk production in the subsequent lactation. Lastly, in the prepubertal heifer long days will promote an increased development of parenchymal cells (Dahl et al., 2000, Dahl and Petitclerc, 2003, Dahl et al., 2011).

The circadian rhythm of milk synthesis has not been specifically investigated in the dairy cow, but the presence of the rhythm is supported as discussed above. This combined with discoveries at the molecular level provide the framework for a link between the circadian system and the mammary gland.

2.2.6 Known Circadian Patterns in the Cow

Daily patterns are generally difficult to study because high resolution is needed for circadian analysis. The main variables tested for the presence of a circadian pattern in the dairy cow are reported in Table 2.1a. An automated blood sampling system was extensively used at the USDA Beltsville Laboratory that continuously collected blood providing a high sampling rate over an extended period. This system was used to investigate the concentration of important blood hormones and metabolites in the dairy cow. Mathematical models were used to determine if the plasma variables followed a circadian or ultradian rhythm. Most variables observed followed both ultradian and circadian rhythms in that they were pulsatile in nature, but a sine wave could also be fitted to the overall data. The high sampling rate allowed for the detection of ultradian rhythms, which most studies overlook with a lower sampling rate. Additionally Giannetto and Piccione (2009) tested twenty-five physiological variables and found eleven were rhythmic (Table 2.1b).

Many additional publications do not analyze data for circadian rhythms, but do report values across the day. For example, Blum et. al (2000) conducted a study investigating the effect fat supplementation and sampled blood every hour for 30 hours in week nine and nineteen of lactation, glucose peaked between 2400 and 0700 h and between 0700 and 0800 h in week nine and nineteen, respectively. Plasma NEFA peaked between 0700 and 0800 h at both sampling periods, but NEFA had a higher amplitude during week nine. Lastly, insulin had higher concentrations between 1200 and 1600 h during week 9 and between 1600 and 0800 h during week 19. Although circadian rhythms have been specifically analyzed in a limited number of publications it is clear that metabolic parameters follow a circadian rhythm in the lactating dairy cow and that this rhythm is dynamic.

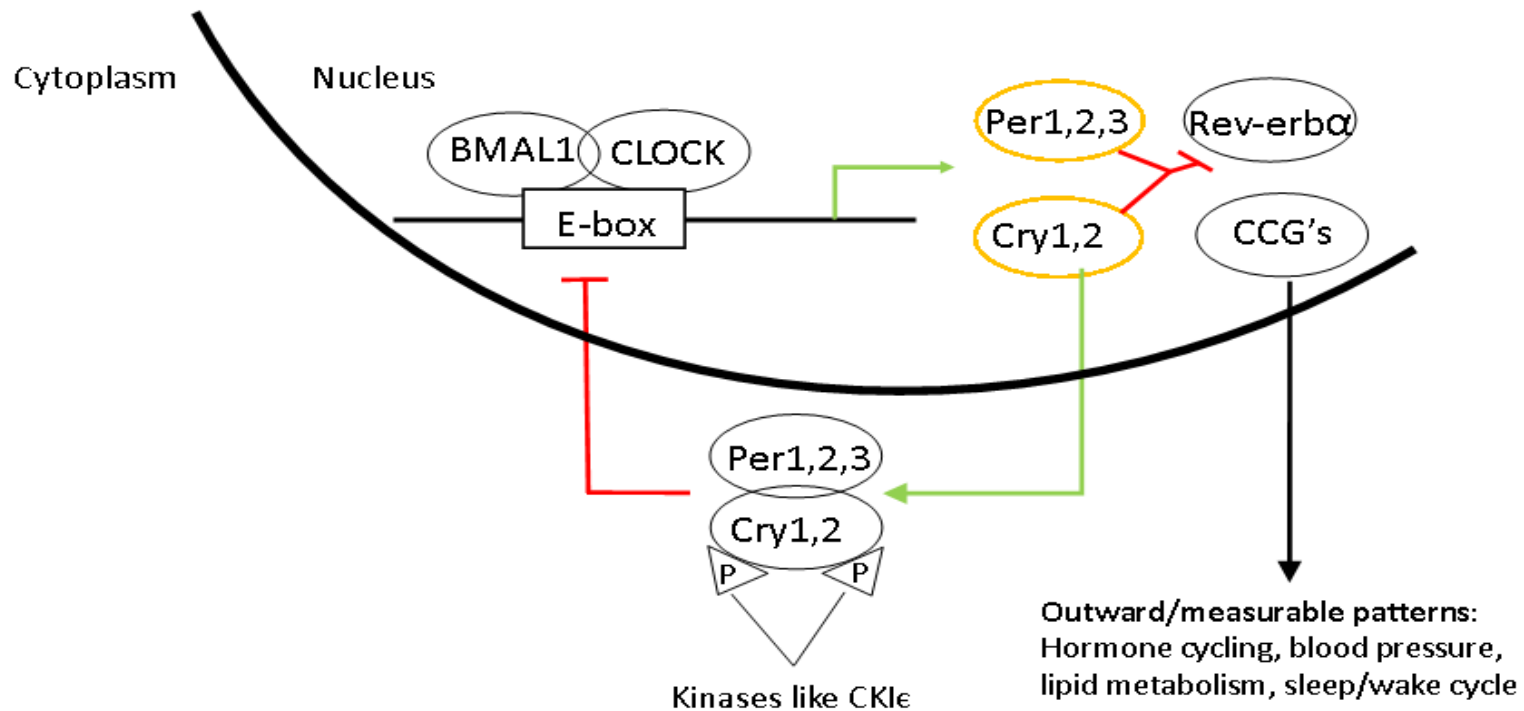


Figure 2.1a Diagram of the Primary Loop of the Circadian System

The primary loop of the circadian system consists of transcription factors that interact to create 24 h cycles. Clock and Bmal1 dimerize and bind to an E-box response element that activates transcription of the second part of the primary loop, the periods (Per) and cryptochromes (Cry). Per and Cry mRNA translocates to the cytoplasm where they are translated. When the concentration of Per and Cry rise to a certain threshold they dimerize and relocate to the nucleus. In the process of moving they are progressively phosphorylated which affects their degradation rate. Once in the nucleus they inhibit the Clock:Bmal complex which ultimately leads to feedback inhibition of their own transcription. Aside from activating Per and Cry, the Clock:Bmal complex activates the transcription of other genes including the secondary loop of the circadian system such as Rev-erbα (Figure 2.1b) and other clock controlled genes (CCGS). The CCG's are responsible for the outward measurable 24 h patterns such as plasma hormone concentrations, blood metabolite concentrations, core body temperature, etc.

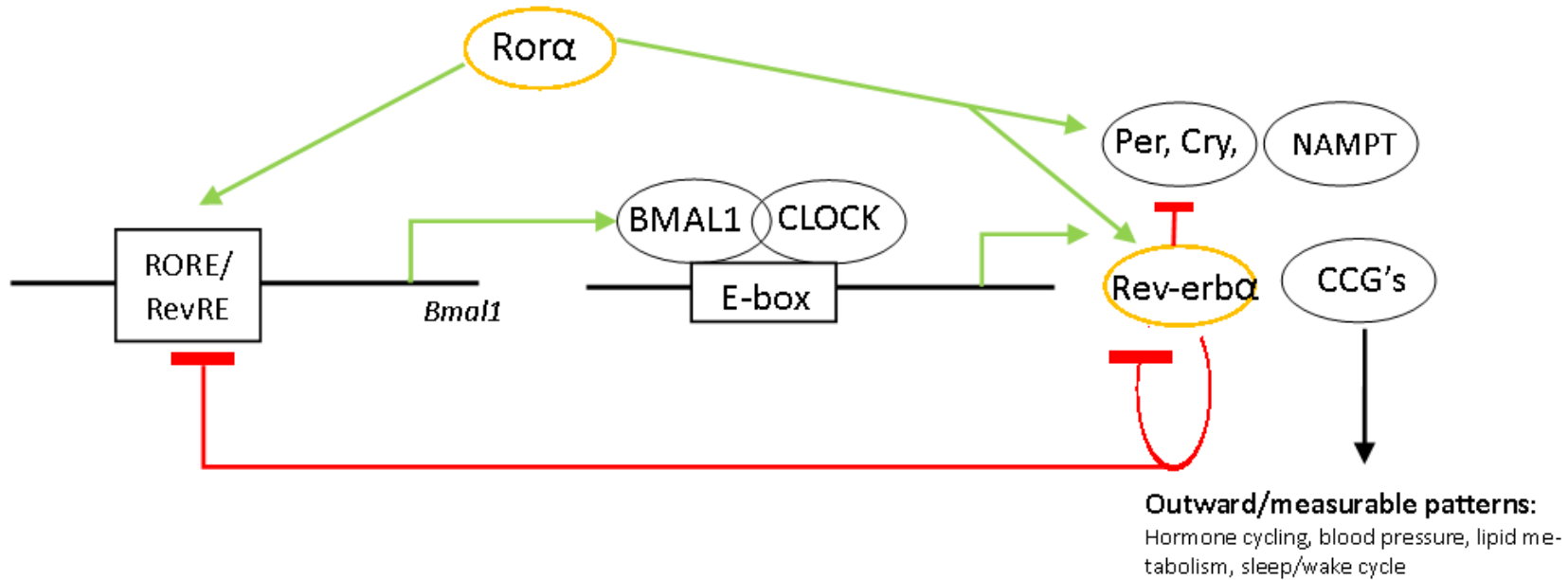


Figure 2.1b Diagram of the Secondary Loop of the Circadian System

The secondary loop is commonly thought to be responsible for the robustness of the circadian rhythm. Similar to the primary loop, the Clock: Bmal complex also activates transcription of the second portion of the secondary loop. An example of the one of the participants of the secondary loop is Rev-erba, which once transcribed and translated, will inhibit itself as well as the RORE/RevRE promoter which activates transcription of Bmal1. A decrease in Bmal1 will decrease the abundance of the Clock: Bmal complex which decreases the probability of the complex to bind the activating E-BOX, thus ceasing the rest of the loop. Rora antagonizes Rev-erba, activates the RORE/RevRE promoter, and perpetuates the transcription of Bmal1 and downstream targets. The secondary loop creates an important link with metabolism. For example, NAMPT, a rate limiting enzyme to NAD⁺ synthesis, is activated by the Clock: Bmal complex. NAD⁺ is crucial energy transfers in the cell, regulates the activity of the citric acid cycle of the cell, and is an indicator of cellular energy balance.

Table 2.1a Known circadian patterns in the cow

Parameter	Zenith	Nadir	Amplitude	Type	Period
	Mean (time)	Mean (time)	Mean (% of mean)	Cir/Ultra ¹	Time, h
Leftcourt Series²					
Lipid, mg/dl					
CE			33.8 (19.2%)	Ultra	2.6
C			3.7 (17.5%)	Ultra	2.6
TG			3.3 (55.8%)	Ultra	2.7
FFA			1.8 (68.2%)	Ultra	2.7
GH, ng/ml	5.31 (0632)	4.12 (1821)		Cir/Ultra	NR
Prolactin, ng/ml					
Group 1	14.84 (1904)	28.9 (0730)		Cir/Ultra	NR
Group 2	28.3 (0840)	40.1 (1917)			
Temperature, °C	39.1 (2323)	38.8 (1550)		Cir/Ultra	NR
Insulin, ng/ml	1.33 (1743)	0.59 (0632)		Cir/Ultra	NR
Urea, mM	15 (1034)	11.21 (2239)		Cir/Ultra	NR
Thyriod, ng/ml			% of mean		
T ₃	1.58 (1846)	0.94 (0703)	25.1	Cir/Ultra	NR
T ₄	50.27 (2054)	41.87 (1000)	9.1	Cir/Ultra	NR
Cortisol	4.48 (536)	3.10 (1757)		Cir/Ultra	2

¹A circadian rhythm which has a period of 24 h; an ultradian rhythm which has a period less than 24h

² Series of papers published from USDA Beltsville (Bitman et al., 1994, Bitman et al., 1990, Lefcourt et al., 1994, Lefcourt et al., 1993, Lefcourt et al., 1995, Lefcourt et al., 1999)

³ (Giannetto and Piccione, 2009)

⁴ Arbitrary Units

NR: not reported

Table 2.1b Known circadian patterns in the cow

Parameter	Zenith	Nadir	Amplitude	Type	Period
	Mean (time)	Mean (time)	Mean (% of mean)	Cir/Ultra ¹	Time, h
Giannetto and Piccione³					
	Time		(Max-min)		
Activity, a.u. ⁴	1302	NR	1161	NR	NR
Temperature, °C	2037	NR	0.32	NR	NR
Hb, g/dl	0409	NR	1.31	NR	NR
Glucose, mmol/l	2309	NR	1.45	NR	NR
Creatinine, umol/l	1244	NR	53.43	NR	NR
Urea, mmol/l	0835	NR	0.72	NR	NR
Cholesterol, mmol/l	0014	NR	0.96	NR	NR
Total Lipids, mmol/l	2355	NR	1.38	NR	NR
NEFA, mg/dl	1114	NR	42.27	NR	NR
Phosphorus, mmol/l	0800	NR	0.16	NR	NR
Magnesium, mmol/l	0220	NR	0.40	NR	NR

¹A circadian rhythm which has a period of 24 h; An ultradian rhythm which has a period less than 24h

²Series of papers published from USDA Beltsville (Bitman et al., 1994, Bitman et al., 1990, Lefcourt et al., 1994, Lefcourt et al., 1993, Lefcourt et al., 1995, Lefcourt et al., 1999)

³(Giannetto and Piccione, 2009)

⁴Arbitrary Units

NR: not reported

Chapter 3

Effect of the timing of feed intake on the circadian pattern of milk synthesis

3.1 Abstract

The rate of feed intake varies over the day and is expected to create a daily rhythm of nutrient absorption. The objective of this study was to characterize the effect of the timing of feed intake on the pattern of milk synthesis. Twenty Holstein cows were used in a crossover design with 21d periods. Treatments were feeding a TMR once daily (1x fed) or in 4 equal meals every 6 hours (4x fed). All cows were fed ad libitum at 105% of daily DMI. Cows were milked every 12 h from d 1 to 14 and every 6 h on d 15 to 21 of each period. Milk samples were collected at each milking on d 14 and d 18 to 21. Blood samples were collected 6 times on d 20 to 21. There was no treatment or treatment by time interaction for daily milk yield. Milk yield was different by time ($P < 0.001$) with peak yield at 0200 h and 2000 h and a nadir at 1400 h. There was a treatment by time interaction for milk fat percent, but 4x fed resulted in higher milk fat percent at all time points compared to 1x fed (0.22 to 0.45% higher; $P < 0.05$). Daily milk fat yield was increased 0.13 kg/d by 4x feeding ($P < 0.001$). However, milk protein percent and daily yield were higher in 1x fed (0.1% and 0.05kg/d; $P < 0.001$) compared to 4x fed. Plasma non-esterified fatty acids had a daily pattern with a predominant peak at 0700 h and a second, smaller peak at 1800 h in both treatments (Time $P < 0.001$). Interactions were detected between treatment and time for plasma glucose, insulin, and blood urea nitrogen. Plasma glucose nadirs occurred at 0200, 1400, and 2200 h for 4x fed whereas 1x fed showed a single, higher nadir at 1000 hours (P

< 0.01). More peaks were observed, and the daily range of plasma insulin was greater for 1x fed (21.56 vs. 11.43 μ IU/mg). Blood urea nitrogen peaked at 1400 h in 4x fed compared to 1000 h in 1x fed, but similar daily ranges and nadirs were observed. In conclusion, dairy cows have a circadian pattern of milk synthesis that is responsive to the timing of feed intake.

3.2 Introduction

Many behavioral activities and physiological processes have a daily rhythm with a twenty-four hour cycle (Ralph et al., 1990, Reppert and Weaver, 2002, Rusak and Zucker, 1979). Reoccurring rhythms with a 24 h cycle are called circadian rhythms and are adaptive because they allow an organism to anticipate food availability and to synchronize nutrient absorption with metabolism (Panda et al., 2002b). Circadian rhythms are synchronized or entrained to environmental factors. A master circadian regulation center is located in the (SCN) of the brain, but more recent research has described circadian timekeeping mechanisms in peripheral tissues (Dibner et al., 2010). Light is a strong entrainer of rhythms, but the timing of feeding also entrains metabolic and behavioral rhythms, and peripheral rhythms are especially responsive to entrainment by feeding (Damiola et al., 2000). Interestingly, the timing of food intake can alter the synchronization between the central master timekeeper and peripheral clocks, resulting in development of numerous disorders including obesity, insulin resistance, and metabolic diseases (Takahashi et al., 2008a). For example, mice fed only during the light phase of the day shift their activity and physiology to the light phase and in some instances become obese and insulin resistant (Honma et al., 1983a). Entrainment of circadian rhythms by feeding has not been specifically investigated in the cow.

The dairy cow has a well-recognized natural daily pattern of feed intake and milk synthesis. However, these rhythms are poorly quantified and their regulation has not been well

described in the literature. Dairy cows show distinct patterns of intake over the day (DeVries and von Keyserlingk, 2005) that is normally described as crepuscular with high intake periods occurring at dawn and dusk (Albright, 1993). Using frequency of cows at the feed bunk, the timing of feeding has been shown to be altered by feed delivery time, feeding frequency, and diet composition (Beauchemin, 1991, DeVries and von Keyserlingk, 2005, DeVries et al., 2005) but has not been statistically tested. The daily pattern of milk synthesis is also not well described, but appears to follow a daily rhythm. Quist et al. (2008) reported a higher fat percent in the evening compared to the morning in commercial herds milked two and three times per d, and Van Der Iest and Hillerton (1989) showed a distinct pattern of milk synthesis in cows milked every four h for two days.

We propose that the dairy cow has a circadian rhythm of milk synthesis that is dependent on the timing of nutrient absorption. Our objective was to characterize the pattern of milk synthesis and demonstrate that it is dependent on the timing of feed intake. To achieve this we observed milk synthesis by milking every 6 h when cows were fed ad libitum one time per day or in equal feedings every 6 h.

3.3 Materials and Methods

3.3.1 Animals and Experimental Design.

Twenty multiparous Holstein cows (115 ± 41.4 DIM, mean \pm SD) from the Pennsylvania State University dairy herd were randomly assigned to one of two treatments in a crossover design with 21 d periods. Animal care and all procedures were approved by the Pennsylvania State University Animal Care Committee. Three cows were removed from the experiment due to mastitis and respiratory disease. Cows were fed the same TMR ad libitum either once a day (1x

fed) or in four equal feedings (4x fed, Table 1). The 1x fed cows were fed at 0800 h at 105% of expected intake and the 4x fed cows were fed equal amounts at 0700, 1200, 1800, and 2400 h to 105% of the previous days total intakes. All TMR was mixed at 0800h, stored at ambient temperature, and were compacted by hand into plastic trash cans. Refused feed was removed before delivery of new feed at each feeding. All cows were milked at 0500 and 1700 h from d 1 to 14 of each period (2 x/d) and at 0500, 1100, 1700, and 2300 h from d 15 to 21 (4 x/d) of each period. Manually controlled lights in the barn were turned on at approximately 0430 h and off at approximately 2400 h. Fluorescent light bulbs were used in over head lamps attached to the ceiling of the tie-stall barn. The average temperature was 73 ° F with highs of 84° and lows of 54° F (September 2009 Climate Summary).

3.3.2 Milk and TMR Sampling

On d 18-21, milk samples were collected at each of the four milkings, preserved with 2-bromo-2-nitropropane-1,3 diol and analyzed for milk fat and true protein by Dairy One DHIA (State College, PA) using infrared spectrophotometry [Fat by Filter B; [(AOAC, 2005); Fossomatic 400; Foss Electric, Hillerød, Denmark]. An additional milk sample was collected at each milking on d 21 and stored at -20 °C for fatty acid (FA) analysis. Briefly, lipids were extracted by hexane:isopropanol (Hara and Radin, 1978) and transmethylated by sodium methoxide according to Chouinard (1999). The methyl esters were quantified using a gas chromatograph with a flame ionization detector (Agilent 6890; Agilent Technologies, Santa Clara CA) equipped with a SP-2560 fused-silica capillary column [100 m x 0.25 mm (i.d.) with 0.2- μ m film thickness; Sigma-Aldrich, St. Louis MO]. Initial oven temperature was 80 °C and immediately ramped 2° C/min to 190° C and held for 15 min. Inlet and detector temperatures were 250° C with a 100:1 split ratio. Gas constant flows held hydrogen carrier at 1 ml/min,

detector hydrogen at 5 ml/min, airflow at 400 ml/min, and carrier plus nitrogen make-up at 40 ml/min. Net energy corrected milk (NeCM) was calculated according to the NRC (2001). Samples of TMR were collected twice per period using the quartering method and immediately frozen at -20 °C. Feed samples were dried at 55 °C in a forced air oven, ground through a 1mm screen using a Wiley mill (Arthur A. Thomas Co., Philadelphia, PA), and analyzed for NDF, ADF, CP, and ash by wet chemistry methods (Cumberland Valley Analytical Services Hagerstown, MD).

3.3.3 Blood

Blood samples were collected six times from the tail vein on d 19 through 21 using potassium EDTA vacuum tubes to represent every four hours of the day. The collections occurred at 0200, 0700, 1000, 1400, 1800, and 2200 h. Blood was immediately placed on ice, centrifuged within 1 h, and plasma stored at -20 °C until further analysis. Plasma samples were analyzed for insulin [Coat-a-count insulin kit (TKIN5); Siemens Healthcare Diagnostics, Loas Angeles CA], glucose [PGO Enzyme procedure no. P 7119; Sigma-Aldrich; (Raabo and Terkildsen, 1960b)], blood urea nitrogen [BUN; Modified Enzymatic Urea Nitrogen (Procedure No. 2050); Stanbio Laboratory, Boerne TX], and non-esterified fatty acids [(Wako HR Series NEFA-HR kit; Wako Chemicals USA Inc. according to Ballou et al. [(2009); HR Series NEFA-HR(2) kit; Wako Chemicals USA Inc., Richmond Va].

3.3.4 Body Temperature

On d 8 to 11 and 18 to 21 core temperature was recorded every ten minutes using an intravaginal temperature probe. Briefly, miniature plastic coated temperature recording devices

[(iBCod; Alpha Mach Inc.;Mont-St.-Hilaire, Oc Canda; (Hasselberg et al.)] were fastened to a T-shaped device (14cm x 17cm) constructed of Tygon tubing (0.95cm ID, 1.43 cm OD, 0.24 cm wall thickness; Tygon Tubing, U.S. Plastic Corp. Lima OH) and fastened with nylon barbed T fittings. Temperature probes were placed centrally in the vagina using a 3.8 cm PVC tube. Body temperature rhythms were determined by fitting a Fourier-curve to the data using CircWave V1.4 software. Usually one sine wave and one or two harmonic waves are fitted to the data, and F-tests are used to denote significance (R.A. Hut, Unpublished; www.euclock.org).

3.3.5 Statistical Analysis

Production and plasma variables were analyzed using the MIXED procedure of SAS with repeated measures (SAS Institute 2003). Fixed effects were treatment, time, and the interaction of treatment and time. Random effects were cow and period, repeated variable were time and day, and subject was cow x period. Daily production, DMI and body temperature were analyzed using a reduced model with cow and period as random effects and treatment as the fixed effect. Studentized residuals of greater than 3 or less than -3 were considered outliers and were removed. Effects were declared significant at $P < 0.05$ for main effects and $P < 0.10$ for interactions since interactions are more difficult to detect. Preplanned contrasts were the effect of treatment at each time point, and the Kenward Rogers adjustment for the denominator degrees of freedom was applied to the model. All production data are reported as the median of the milking interval (MI) to represent the mean time of synthesis.

3.4 Results

3.4.1 Production

When cows were milked 4 x/d there was a treatment by time interaction for milk yield ($P = 0.07$; Figure 3.1A). Milk yield was highest for both treatments during the first milking interval (MI) and decreased during the second and third milking intervals. In the fourth MI 1x fed milk yield was 0.7 kg higher than 4x fed ($P < 0.01$). There was no treatment effect on total milk yield over the day ($P = 0.941$; Table 3.4)

There was a tendency for a treatment by time interaction for milk fat concentration ($P = 0.11$; Table 3.4). Milk fat concentration was lowest at the first MI, increased during the second and third MI, and finally decreased during the fourth MI. Overall, 4x fed cows had higher fat concentration at all time points (Figure 3.1C; $P < 0.05$). There was an interaction of treatment and time for milk fat yield with 4x fed cows having higher yields during the first MI and tending to have higher yields during the second MI as fat yield progressively decreased during the day (Figure 3.1B). Once a day feeding had the lowest fat yield in the first MI, peaked during the second MI, and decreased slightly during the third and fourth MI (Figure 3.1B). Daily milk fat yield was 124 g higher and milk fat percent was 0.27 percentage units higher for 1x fed ($P < 0.001$; Table 3.4).

Milk FA profile also varied throughout the day (Table 3.3). There was a treatment by time interaction for the concentration of FA less than 16 carbons with no difference between treatments during the first, third, and fourth MI, but during the second MI the 4x fed had a higher concentration of FA less than 16 carbons ($P < 0.05$; Figure 3.2A). Similarly, there was a treatment by time interaction for the concentration of FA greater than 16 carbons with no

difference between treatments at the first, third and fourth milking intervals, but 1x fed cows had higher preformed fatty acids during the second MI ($P < 0.01$; Figure 3.2B).

There was a main effect of treatment on milk fat *trans*-10 18:1, an indicator of the altered pathway of ruminal FA biohydrogenation. Milk *trans*-10 18:1 was at least 0.40 percentage units higher in 1x fed at all time points ($P < 0.05$; Figure 3.3A). However, there was a treatment by time interaction for *cis*-9, *trans*-11 conjugated linoleic acid (CLA), an intermediate in the normal biohydrogenation pathway. The 1x fed had higher milk fat *cis*-9, *trans*-11 CLA at the second MI, while *cis*-9, *trans*-11 CLA remained consistent throughout the day in 4x fed (Figure 3.3B).

There was a treatment by time interaction for milk protein percent with no difference between treatments during the first MI, but 4x fed cows had higher milk protein concentration in the second, third, and fourth MI (Figure 3.4A). Daily weighted average milk protein concentration was 0.07 percentage units higher in 1x fed cows ($P < 0.001$; Table 3.4). There was a treatment by time interaction for protein yield with the lowest yield for both treatments occurring during the third MI (Figure 3.4B). There was no effect of treatments during the first three MI, and a 25 g increase for 1x fed during the fourth MI. However, total daily milk protein yield was not different between treatments (Table 3.4).

There was a tendency for a treatment by time interaction for ECM with the 4x fed cows tending to produce more ECM during the first milking interval (8.1 vs 7.5 Mcal NE_L; Figure 3.4C; Table 3.4). After the first MI, the 4x fed cows gradually produced less ECM whereas the 1x fed remained more consistent throughout the day.

There appears to be a treatment by milking frequency interaction on dry matter intake, although the study was not designed to test for this interaction (Figure 3.5). There was no effect of treatment on DMI during 2 x/d milking, but during 4 x/d milking the 4x fed progressively increased DMI starting on d 17 ($P < 0.01$; Table 3.4). There was no difference in feed efficiency between treatments during the 4 x/d milking period (Table 3.4).

There was an effect of time for milk yield during 2x milking ($P < 0.01$) with the morning milking higher in both treatments. There was an effect of treatment and time for milk fat concentration and yield ($P < 0.001$), although there was no treatment by time interaction (Table 3.4). The 4x fed had higher concentrations and yields of milk fat than 1x fed and the fat concentration and yield were lower during the morning milking in both treatments. There was a treatment by time interaction for milk protein concentration ($P < 0.01$) with both treatments having higher concentrations during the evening milking (0.1 percentage units; Table 3.4). Overall, 1x fed had higher milk protein concentrations compared to 4x fed (2.99 and 2.93 respectively; Table 3.4). There was a tendency ($P = 0.09$) for an effect of time on milk protein yield during 2x/d milking with higher yields during the morning milking. Lastly, there was an effect of treatment on NeCM during 2x/d milking with 4x fed cows producing 1.3 more kilograms of energy corrected milk than 1x fed ($P < 0.001$; Table 3.4).

3.4.2 Blood

There was a main effect of time, but no effect of treatment or interaction of treatment and time on plasma NEFA. Both treatments peaked at 0600 h, decreased immediately afterwards, and remained low for the rest of the day ($P < 0.001$; Figure 3.6A). There was a treatment by time interaction for BUN with the 1x fed cows peaking at 1000h and the 4x fed cows peaking at 1400h, but there were no difference between treatments at any time points ($P = 0.02$; Figure 3.6B).

There was a treatment by time interaction for plasma glucose ($P = 0.0002$). The 1x fed peaked between 2200 and 0600 h and fell to a nadir at 1000 h whereas 4x fed peaked at 0600 and 1800 h and fell to a nadir at 1400 h (Figure 3.7A). There tended to be a treatment effect on plasma glucose at 0200 h and were different at 1400 and 2200 h. Like glucose, there was a

treatment by time interaction for plasma insulin with 4x fed having higher plasma levels at 0200 and 0700 h and lower plasma levels at 1000 and 1800 h ($P < 0.001$; Figure 3.7B).

3.4.3 Temperature

There was a treatment effect for phase (time of peak) of core body temperature (CBT; $P < 0.05$). Core body temperature of 1x fed peaked around 1400 h whereas 4x fed cows peaked around 1030 h (Table 3.5). Core body temperature was observed during both 2 x/d and 4 x/d milking and a difference was observed in core body temperature phase between milking frequencies ($P < 0.001$). During 2x/d milking CBT peaked at 1500 h, while CBT peaked at 1100h when the cows were milked 4x/d (Table 3.5; Figure C.1, C.2, C.3).

3.5 Discussion

Circadian rhythms are entrained and modified by numerous environmental signals. Possible entraining variables for the dairy cow are the light/dark cycle and the timing of feeding, milking, and other daily management activities. The current experiment was designed to investigate the circadian rhythm of the dairy cow and the effect of the natural pattern of feed intake on the circadian rhythm. The 1x fed treatment allowed cows to consume feed naturally over the entire day with minimal influences. The natural pattern of intake of cows fed 1x per day is expected to be influenced by the timing of feed delivery and milking (DeVries and von Keyserlingk, 2005). A morning feed delivery time was selected based on the typical feeding time on commercial dairy farms. Feeding cows frequently at evenly spaced intervals equalizes intake over the day. Intensive experiments seeking establishment of steady state conditions will commonly use hourly or bi-hourly feed delivery. Feeding every six h in four equal meals was

selected in the current experiment to minimize the disturbance of natural behaviors while still spreading intake over the day. Lastly, the diet composition is similar to that commonly fed on dairy farms in the United States. An interaction of diet composition and circadian rhythms is not known in the dairy cow and represent logical follow-up experiments.

The cows were housed together in a tie stall barn with ten cows on one side and the remaining ten cows directly across the isles. The treatments were randomly assigned to the stalls so treatments were interspersed among each other. This may have initially caused cross social entrainment, especially during the when treatments crossover between periods. However, cows quickly adapted to treatment and by the sampling days the cows were disturbed very little by the feeding of the other treatment. To maintain consistency all feed was mixed at 0800 h. The 0600 h for the 4x fed treatment was mixed the previous day and feed was up to 28 h old by the 1200 h feeding. Feed was compacted in barrels to reduce heating, but some heating of feed was noticed during period 2. The experimental design required the 4x fed cows to be fed in equal meals, but also to be fed ad libitum. If refusals are uneven over the day the treatment is compromised. The ability of the cow to create uneven refusals is dependent on the total daily refusals allowed so care was taken to control the total daily refusals without limiting daily intake. The last environmental confounding factor is that the lights were not controlled by a timer. Because the lights were manually controlled the lights were sometimes left on all night. Also, depending on the preference of the overnight push-up crew, a combination in the number of lights left on changed from night to night. This affected the intensity of light the cows were exposed to and it could vary greatly between locations in the barn. This might have caused issues with the entrainment to the light/dark cycle may explain the variability in the core body temperature.

The 4x fed treatment is also expected to have limited the ability of cows to sort feed during the early part of the day. Cows preferentially sort for short particles (Leonardi and Armentano, 2003) and the opportunity for sorting is dependent on the amount of feed available to

sort. It is reasonable to expect that some of the 1x fed cows consumed not only more feed after feeding, but also feed higher in starch and lower in fiber. Conversely, it is expected that the 1x fed cows consumed feed higher in fiber as the day progressed (Leonardi and Armentano, 2003). The 4x fed would also be expected to sort at each feeding, but the magnitude of sorting would be limited by the amount of feed available. Since the objective of design was to compare uneven nutrient intake to more even nutrient intake, increased sorting in 1x fed cows would benefit this design by further exasperating differences in fermentable organic matter intake over the day.

An interesting interaction was observed where DMI was not different between treatments during 2 x/d milking, but was increased by 4x fed during the 4 x/d milking portions of the experiment. Milking and providing fresh feed appear to stimulate feeding behavior (DeVries and von Keyserlingk, 2005, DeVries et al., 2005), and 4x fed cows received fresh feed after returning from the parlor. It appears that the combination of milking and fresh feed increased intake in 4x fed cows, however they did not increase energy corrected milk. The increased intake is expected to be a short-term effect that would subside over the long-term. On the other hand, the change in intake may be a long-term adaptation to feeding frequency that happened to occur at the time of 4x/d milking.

Observing the daily pattern of milk and component synthesis is not well studied in the lactating dairy cow and is limited by twice daily milking commonly used in research experiments. Milking every six hours allowed a higher resolution of milk synthesis over the day. Milking every 4 h has been reported in the literature, but was not selected due to the increased time away from feed and influence on natural behaviors and rhythms. In addition, cows were milked at the increased frequency for only the last week of each period. The first two weeks allowed diet adaptation and observation without the influence of increased milking. The cows quickly adapted to the increased milking frequency and no major differences were observed in milk and milk component treatment responses during 4x milking.

Dairymen commonly recognized that morning and evening milking differ in milk yield and composition. In a 12 h milking interval, Gilbert et al. (1973) reported an increase of 0.65 kg of milk at the morning milking and an increase in milk fat and protein by 0.32 and 0.09 percentage units during the evening milking, respectively. Milk yield at individual milkings was extensively modeled with the development of AM/PM Dairy Herd Improvement Association (DHIA) sampling method in the 1960's where only one milking per month is sampled (Everett and Wadell, 1970a). The differences between morning and evening milking were found to be dependent on the milking interval and days in milk. For example, there was up to a 1.71 kg more milk at morning milking for cows in early lactation Holstein cows (Everett and Wadell, 1970b). However, difference in milk yield and composition when cows are milked twice per day is highly influenced by the timing of milking, which was not considered.

More recently Quist et al. (2008) conducted a large survey of the milking-to-milking variation in milk yield and composition on 16 dairy farms. Milk yield and milk fat concentration showed a clear repetitive daily pattern over 5 d in herds that were milked 2 and 3 x/d. Surprisingly milk yield was highest and milk fat lowest in the AM milking of herds milked 2 x/d, but milk yield and milk fat concentration was lowest at the AM milking and highest at the night milking of herds milked 3 x/d. The difference in these rhythms may be due to differences in the milking interval or timing of milkings, neither of which were reported. However, their data demonstrated a rhythm of milk and milk fat and a possible effect of milking times. The current experiment demonstrates an effect of time of day on milk and milk fat yield and milk fat and protein concentration in cows milked every 6 h under well-controlled conditions. Additionally, a difference in key interaction of the timing of feed intake is reported. The ability of the 1x fed cows to maintain their natural pattern of feed intake could explain the increase in milk yield during the fourth milking interval. The 1x fed cows had the feed available to consume more DM

prior to the fourth milking interval unlike the 4x fed because the last bout of feeding occurred after the fourth milking interval.

Milk fat synthesis is well known to be responsive to nutrition in the cow. According to the biohydrogenation theory of milk fat depression, specific fatty acid isomers decrease milk fat synthesis in the mammary gland by inhibiting enzymes involved in lipid synthesis (Harvatine et al., 2009). Interpretation of the milk fat response to 4x fed is difficult because of changes in both rumen fermentation and the temporal pattern of nutrient absorption. *Trans*-10 C18:1 is an indicator of the altered pathway of biohydrogenation and has been associated with milk fat depression (Harvatine et al., 2009). Higher concentrations of this isomer were observed at all timepoints in 1x fed which is in agreement with the lower fat percent at all milkings and decreased fat yield. However, it appears that changes in absorption of biohydrogenation intermediates over the day does not explain the circadian pattern because the concentration of *trans*-10 C18:1 fatty acids was reasonably constant in milk over the day. Peak transfer of FA into milk is expected by 4 to 6 h after absorption (Harvatine and Bauman, 2011), so the 6 h resolution of milking would be reasonable to observe differences in *trans*-isomer absorption over the day. Additionally, the decreased *de novo* and increased preformed FA in milk at the second milking interval coincided with increased *cis*-9, *trans*-11 CLA and may indicate a modification of mammary lipid metabolism during this milking interval. Milk *cis*-9, *trans*-11 CLA is predominantly synthesized in the mammary gland by desaturation of *trans*-11 C18:1 by the stearyl CoA desaturase enzyme (Griinari et al. 2000 J Nutr 130:2285). The desaturase enzyme is highly responsive to nutrition and is increased in some cases of diet induced MFD. Additionally, *de novo* FA synthesis is more sensitive to the bioactive FA that cause MFD. Increased *cis*-9, *trans*-11 CLA and decreased *de novo* synthesized FA indicates an additional effect of bioactive FA at the second milking interval. Interestingly, the largest difference between treatments occurred at the first milking interval and the range in milk fat concentrations over the day was

reduced by about half with 4x fed indicating a clear effect of nutrition beyond rumen bioactive FA on the amplitude of the rhythm.

Milk protein is also responsive to nutrition, but the magnitude of change is much smaller than milk fat. Milk protein concentration and yield showed daily patterns that were responsive to the timing of feed intake. This may be partially explained by changes in insulin. Exogenous administration of insulin increases milk protein presumably through stimulation of IGF-1 (Griinari et al., 1997). Increased insulin at 1800 h corresponds to the timing of increased milk protein synthesis during the last MI and continuing into the first MI. The timing of amino acid absorption may also have an impact on milk protein synthesis. Although it has not been directly measured, duodenal flow of microbial protein would be expected to peak later in the day following peak ruminal fermentation and microbial growth.

Energy corrected milk is dependent on milk and milk component yields, so it was reasonable to expect a change over the day since both milk yield and milk fat vary over the day. The pattern occurs due to the higher fat content of the milk for the 4x fed cows during the first MI and the drop in milk yield and protein during the fourth MI. The 1x cows produced higher milk yields and protein yields during the fourth MI, which accounts for the gradual increase in ECM over the day.

Plasma NEFA is commonly observed to peak immediately before feeding in experiments when cows are fed in the morning. Nikkhah et al. (2008) reported peak NEFA before feeding in cows fed at 0900 h and peak NEFA 6 to 8 h before feeding in cows fed at 2100 h. In the current experiment NEFA peaked in the morning in both the 1x and 4x fed suggesting that NEFA is tightly regulated by a different mechanism than the timing of nutrient availability. Plasma NEFA are an indicator of lipid mobilization from adipose tissue and are quickly cleared from the blood making them a sensitive indicator of temporal changes. Therefore, the daily pattern of fat

mobilization appears to be explained by its association with the circadian system similar to that observed in rodents (Froy, 2011) and in the cow is not as tightly associated with feeding as commonly thought.

The ruminant absorbs little glucose and conducts constant gluconeogenesis. Changes in plasma glucose indicate either changes in the rate of gluconeogenesis or rate of plasma glucose clearance. Since the daily pattern of glucose was modified by 4 x fed, this indicates possible changes in propionate availability or circadian regulation of gluconeogenesis or glucose disposal. It has been recently reported that the pancreas has a functioning clock regulating insulin production thus providing a link between circadian rhythms and regulation of plasma glucose (Marcheva et al., 2011). The pattern of insulin was modified by feeding schedule, but the changes in insulin do not correspond to the expected changes in plasma glucose. The ruminant absorbs ammonia through the rumen epithelium and the rate is increased when rumen ammonia is high. Rumen ammonia is high when the rate of protein degradation is greater than the ammonia use by the microbes. The change in BUN over the day may indicate changes in the amount of protein degraded over the day presumably due to changes in the rumen degradable protein pool or microbial proteolysis capacity (Reynolds and Kristensen, 2008).

A circadian pattern of CBT is conserved among most species. It is commonly used in circadian biology as a marker for the intrinsic circadian rhythm (Refinetti, 2010a). The differences in the phase of CBT indicate differences in the phase and amplitude of the core circadian rhythm. Plotting the normalized fitted curve shows variability in both treatments which could skew the phase. There may have been a lack of continuity with the lighting schedule, so this could affect the phase of the core circadian rhythm. Depending on the position in the barn, some cows could have been exposed to higher light intensity than other which would explain the variability in the phases (Altimus et al., 2010). Drinking bouts have also been shown to noticeably decrease rumen and reticular temperatures, and more importantly takes the rumen or

reticulum anywhere from 20 to 120 minutes to recover from the temperature decrease (Simmons et al., 1965). Rectal temperature, on the other hand, does not seem to be affected by drinking bouts, which gives an indication that vaginal temperature also should not be affected by drinking bouts (Bewley et al., 2008).

3.6 Conclusion

There is a pattern to milk yield and milk component synthesis that is influenced by the timing of feed intake. Altering only the timing of feed intake changed the overall yield of both milk and milk components. This could prove important to the industry because there was no extra cost to the ration. Both treatments followed the same basic pattern in temporal milk production and plasma metabolites and the timing of feed intake altered the amplitudes of the rhythm which may be an indication of an underlying circadian pattern responsive to non-feeding factors. More research will need to be done to understand the natural rhythm of the mammary gland and the entrainment by feeding. Less labor-intensive strategies such as the time of the day to feed once a day may have similar impacts. These discoveries highlight practical opportunity to improve rumen fermentation, cow health, and farm efficiency.

Table 3.1 Ingredient and nutritional composition of experimental diet

	Composition, % DM	SEM
Dietary Ingredients		
Corn silage	42.3	
Alfalfa haylage	13.3	
Ground corn	10.8	
Soybeans	7.5	
Canola meal	6.6	
Cookie meal	7.4	
Sugar	4.2	
Grass/Straw Hay	4.4	
Min-Vit Mix ¹	3.1	
Optigen ²	0.4	
Nutrient		
NDF	31.8	0.24
ADF	21.0	0.29
CP	16.1	0.13
NFC ³	42.3	0.40
Ash	7.1	0.29
TDN ⁴ (% of DM)	32.8	0.18
Ne _L ⁵ (Mcal/lb)	0.34	0.00

¹ Contained: 11% CP, 18% NDF, 5.1% fat, 14% Ca, 0.35% P, 4.6% Mg, 0.42% K, 0.3% S, 1,071 ppm Mn, 357 ppm Cu, 1,085 ppm Zn, 6.66 ppm Se, 6.4% salt (DM basis), 262,101 IU vitamin A, 65,421 IU/kg vitamin D, and 1,972 IU/kg vitamin E (DM basis).

² Nonprotein N from Alltech Inc. Nicholasville, KY (243% CP, DM basis).

³ Non fiber carbohydrates = 100 – (CP + NDF + EE + ASH-NDIP)

⁴ Calculated by Cumberland Valley Analytical Services; % TDN = 93.53 – (1.03 x ADF)

⁵ Calculated by Cumberland Valley Analytical Services; Ne_L = (TDN x 0.0234) – 0.5448

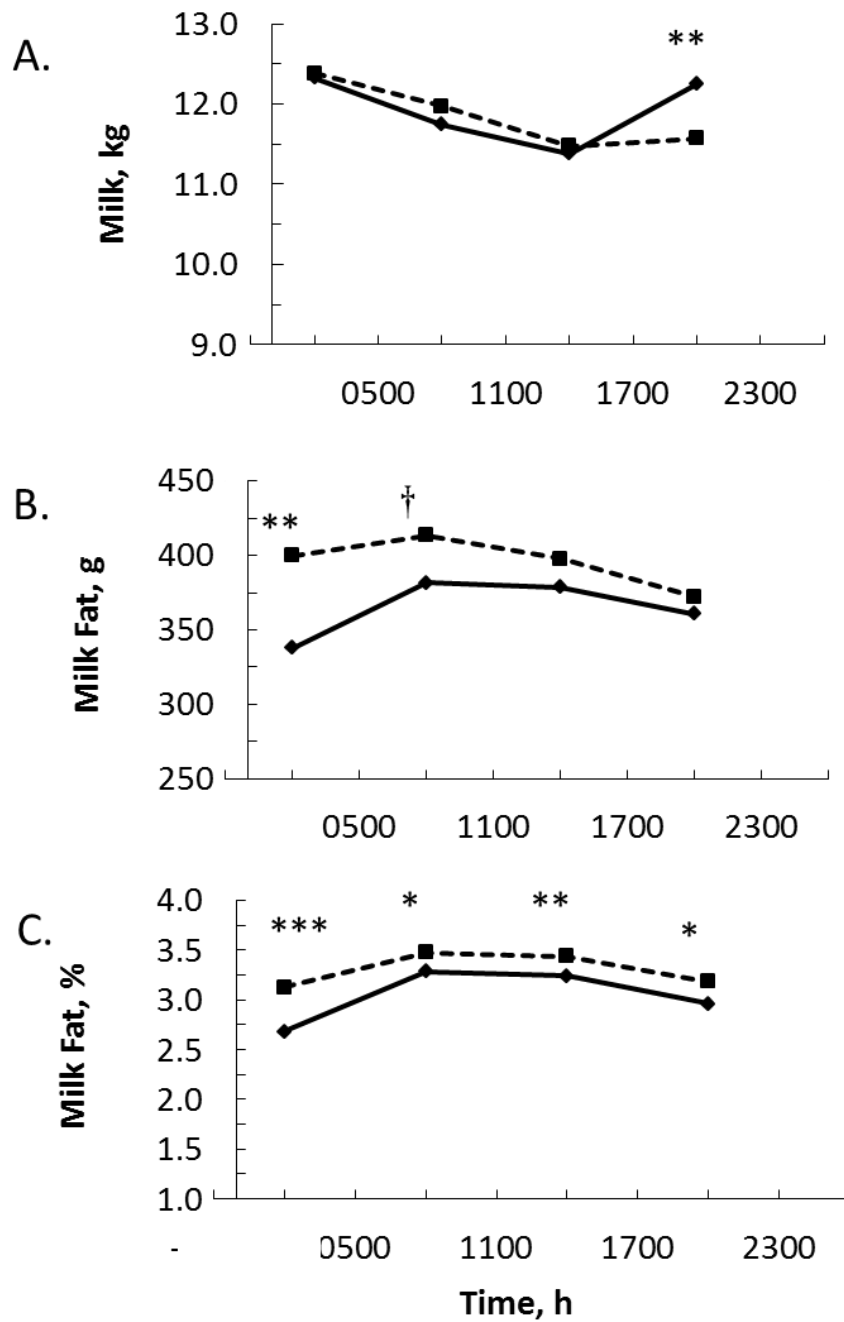


Figure 3.1 Temporal Pattern of Milk Yield and Milk Fat Yield Percent

Temporal pattern of milk yield and milk fat yield and percent in cows fed once per day (solid line) or in four equal meals every six h (dashed line). Cows were milked every 6 h and data is plotted as the mean of the milking interval. Panel A: Milk yield (Treatment x Time $P = 0.07$), Panel B: Milk fat yield (Treatment x Time $P < 0.001$), and Panel C: Milk fat percent (Treatment $P < 0.001$, Time $P < 0.001$, Treatment x Time $P = 0.11$). Preplanned contrasts tested the effect of treatment at each milking ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

Table 3.2 Milk fatty acid profile of cows fed once per day or in four equal meals every six hours.

Milk	1x Fed ¹	4x Fed	SEM	P-value		
	g/d			Trt	Time	Trt*Time
4:0	3.72	3.96	0.11	<0.01	0.32	0.93
6:0	2.24	2.39	0.08	<0.01	0.39	0.35
8:0	1.23	1.29	0.05	<0.05	0.05	0.03
10:0	2.90	2.97	0.12	0.37	0.01	<0.01
12:0	3.42	3.41	0.11	0.87	<0.01	<0.001
14:0	11.21	11.29	0.18	0.50	0.01	<0.01
14:1	1.23	1.11	0.18	0.19	0.61	0.31
15:0	1.20	1.08	0.07	<0.01	<0.001	0.54
16:0	28.10	28.58	0.56	0.10	0.034	<0.001
16:1	1.47	1.43	0.10	0.57	0.97	0.24
17:0	0.52	0.49	0.01	<0.01	0.42	0.26
18:0	8.97	9.34	0.59	0.21	<0.01	0.032
18:1 t4	0.01	0.01	0.01	0.76	0.01	0.77
18:1 t5	0.01	0.01	ND ²	0.56	0.03	0.48
18:1 t6-8	0.47	0.41	0.03	<0.001	0.46	0.03
18:1 t9	0.34	0.31	0.01	<0.001	0.53	<0.01
18:1 t10	1.27	0.80	0.31	<0.01	0.50	0.13
18:1 t11	1.15	1.13	0.06	0.78	0.25	0.30
18:1 t12	0.65	0.60	0.02	<0.01	<0.05	0.07
18:1 c9	18.23	18.30	0.48	0.82	<0.01	<0.001
LA ³	3.33	3.25	0.14	0.11	<0.01	<0.01
20:0	0.11	0.11	0.01	0.42	0.06	0.05
LN ⁴	0.53	0.53	0.02	0.82	<0.01	0.03
CLA 9,11 ⁵	0.66	0.64	0.04	0.24	0.01	<0.01
CLA 10,12 ⁶	ND	ND	ND	ND	ND	ND
Fatty acids by source ⁷						
< 16 Carbons	36.27	35.95	0.68	0.41	0.020	<0.01
16 Carbons	29.67	30.09	0.59	0.17	<0.01	<0.001
> 16 Carbons	27.16	27.43	0.49	0.36	<0.001	<0.001
Desaturase Indexes ⁸						
C14 Index	0.10	0.09	0.01	0.16	0.15	0.09
C16 Index	0.05	0.05	0.00	0.20	0.33	<0.01
CLA Index	0.38	0.36	0.36	0.07	<0.01	<0.001

¹ LS means for the treatment of cows fed once a day (1x Fed) or in four equal meals every six hours (4x Fed)

² ND = below the level of detection (< 0.01 g/d)

³ LA = Linoleic Acid

⁴ LN = alpha Linolenic Acid

⁵ CLA 9,11 = cis-9, trans-11 conjugated linoleic acid

⁶ CLA 10,12 = trans-10, cis-12 conjugated linoleic acid

⁷ Fatty acids < 16 carbons originate from mammary de novo synthesis, fatty acids > 16 carbons originate from extraction from plasma, and 16 carbon fatty acids originate from both sources.

⁸ C14 index calculated as C14:1 / (C14:0 + C14:1), C16 Index calculated as C16:1 / (C16:0 + C16:1), CLA index calculated as cis-9 C18:1 / (C18:0 + cis-9 C18:1)

Table 3.3 Milk fatty acid yields of cows fed once per day or in four equal meals every six hours.

Milk	1x Fed ¹	4x Fed	SEM	P-value		
	g/100g			Trt	Time	Trt*Time
4:0	26.93	31.26	3.25	0.01	0.27	0.61
6:0	16.28	18.94	1.86	<0.01	0.15	0.82
8:0	8.97	10.20	1.00	0.01	0.38	0.75
10:0	21.19	23.25	2.17	0.02	0.27	0.65
12:0	24.75	26.73	2.15	0.09	0.17	0.56
14:0	80.45	89.00	7.24	0.12	0.20	0.69
14:1	8.27	8.51	0.63	0.04	0.81	0.12
15:0	8.30	8.28	0.81	0.63	0.05	0.60
16:0	201.15	224.17	22.37	0.96	0.12	0.73
16:1	10.68	11.07	0.86	0.03	0.37	0.09
17:0	3.66	3.78	0.35	0.57	0.22	0.48
18:0	64.76	73.38	8.61	0.06	0.35	0.81
18:1 t4	ND ²	ND	ND	ND	ND	ND
18:1 t5	ND	ND	ND	ND	ND	ND
18:1 t6-8	3.24	3.17	0.16	0.68	0.47	0.14
18:1 t9	2.41	2.37	0.15	0.78	0.37	0.09
18:1 t10	8.00	5.90	0.99	<0.01	0.32	0.02
18:1 t11	8.30	8.98	1.12	0.27	0.68	0.48
18:1 t12	4.59	5.00	0.29	0.02	0.36	0.06
18:1 c9	128.96	139.74	12.30	0.12	0.19	0.04
LA ³	23.06	25.60	1.31	0.04	0.34	0.05
20:0	0.78	0.87	0.10	0.06	0.15	0.86
LN ⁴	3.80	4.10	0.23	0.14	0.11	0.16
CLA 9,11 ⁵	4.67	4.87	0.51	0.56	0.19	0.03
CLA 10,12 ⁶	ND	ND	ND	ND	ND	ND
Fatty acids by source ⁷						
< 16 Carbons	192.80	213.13	17.34	0.05	0.20	0.74
16 Carbons	211.87	236.72	20.86	0.03	0.15	0.72
> 16 Carbons	251.89	268.77	23.87	0.21	0.33	0.17
Desaturase Indexes ⁸						
C14 Index	0.09	0.09	0.01	0.35	0.08	0.04
C16 Index	0.05	0.05	0.00	0.35	0.41	<0.01
CLA Index	0.51	0.50	0.01	0.70	0.14	0.07

¹ LS means for the treatment of cows fed once a day (1x Fed) or in four equal meals every six hours (4x Fed)

² ND = below the level of detection (< 0.005 %)

³ LA = Linoleic Acid

⁴ LN = alpha Linolenic Acid

⁵ CLA 9,11 = *cis*-9, *trans*-11 conjugated linoleic acid

⁶ CLA 10,12 = *trans*-10, *cis*-12 conjugated linoleic acid

⁷ Fatty acids < 16 carbons originate from mammary de novo synthesis, fatty acids > 16 carbons originate from extraction from plasma, and 16 carbon fatty acids originate from both sources.

⁸ C14 index calculated as C14:1 / (C14:0 + C14:1), C16 Index calculated as C16:1 / (C16:0 + C16:1), CLA index calculated as *cis*-9 C18:1 / (C18:0 + *cis*-9 C18:1)

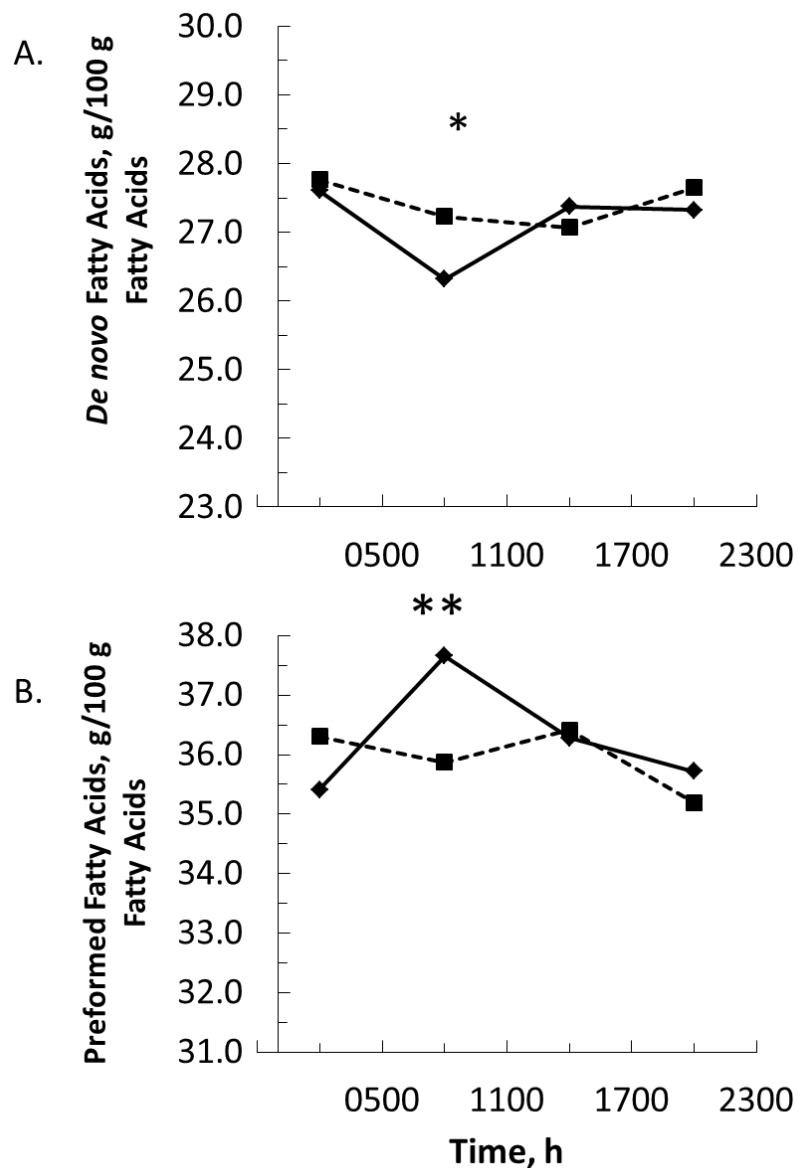


Figure 3.2 Temporal Pattern in *de novo* and Preformed Fatty Acids

Temporal pattern of concentrations *de novo* and preformed fatty acids in milk fat of cows fed once per day (solid line) or in four equal meals every six h (dashed line). Cows were milked every 6 h and data is plotted as the mean of the milking interval. Panel A: *De novo* Fatty Acids are the sum of fatty acids less than 16 carbons (Treatment x Time $P = 0.004$), and Panel B: Preformed Fatty Acids are the sum of fatty acids greater than 16 carbons (Treatment x Time $P < 0.001$). Preplanned contrasts tested the effect of treatment at each milking ($\dagger P < 0.10$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

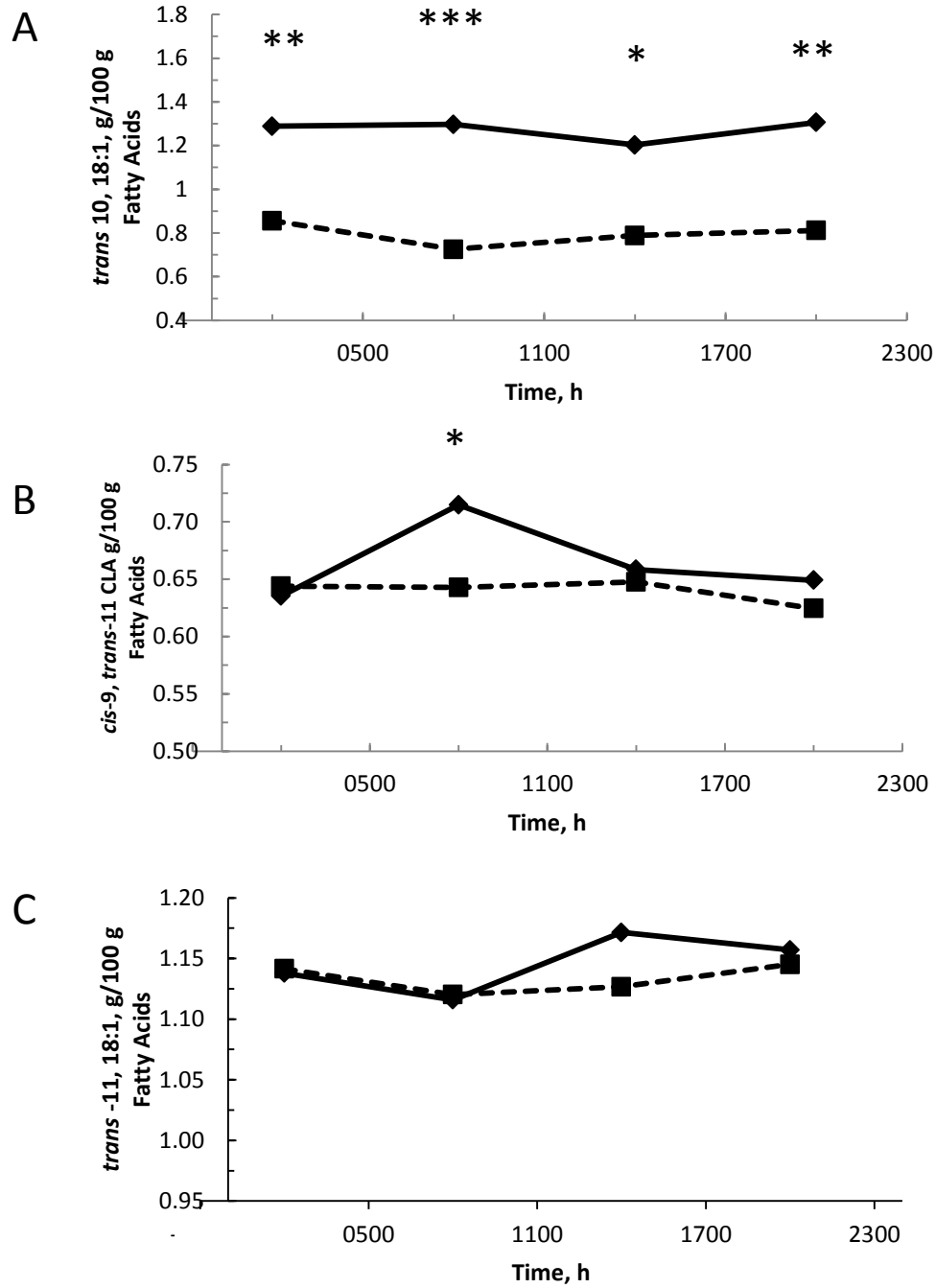


Figure 3.3 Temporal Patterns of Milk Trans Fatty Acid Concentration

Temporal pattern of selected milk fat trans fatty acids concentration in cows fed once per day (solid line) or in four equal meals every six h (dashed line). Cows were milked every 6 h and data is plotted as the mean of the milking interval. Panel A: *Trans*-10, 18:1 (Treatment x Time $P < 0.13$), Panel B: *cis*-9, *trans*-11 CLA (Treatment x Time $P < 0.01$), and Panel C: *trans*-11, 18:1 (Treatment $P = 0.78$, Time $P = 0.25$, Treatment x time $P = 0.30$). Preplanned contrasts tested the effect of treatment at each milking ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

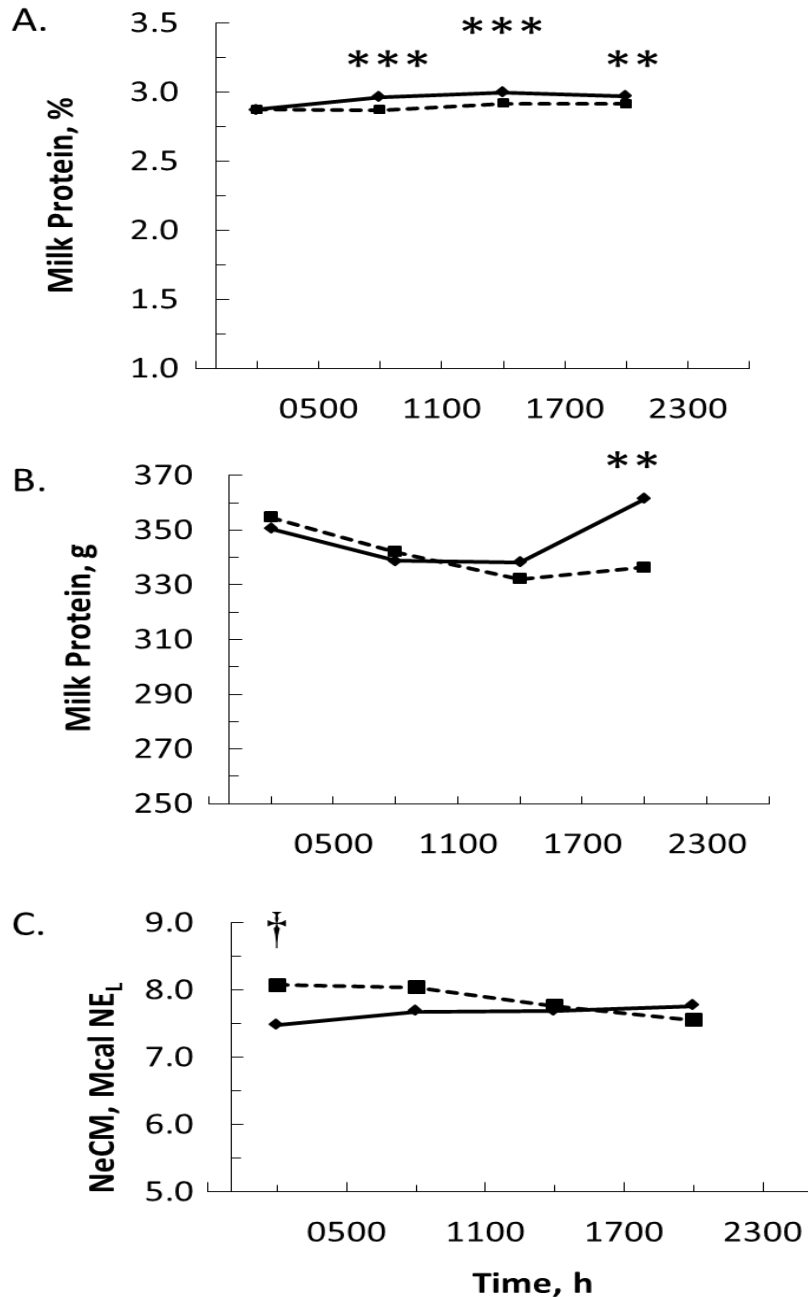


Figure 3.4 Temporal Pattern of Milk Protein and NeCM

Temporal pattern of milk protein yield and concentration and energy correct milk (NeCM) in cows fed once per d (solid line) or in four equal meals every six h (dashed line). Cows were milked every 6 h and data is plotted as the mean of the milking interval. Panel A: Milk protein percent (Treatment x Time $P < 0.001$), Panel B: Milk protein yield (Treatment x Time $P < 0.001$), and Panel C: NeCM (Treatment x Time $P = 0.14$). Preplanned contrasts tested the effect of treatment at each milking ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

Table 3.4 Daily milk production and daily intakes in cows fed once per day (1x Fed) or in four equal meals every six h (4x Fed) and milked every six h.

Parameter	Treatment		SEM	P-values		
	1x Fed ¹	4x Fed		trt	time	trt*time
During 4x/d milking (d 17-21)						
Milk Yield, Kg	47.7	47.4	1.5	0.57	<0.001	0.072
Fat, %	3.04	3.31	0.15	<0.001	<0.001	0.11
Fat Yield, g/d	1460	1584	320	<0.001	0.02	<0.001
Protein, %	2.95	2.89	1.07	<0.001	<0.001	<0.001
Protein Yield, g/d	1388	1364	0.05	0.14	0.01	0.05
NeCM ² , Mcal NE _L	30.6	31.4	1.2	0.07	0.67	0.14
DMI, kg	28.2	30.4	1.0	<0.01	-	-
During 2x/d milking (d 10-14)						
Milk Yield, Kg	45.65	45.59	1.24	0.94	<0.01	0.55
Fat, %	2.98	3.28	0.16	<0.001	<0.001	0.39
Fat Yield, g/d	1351	1491	0	<0.001	<0.001	0.97
Protein, %	2.99	2.93	0.03	<0.001	<0.001	0.01
Protein Yield, g/d	1362	1335	0	0.23	0.09	0.99
NeCM ³ , Mcal NE _L	41.50	44.03	2.15	<0.001	0.14	0.59
DMI, kg	27.8	28.2	1.8	0.54	-	-

¹LS means for the treatment of cows fed once a day (1x Fed) or in four equal meals every six hours (4x Fed)

²Energy corrected milk

³Calculated as DMI/ECM

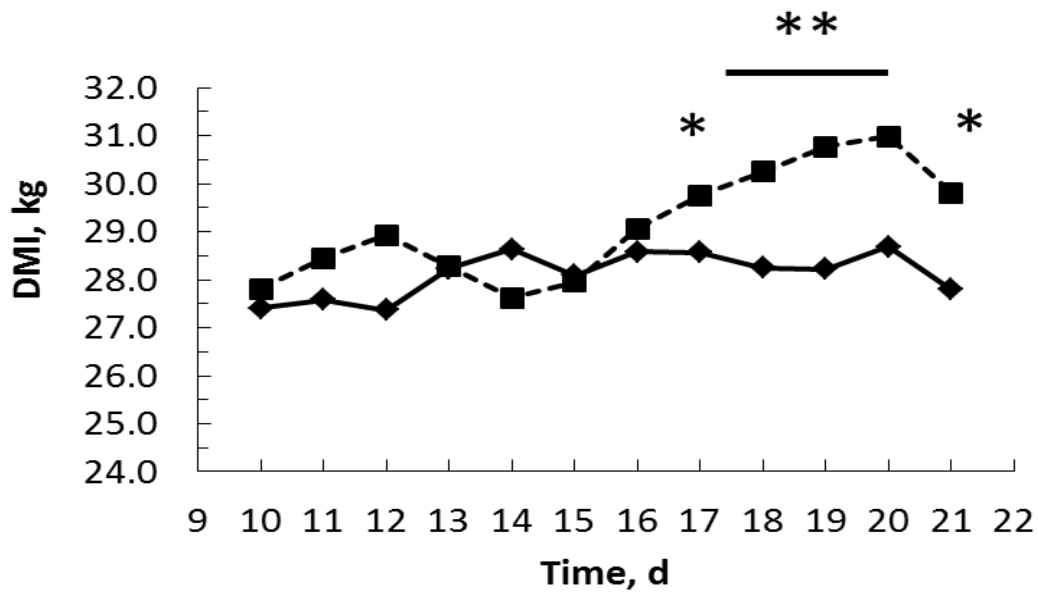


Figure 3.5 Daily Dry Matter Intake for Cows Milked Twice a Day and Four Times a Day and Fed Once a Day or in Four Equal Meals

The dry matter intake in cows fed once per day (solid line) or in four equal meals every six h. Cows were milked every 6 h (dashed line) in days 15 through 21 (Treatment x Day $P < 0.01$) and milked twice a day in days 10 through 14 (Treatment x Day $P = 0.54$). Preplanned contrasts tested the effect of treatment at each milking. († $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

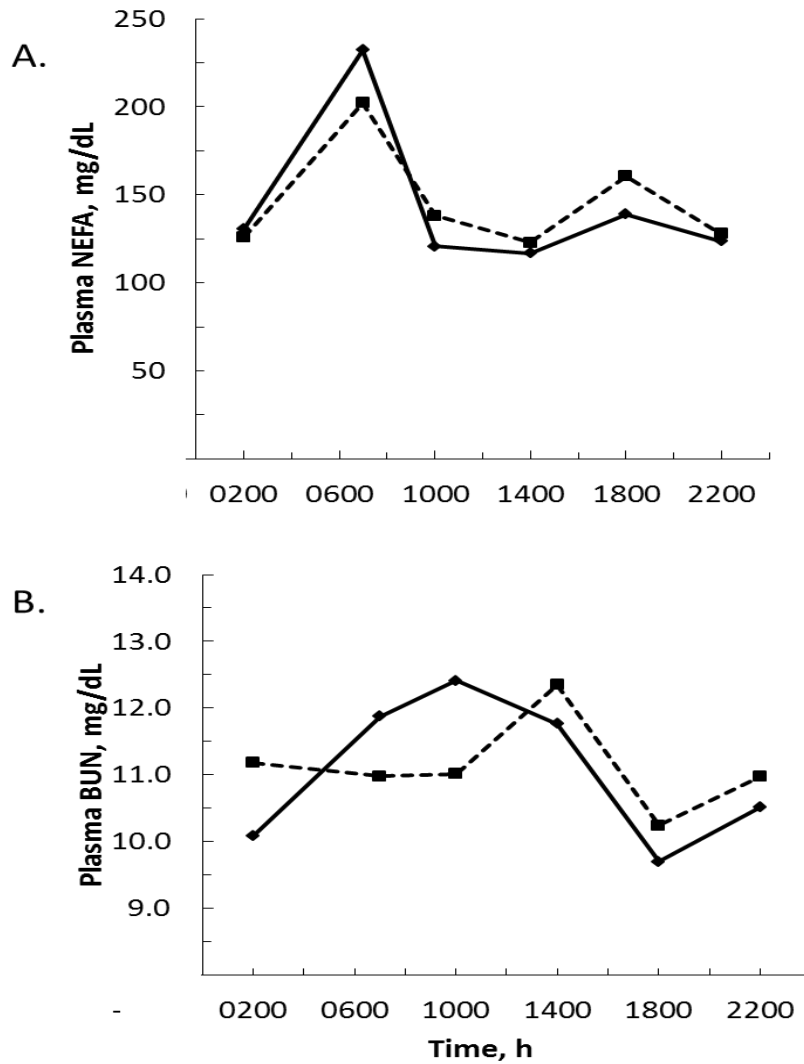


Figure 3.6 Temporal Pattern of Plasma NEFA and BUN

Temporal pattern of plasma concentrations of NEFA and BUN in cows fed once per day (solid line) or in four equal meals every six h (dashed line). Blood was taken every 4 h. Panel A: Plasma NEFA (Treatment $P < 0.81$, Time $P < 0.001$), and Panel B: Plasma BUN (Treatment x Time $P = 0.02$). There was no difference between treatments at any time point. Preplanned contrasts tested the effect of treatment at each milking. ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

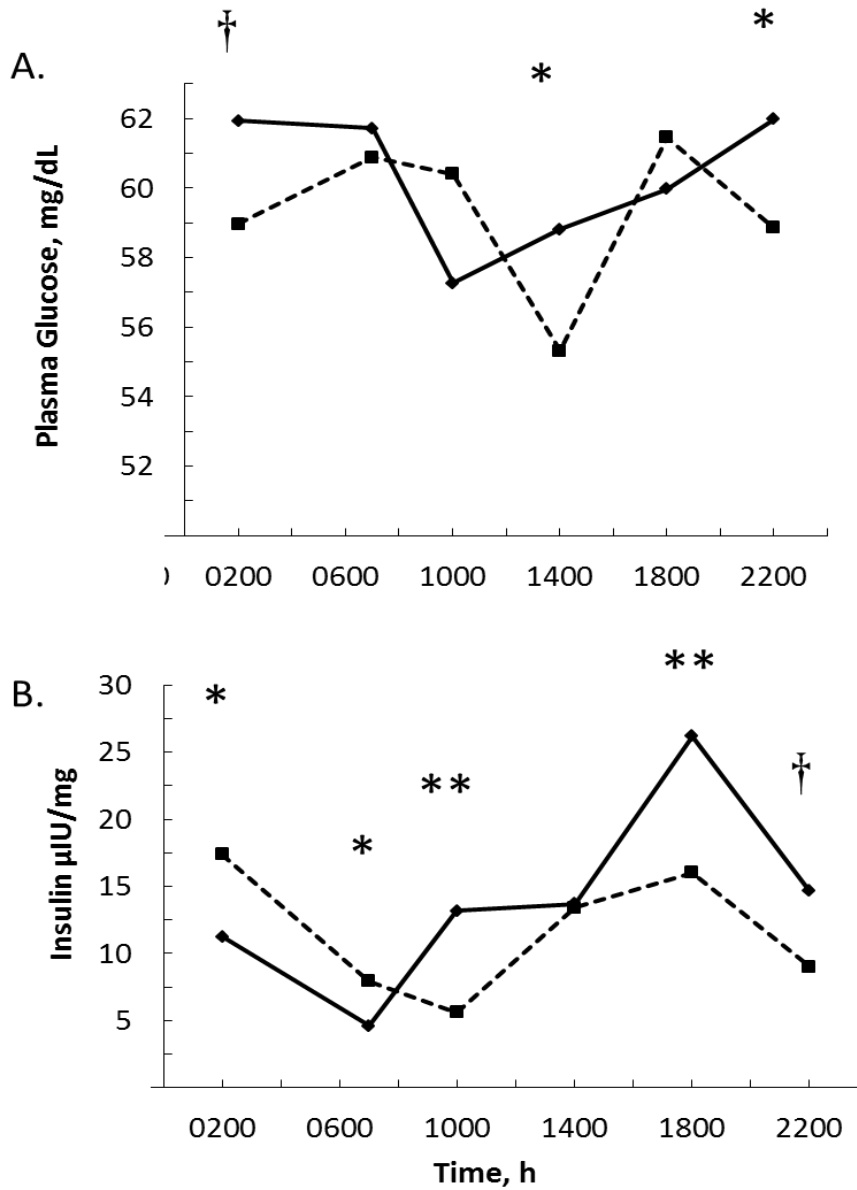


Figure 3.7 Temporal Pattern of Plasma Glucose and Insulin

Temporal pattern of plasma concentrations of glucose and insulin in cows fed once per day (solid line) or in four equal meals every six h (dashed line). Blood was collected every 4 h. Panel A: Plasma glucose (Treatment x Time $P = 0.002$), and Panel B: Plasma insulin (Treatment x Time $P < 0.001$). Preplanned contrasts tested the effect of treatment at each milking. ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

Table 3.5 The phase of daily core body temperature in cows fed once per day (1x Fed) or in four equal meals every six h (4x Fed) and during twice a day milking (2x milk) and four time a day milking (4x Milk).

Trt ¹	Phase (h) ²	SEM	<i>P</i> -value
Feeding Frequency			
1x Fed	1400	2.80	0.05
4x Fed	1030	2.80	
Milking Frequency ³			
2x Milk	1500	0.70	<0.001
4x Milk	1100	0.70	

¹ LS means for the treatment of cows fed once a day (1x Fed) or in four equal meals every six hours (4x Fed)

² Phase of temperature corresponding to time of day as given through Circwave

³All cows were milked either twice a day (2x milk) or in four equal milking intervals (4x milk) as explained in materials and methods

Chapter 4

The Effects of Feeding Regimens that Feed Two Rations Over the day on Production and Ruminal Fermentation in Dairy

4.1 Abstract

There is circadian pattern of feed intake and milk synthesis in the dairy cow and feeding a single total mixed ration over the day may not synchronize ruminal fermentation, nutrient absorption, and milk synthesis. The object of this study was to determine if feeding multiple TMRs over the day that complement the pattern of feed intake would stabilize ruminal fermentation and synchronize nutrient absorption with milk synthesis. Nine ruminally cannulated cows were used in a 3 x 3 Latin Square design with 23 d periods. Diets were a control (Con; 30.1% NDF), a low forage diet (L; 26.9% NDF), and a high fiber diet (H; 31.8% NDF). The L and H diets were balanced to provide the same nutrient composition as the Con diet when cows were fed three parts of L and 7 part of H. The control treatment (Con) was fed at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. All treatments were fed at 110% of daily intake. Preplanned contrasts compared Con to HL and HL to LH. DMI was decreased 1.9 kg by HL compared to Con ($P < 0.01$), but intake did not differ between HL and LH. There was no difference between Con and HL for milk yield and composition, but LH tended to reduce milk fat yield compared to HL ($P = 0.06$). There was no effect of treatment on milk *trans*-10 C18:1, *trans*-11 C18:1, and fatty acids less than 16 carbons. There was no difference between treatments in empty body weight gain, plasma insulin, glucose, and NEFA, and rumen VFA concentration.

There was an effect of time with regards to plasma metabolites ($P < 0.001$) and VFA production ($P < 0.001$). There was a tendency for a treatment by time interaction for rumen volume ($P < 0.09$) with LH having a larger rumen pool than Con and tending to contain more HL. Feeding multiple rations over the day decreased intake without decreasing milk or body weight gain. Feeding multiple rations over the day reduced intake with no impact on milk yield or body weight gain, but had little impact on other production and rumen parameters.

4.2 Introduction

Total mixed rations (TMRs) are used to minimize sorting, supply a constant composition of nutrients to the rumen, and minimize the detrimental effects of high starch diets (Coppock et al., 1981). Typically, the same TMR recipe is fed one to three times per day. The environmental factors influencing feeding behavior have recently been investigated including the frequency and timing of feed delivery, stocking density, and cow parity (von Keyserlingk and Weary, 2010). Increasing the number of times a TMR is fed is associated with stimulating feed intake and altering its circadian pattern (DeVries et al., 2003). Furthermore, the timing of nutrient intake is at the center of the issue of sorting where cows tend to consume a more fermentable diet for the first part of the day and a less fermentable diet the later part of the day. We propose that the high intake period of the day results in a similar dynamic in fermentable carbohydrate intake and that feeding multiple TMRs of different composition over the day may be beneficial.

Cattle have naturally occurring patterns of feed intake with higher rates of intake occurring around dawn and dusk (Albright, 1993). These varying rates of intake cause fluctuations in the rate of nutrients entering the rumen and change the composition of rumen digesta (Allen, 1997). Nutrient digestion rates vary depending on the concentration and overall

composition of nutrients within a given diet (Firkins et al., 1998), and thus may vary over the day with changes in rumen digesta composition. Feed intake increases passage rates and may modify the overall ruminal performance (Girard, 1990). This directly contradicts the steady state environment assumed by many nutritionists and when modeling digestion kinetics (Mertens, 1987). Taken together, the natural and/or management influenced circadian pattern of feed intake, feed delivery, and sorting create a pattern of nutrient intake that is not consistent throughout the day and should be taken into account when formulating rations.

The timing and length of milking intervals often confounds observing a daily pattern of milk synthesis that is dependent of the timing of feed intake has been observed (Chapter 3). Generally, higher milk yields are observed in the morning compared to the evening, while fat percent is higher in the evening compared to the morning (Everett and Wadell, 1970a, Gilbert et al., 1973). The circadian pattern to milk synthesis and the diurnal pattern of feed intake may create a portion of the day that milk synthesis is limited due to the lack of nutrients available to the mammary gland (Figure 4.1).

We propose that the natural pattern of feed intake creates a large variation in the amount of fermentable carbohydrate entering the rumen over the day. This may not be ideal for stable rumen fermentation and maximal milk synthesis over the entire day. Our hypothesis is that feeding a higher fiber diet during the high intake period of the day and a lower fiber diet during the low intake period of the day will stabilize ruminal fermentation and availability of nutrients for milk synthesis. The objective of this experiment was to characterize the intake, milk production, and rumen response to feeding two TMRs over the day that differ in their forage to concentrate ratio.

4.3 Materials and Methods

4.3.1 *Animals and Experimental Design*

Nine multiparous Holstein cows (158 ± 48 DIM, mean \pm SD) from the Pennsylvania State University Dairy Herd were randomly assigned to one of three treatments in a 3x3 Latin Square design with 23 d periods. Animal care and procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Three diets used were a control (30.1% NDF), a low forage diet (L; 26.9% NDF), and a high fiber diet (H; 31.8% NDF). The L and H diets were balanced to provide the same nutrient composition as the Con diet when cows were fed three parts of L to seven part of H. The control treatment (Con) was fed the control TMR at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. All treatments were offered at 110% of intake. Refused feed was removed before delivery of new feed at each feeding. All cows were milked at 0500 h and 1700 h.

The times were determined by preliminary analysis of feeding behavior observed in a similar automated observation system and manual observation during a previous experiment (Chapter 3). In the previous experiment it was noted that on an ad libitum ration most cows were finished eating before midnight and ate very little from 2400 to 0500h. Therefore we decided to define this as a period of low intake. Additionally, analysis of unpublished feeding behavior data indicated that over 16% of total intake was consumed two hours after feeding. This was then defined as the high intake period of the day. The timing of the first feeding was determined by the farm-feeding schedule. The second feeding time for the LH group was determined from the analysis of previous data with adjustment based on the rate of intake in the first days of the

experiment. The second feeding for the HL group was determined from the observations from Chapter 3.

4.3.2 Milk and TMR Sampling

Milk samples were collected at each milking on d 18 to 20 using an automatic sampler attached to the milking apparatus, preserved with 2-bromo-2-nitropropane-1,3 diol, and analyzed for milk fat and true protein by Dairy One DHIA (State College, PA) using infrared spectrophotometry [(AOAC, 2005); Fossomatic 400; Foss Electric, Hillerød, Denmark]. An additional milk sample was collected at each milking on d 20 and stored at -20 °C until analyzed for fatty acid (FA) profile as previously described (Chapter 3). Orts were weighed and subsampled (12.5%) from d 15 to 17. Subsamples were composited by period. Individual feeds and TMR were collected using the quartering method at the end of Period 3 and stored at -20 °C. The sample amounted to 12.5% of the total feed. Feed and ort samples were dried at 55 °C in a forced air oven, ground through a 1mm screen using a Wiley mill (Arthur A. Thomas Co., Philadelphia, PA).

4.3.3 Blood and Rumen

Indwelling jugular catheters were placed on d 14 and blood samples were collected every nine hours on d 15 to 17 to represent every three hours of the day. The collections occurred at 3000, 0600, 0900, 1200, 1500, 1800, 2100, and 2400 h. Blood was treated with potassium EDTA, immediately placed on ice, centrifuged within 1 h, and plasma stored at -20 °C until further analysis. Plasma samples were analyzed for insulin [(Coat-a-count insulin kit (TKIN5); Siemens Healthcare Diagnostics, Los Angeles CA)], glucose [PGO Enzyme procedure no. P

7119; Sigma-Aldrich;(Raabo and Terkildsen, 1960a)], blood urea nitrogen [BUN; Modified Enzymatic Urea Nitrogen (Procedure No. 2050); Stanbio Laboratory, Boerne TX], and non-esterified fatty acids according to Ballou et al. [(2009); HR Series NEFA-HR(2) kit; Wako Chemicals USA Inc., Richmond Va].

Eight rumen samples were also collected on d 15 through 17 during the same times as the blood. Samples were taken from five different locations in the rumen, pooled, mixed. Rumen contents were squeezed through nylon screen (1-mm pore size). Rumen fluid samples were poured into a sample vial and stored at -20 °C until further analysis. Immediately upon thawing, the rumen fluid was acidified and analyzed for volatile fatty acids (VFA) by gas chromatography (Yang and Varga, 1989). Ruminal contents were completely manually evacuated through the ruminal cannula at 0800 h on d 21, 1500 h on d 22, and 0100 h on d 23. The contents were collected in a large barrel, weighed, and height was determined with a tape measure. The volume was calculated based on calibration with water. Total ruminal content mass and volume were calculated using these weight and volumes.

4.3.4 Rumen pH

Ruminal pH was monitored every ten minutes between d 17 to d 24 by indwelling pH data logger (Model 2xKB5; Kahne Animal Health, Auckland NZ). They were placed centrally in the rumen but migration did occur. The probes were removed every morning prior to the morning milking, cleaned, data retrieved, and recalibrated. A total of five probes were rotated between animals to allow collection of approximately three days per cow per period.

4.3.5. Statistical Analysis

Daily production parameters of intake, milk yield and composition, body weight change, and rumen pH variables were analyzed by the PROC Mixed statement of SAS (SAS Institute 2003). The model included the random effect of cow and period and the fixed effect of treatment. Preplanned contrasts were Con vs. HL and HL vs. LH. Time course response variables including milk production by milking, rumen contents variables, and plasma metabolite and hormones were analyzed using the MIXED procedure of SAS with repeated measures (SAS Institute 2003). Fixed effects were treatment, time, and the interaction of treatment and time. Random effects were cow and period, repeated variables were time and day, and subject was cow x period. Preplanned contrasts were Con vs HL and HL vs LH at each timepoint and the Kenward Rogers adjustment for the denominator degrees of freedom adjustment was used. Studentized residuals of greater than 3 or less than -3 considered outliers and were removed.

4.4 Results and Discussion

4.4.1 Experimental Conditions

The study was designed to test feeding multiple rations over they day. However, to provide an appropriate control that consumed the same nutrient profile, it was essential to feed the L and H rations at a set ratio so that their combination provided the same nutrients as the control. This limited how different that L and H rations could be in their starch and fiber concentration and how much of each could be fed. It is difficult to formulate high and low forage diets without multiple confounding factors. The forage to concentrate ratio was changed by a simple substitution of forage for corn grain while keeping the protein and other concentrate portions of

the diet the same. With any substitution of feed ingredients multiple nutrients and nutrient fractions are affected. The ration was manipulated in manner that would allow adjustment of dietary starch with minimal changes in protein. Substitution of forage for ground corn reduces the effective fiber and substitutes a less fermentable feedstuff with a more fermentable feedstuff. This could increase rate of passage as well as modify microbial protein synthesis. Other possible methods to change diet fermentability would include substituting a more fermentable feedstuff for a less fermentable feedstuff with a similar chemical composition. For example, substituting steam flaked corn for cracked corn would create different rates of fermentation without changing nutrient composition (San Emeterio et al., 2000).

Cows have a naturally occurring daily pattern to feed intake (DeVries et al., 2003) with higher and lower rates of intake over the day (Tolkamp et al., 2011). The highest rate of intake occurs after the morning feeding, but establishment of feeding times required an estimation of when the provided feed would be finished. The high forage portion of the LH group was supposed to be fed after this initial high intake part of the day, but there is a second period of high intake in the evening around milking that occurred after cows have consumed the first 30% of their daily feed. A similar issue arose for determination of feeding times in the HL treatment. Ideally, analyzing feeding behavior data of the cows prior to the beginning of the experiment might have given insight into a more reliable feeding time. Also, looking at purely consumption and rates of intake may not have been the best way to determine when to feed the different rations. A more complete understanding may be gained from modeling rumen nutrient concentration based on the integration of the rate of intake and rates of digestion and passage.

A further complication is that offering fresh feed is a known stimulus of feeding behavior. This makes it impossible to feed multiple rations over the day without modification of the natural pattern of intake. Overall there may have been a more ideal time to feed the other portions of the feeding regimen which also brings into question the impact of the different

feeding times of the two treatments. We did not control for the different feeding times in the treatments, and hindsight suggests we should have stimulated both treatments at all feeding times. This could have been achieved by dumping more of the same ration at the times where the other treatments are being fed the new portion. Also, cows were housed in consecutive stalls and the treatments were randomly assigned to stalls. The natural behavior of the cows in the other treatments may have been disrupted due to the feeding times.

4.4.2 Milk Production

There was an overall effect of milking time (AM vs. PM) on milk yield and composition, but there was no treatment or treatment by milking interactions. Milk yield, fat yield, and protein yield were higher at the morning milking, whereas fat concentration was higher during the evening milking (Table 4.3). This could be explained by the longer interval from the evening to the morning milking since the cows had a longer overnight milking interval, however higher milk yield and lower milk fat percent are normally reported for AM milkings (Quist et al. 2008). Both fat corrected milk (FCM) and energy corrected milk (ECM) yields were higher during the morning milking (Table 4.3). There was a tendency for an effect of treatment on daily fat yield and FCM ($P = 0.07$ for both). The HL cows produced 126 g and 3.1 kg less total fat and FCM than the LH, respectively ($P < 0.02$ and $P < 0.03$; Table 4.3). Due to the tendency for a higher fat yield in LH along with higher milk yields in LH explains the tendency for FCM to be higher for LH. Lower milk fat yield was not expected with HL compared to LH as the HL treatment was expected to stabilize rumen fermentation. It appears that the rumen may be more susceptible to starch during the late evening and less susceptible to starch during the earlier part of the day.

4.4.3 Milk Fatty Acid Profile

There is a tendency for a treatment difference for preformed fatty acids with Con being 1.7 percentage units lower than HL (Table 4.4; $P = 0.02$). In terms of the fatty acid profile, there is a tendency for a treatment by milking interaction for both preformed and *de novo* fatty acids ($P = 0.09$; $P = 0.11$ respectively, data not shown). LH tended to contain 21 grams more preformed fatty acids than HL ($P < 0.08$). Con compared to HL and HL compared to LH both synthesized roughly 18 g more fatty acids in the mammary gland as indicated by *de novo* fatty acid yield ($P = 0.04$ and $P = 0.03$ respectively). The *trans*-10 and *trans*-11 isomers of C18:1, indicators of the biohydrogenation pathway in the rumen, both showed a milking by treatment interaction ($P = 0.01$ and $P < 0.01$). *Trans*-10, C18:1 was higher in Con compared to HL ($P < 0.001$) at the morning milking whereas there was no difference during the evening milking. The same pattern was discovered for *trans*-11, C18:1 with Con having higher yields during the morning milking ($P < 0.001$; Table 4.4). The lack of a milk fat response could be due to the fact that there was an increase in both of the *trans* intermediates of both biohydrogenation pathways which indicates that neither pathway was taking over. This could be an indication that there are times over the day where the concentration of readily fermentable carbohydrates is too high and the alternative biohydrogenation pathway takes over but not long enough to see a milk response before the normal pathway is restored (Harvatine et al., 2009)

4.4.1 Feed Intake and Rumen Pool Size

There was a treatment effect on intake with a higher DMI for the control group than HL ($P < 0.02$) however this did not result in any differences in empty body weight gain ($P < 0.30$; Table 4.2). Body weight observations are difficult in ruminants because of the variation in rumen

fill. Rumen empty body weights decrease this variation and normally provide adequate power to observe body weight changes even with short periods. There was a treatment by time interaction for rumen empty volume ($P < 0.09$; Table 4.6) with no difference in volume at 0100 h and 0800 h, but at 1500 h LH had a greater volume than Con and tended to have a great volume than HL ($P = 0.006$, $P < 0.06$ respectively; Figure 4.2). The volume was lowest in all treatments at 0800 h (Figure 4.2). Rumen pool sizes are normally averaged over the day, but this observation supports the fact that cows have varying rates of intake over the day with the night being one of the lowest intake periods of the day (DeVries et al., 2003). With decreased intake, but continued digestion and passage, the rumen pool size decreases. There were no main effect differences for rumen empty density (Table 4.6; Figure 4.2).

4.4.2 Ruminal Fermentation

There was no treatment effect on the time spent below pH 6.0, 5.8, or 5.6 (Table 4.7). Numerically, the time pH was below 5.6 ranged from 3 to 4 h/d. Rumen pH below 5.5 could be an indication of sub-acute acidosis and could indicate times of the day where fermentation is hindered (Garrett et al., 1999), which is supported by the presence of *trans*-10 C18 an indicator of the altered pathway of biohydrogenation (Harvatine et al., 2009). A daily pattern to ruminal pH has been reported (Beauchemin, 1991, French and Kennelly, 1990, Kononoff and Heinrichs, 2003, Nocek and Braund, 1985, Shabi et al., 1998). It appears that rumen pH is primarily dependent on the rate of intake since LH and LH had minimal impact.

An effect of time was observed on all VFAs with a peak at 1800 h regardless of treatment. There was a slight tendency for a treatment by time interaction for propionate and valerate ($P = 0.15$ and $P = 0.11$; Table 4.7). Briefly, at 0300 h HL had higher concentrations of propionate compared to LH ($P = 0.07$; Figure 4.3). At 0900 h the three treatments reached a

nadir with Con tending to have higher levels of propionate than HL ($P = 0.05$). Propionate concentration increased from the nadir to a peak at 1800 h, similar to the time course of the other VFA. However, at 2100 h HL tended to have lower levels than LH ($P = 0.08$), but was reversed at 2400 h and Con had higher levels of propionate compared to HL ($P = 0.04$) and tended to have higher levels than LH ($P = 0.06$). Acetate followed a similar pattern (Figure 4.3). There was a time affect for the acetate to propionate ratio with a reverse pattern to propionate. The lowest concentrations occurred from 1800 h to 0300 h and the highest values occurred from 0600 h through 1500 h (Figure 4.3). There was effect of time for butyrate with the nadir occurring at 1800 h and peak at 1800 h (Figure 4.4) and valerate followed a similar time course (Figure 4.4). Lastly there was an effect of treatment on branched chain VFA which include isobutyrate and isovalerate ($P < 0.001$; Figure 4.5) and LH had a higher average concentration compared to HL. Daily fluctuations in VFA production indicate that the rumen undergoes patterns of fermentation over the day. This has been supported by a number of other articles (French and Kennelly, 1990, Gustafsson and Palmquist, 1993, Sutton et al., 1988).

4.4.4 Blood

There was an effect of time for plasma glucose with all three treatments reaching a peak at 0600 h, gradually decreasing over the day to a nadir at 1800 h, and rising slightly at 2100 and 2400 h (Figure 4.6; $P < 0.001$). There was a diurnal pattern to plasma insulin ($P < 0.001$) with a nadir occurring at 0900 h and the peak following nine hours later at 1800 h (Figure 4.6).

There was a treatment by time interaction for plasma blood urea nitrogen (BUN; $P = 0.05$; Figure 4.7). The three treatments reached their peaks at 1200 h with HL tending to have higher concentrations than LH ($P = 0.08$). They gradually decreased to reach their nadir between

2400 h and 0300 h with HL having higher concentrations than Con at 1800 h ($P < 0.01$) and 2400 h ($P < 0.001$). HL also had higher concentrations than LH at 2400 h ($P < 0.001$; Table 4.6).

There was an effect of treatment and time ($P < 0.01$ and $P < 0.001$, respectively) for plasma NEFA (Figure 4.7). Con and LH had approximately a 12 and 9 mg/dl increase in average daily concentrations compared to HL (Table 4.8). NEFAs are released from fat stores when energy needs are not met by diet, so higher levels of NEFA could be an indication that HL had less fat mobilization. All three treatments peaked at 0900 h, but the amplitude of that peak was smaller in HL compared to Con and LH. There was another smaller peak at 1800 h for HL whereas Con peaked again at 2100 h and LH at 2400 h. The nadir occurred around 1500 h for all three treatments. This pattern over the day corresponds nicely to the timing of nutrient intake. The greatest peak corresponds to right before the morning feeding, in which all three treatments were fed and the cows are recovering from the lowest intake part of the day. The additional trough for the HL could be explained by the low forage part of the ration being consumed during this lower intake part of the day. The additional peak could be explained by the evening milking by which the rate of milk synthesis would be high after that milking.

4.5 Conclusion

There is a pattern to rumen fermentation with periods over the day where fermentation does not have the nutrients to operate with maximum efficiency. Feeding multiple mini-rations in a specific feeding regimen designed to stabilize fermentation can decrease dry matter intake with no effect on empty body weight or milk and milk component production.

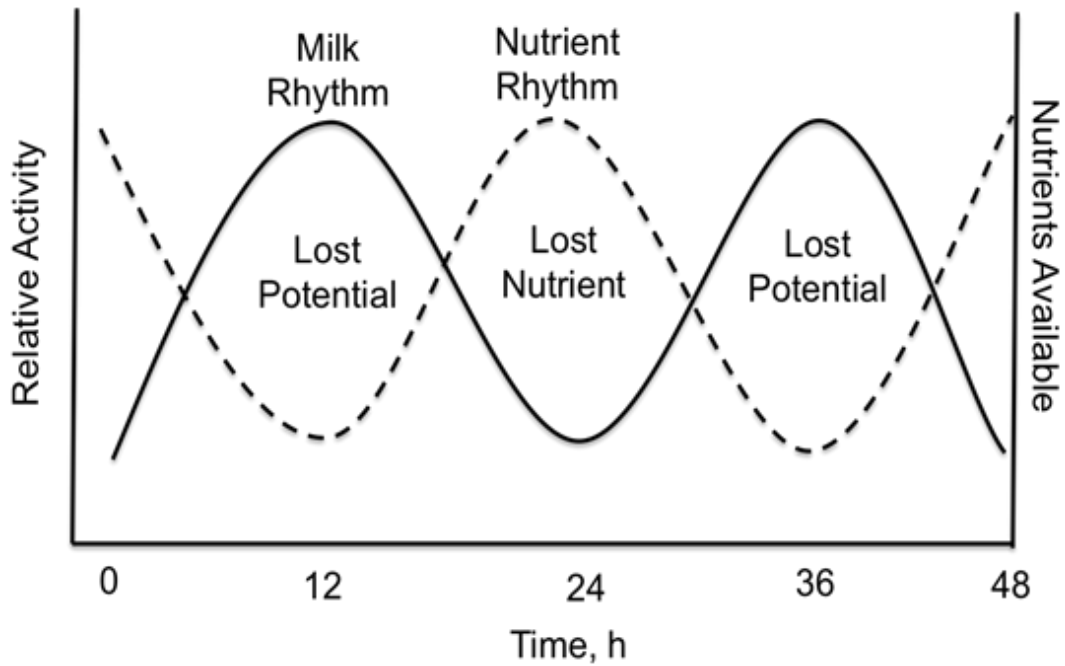


Figure 4.1 Illustration of the impact of unsynchronized rhythms of nutrient absorption and milk synthesis.

The solid line represents the rhythm of milk synthesis and the dotted line represents the rhythm of nutrient availability for milk synthesis. In the extreme case, these rhythms are 180 degrees out of sync. Milk synthesis is most likely limited during those parts of the day where the rhythm is most out of phase from each other, every 12 hours in this example (Modified from Harvatine, unpublished).

Table 4.1 Ingredient and nutritional composition of experimental diet

	Treatment			Stdev.
	Con	L ¹	H	
Dietary Ingredients	% DM			
Corn silage	38.9	32.2	42.5	0.69
Alfalfa haylage	12.6	10.5	13.8	0.22
Ground corn	19.2	29.0	13.8	1.02
Soybeans	7.2	7.2	7.2	-
Canola meal	9.6	9.6	9.6	-
Cookie meal	3.3	3.4	3.3	-
Cottonseed Hulls	1.4	1.5	1.2	0.03
Sugar	N/A	N/A	N/A	-
Grass/Straw Hay	4.2	4.5	3.4	0.07
Mineral and Vitamin Mix ²	3.2	3.2	3.2	-
Optigen ³	0.41	0.41	0.41	-
Nutrients				
NDF	30.1	26.9	31.8	2.49
ADF	20.2	17.7	21.4	1.89
CP	17.5	17.4	17.5	0.06
Starch	33.3	37.8	31.4	3.29
Sol Protein	6.1	5.8	6.2	0.21

¹ L = Low Forage diet and H = High forage diet

² Contained: Contained 11% CP, 18% NDF, 5.1% fat, 14% Ca, 0.35% P, 4.6% Mg, 0.42% K, 0.3% S, 1,071 ppm Mn, 357 ppm Cu, 1,085 ppm Zn, 6.66 ppm Se, 6.4% salt (DM basis), 262,101 IU vitamin A, 65,421 IU/kg vitamin D, and 1,972 IU/kg vitamin E (DM basis).

³ Nonprotein N source from Alltech Inc. Nicholasville, KY (243% CP, DM basis).

⁴ These are theoretical values

Table 4.2 The effect of feeding a single TMR (Con) or feeding two TMR that differ in their forage to concentrate ratio at different times of the day on intake and body weight gain.

Parameter	Treatment			SEM	P-value
	Con ¹	HL	LH		
Dry Matter Intake, kg/d					
Total	26.3	24.5	25.3	1.1	0.01
L Diet	-	8.3 ²	7.3		0.02
H Diet	-	17.0 ³	18.4		0.26
BWC ⁴ , kg/d	11.4	19.5	10.0	7.7	0.30
Feed Efficiency ⁵	1.45	1.52 ^{LH}	1.62	0.14	0.04

¹ The control treatment (Con) was fed at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h.

² T- test using a two-tailed, equal variances model for the difference between the consumption of the low portion of the feeding regimen for the two treatments HL and LH

³ T- test using a two-tailed, equal variances model for the difference between the consumption of the high portion of the feeding regimen for the two treatments HL and LH

⁴ BWC = body weight change

⁵ Calculated as ECM/DMI

^{LH} HL tended to be lower than LH ($P = 0.096$)

Table 4.3 The effect of feeding a single TMR (Con) or feeding two TMR that differ in their forage to concentrate ratio at different times of the day on milk production in dairy cows.

	Treatment				P-values ¹			Daily trt ³
	Con ²	HL	LH	SEM	Trt*MLkg	MLkg	Trt	
Milk Yield, kg								
Daily	39.8	38.7	41.1	2.4				0.14
AM	25.6	24.2	25.2	1.4	0.48	<0.001	0.75	
PM	14.7	15.5	16.0					
Fat Percent, %								
Daily	3.44	3.39	3.45	0.25				0.73
AM	3.01	2.81	2.94	0.28	0.80	<0.001	0.44	
PM	4.19	4.14	4.25					
Fat Yield, kg								
Daily	1.36	1.29 ^{LH}	1.41	0.12				0.07
AM	0.76	0.67	0.74	0.06	0.17	<0.001	0.16	
PM	0.62	0.64	0.68					
Protein Percent, %								
Daily	3.08	3.10	3.10	0.09				0.86
AM	3.07	3.10	3.08	0.10	0.40	0.11	0.88	
PM	3.10	3.10	3.13					
Protein Yield, kg								
Daily	1.23	1.20	1.27	0.07				0.19
AM	0.78	0.75	0.77	0.04	0.45	<0.001	0.68	
PM	0.46	0.48	0.50					
MUN, mg/dL								
Daily	14.94	14.14	14.40	3.88				0.58
AM	11.84	11.22	11.70	2.97	0.80	<0.001	0.65	
PM	12.69	12.40	12.55					
FCM ⁴								
Daily	39.3	37.7 ^{LH}	40.8	2.6				0.07
AM	23.8	22.6	23.4	1.2	0.46	<0.001	0.73	
PM	13.8	14.5	15.0					
ECM ⁵								
Daily	38.7	37.5	40.1	2.3				0.11
AM	24.0	22.5	15.2	1.2	0.29	<0.001	0.46	
PM	15.2	15.9	16.6					

¹ Statistical test of the effect of treatment (trt), milking time (mlkg), and the interaction of treatment and milking time (trt*mlkg).

² The control treatment (Con) was fed at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h.

³ The LSmeans for the daily average or sum of production parameters.

⁴ Fat corrected milk: $(0.4255*MY)+(16.425*((fat\%/100)*MY/2.2))$.

⁵ Energy Corrected Milk: $(0.092*MY+0.0563*protein\%+0.192)*MY$.

^{LH} Contrast between HL and LH (significance at $P < 0.05$)

Table 4.4 The effect of feeding a single TMR (Con) or feeding two TMR that differ in their forage to concentrate ratio at different times of the day on milk fatty acid profile.

	Treatments				P - values		
	C ¹	H	L	SEM	Trt	Time	Trt*Time
Milk	g/100g						
18:1 t10	0.53	0.54	0.53	0.04	0.93	0.75	0.34
18:1 t11	1.22	1.25	1.17	0.11	0.59	0.64	0.25
LA ²	3.57	3.58	3.48	0.22	0.74	0.89	0.64
CLA 9,11	0.69	0.73	0.68	0.08	0.42	0.72	0.42
CLA 10,12	-	-	-	-	-	-	-
c16 + c16.1	27.07	26.44	27.39	1.13	0.20	0.62	0.61
<i>de novo</i> ⁴	27.60	27.46	27.45	0.50	0.94	0.54	0.94
preformed ⁵	37.38	39.15	38.70	0.87	0.06	0.67	0.37
desat 14 ⁶	0.09	0.09	0.09	0.01	0.34	0.40	0.73
desat 16 ⁷	0.04	0.04	0.04	0.00	0.98	0.07	0.49
desat CLA ⁸	0.36	0.37	0.36	0.02	0.76	0.93	0.76

¹The control treatment (Con) was fed at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h.

²LA = Linoleic Acid.

⁴The sum of the fatty acids less than 16 carbons in length.

⁵The sum of the fatty acids greater than 16 carbons in length.

⁶C14 index calculated as C14:1 / (C14:0 + C14:1).

⁷C16 Index calculated as C16:1 / (C16:0 + C16:1).

⁸CLA index calculated as cis-9 C18:1 / (C18:0 + cis-9 C18:1).

Table 4.5 The effect of feeding a single TMR (Con) or feeding two TMR that differ in their forage to concentrate ratio at different times of the day on milk fatty acid yield.

	Treatments				P - values		
	C ¹	H	L	SEM	Trt	Time	Trt*Time
Milk	g/d						
18:1 t10	3.7	3.4	3.7	0.4	0.23	<0.01	0.01
18:1 t11	8.4	8.0	8.3	0.8	0.75	<0.01	<0.001
LA ²	24.4	22.2	24.2	1.5	0.04	<0.001	0.02
CLA 9,11	4.8	4.7	4.8	0.6	0.92	<0.01	<0.01
CLA 10,12	-	-	-	-	-	-	-
c16 + c16.1	191.3	170.1	192.9	21.2	0.02	<0.01	0.24
<i>de novo</i> ⁴	192.1	173.8	192.3	17.2	0.06	<0.001	0.11
preformed ⁵	255.4	246.9	268.6	17.6	0.19	<0.01	0.09
desat 14 ⁶	0.09	0.09	0.09	0.01	0.34	0.40	0.73
desat 16 ⁷	0.04	0.04	0.04	0.00	0.98	0.07	0.49
desat CLA ⁸	0.36	0.37	0.36	0.02	0.76	0.93	0.76

¹ LS means for the treatment of cows fed diet regimens Con, HL, LH

² Linoleic Acid

⁴ The sum of the fatty acids less than 16 carbons in length

⁵ The sum of the fatty acids greater than 16 carbons in length

⁶ C14 index calculated as C14:1 / (C14:0 + C14:1)

⁷ C16 Index calculated as C16:1 / (C16:0 + C16:1)

⁸ CLA index calculated as cis-9 C18:1 / (C18:0 + cis-9 C18:1)

Table 4.6 The effect of feeding a single TMR (Con) or feeding two TMR that differ in their forage to concentrate ratio at different times of the day on rumen empty volume and density.

Time (h)	Treatment				<i>P</i> -value		
	C ¹	HL	LH	SEM	trt	time	trt*time
Volume, L							
800	107	101	109	6.5	0.02	<0.001	0.09
1500	121	128	143	7.6			
2500	127	132	132	6.4			
Density, kg/L							
800	0.85	0.86	0.85	0.02	0.28	0.16	0.96
1500	0.85	0.86	0.85	0.03			
2500	0.83	0.84	0.81	0.02			

¹The control treatment (Con) was fed at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h.

Table 4.7 The effect of feeding a single TMR (Con) or feeding two TMR that differ in their forage to concentrate ratio at different times of the day on rumen volatile fatty acid concentration and profile and rumen pH.

	Treatments				P-values		
	Con ¹	HL	LH	SEM	Trt	Time	Trt*Time
VFA, µM/mL							
Acetate	68.9	68.7	70.5	1.9	0.19	<0.001	0.16
Propionate	26.0	26.3	25.9	1.3	0.82	<0.001	0.12
Butyrate	14.0	13.7	13.8	0.5	0.63	<0.001	0.37
Valerate	2.0	2.0	2.1	0.1	0.17	<0.001	0.07
Branched Chain ²	2.7	2.7	2.9	0.2	0.05	<0.001	0.30
A:P ratio ³	2.7	2.7	2.8	0.2	0.17	<0.001	0.34
Total VFA	113.5	113.1	114.9	2.7	0.60	<.0001	0.10
VFA, Molar Percent							
Acetate	60.9	60.8	61.5	0.9	0.02	<0.001	0.62
Propionate	22.8	23.0	22.4	1.1	0.11	<0.001	0.30
Butyrate	12.3	12.1	11.8	0.4	0.03	<0.001	0.23
Valerate	1.7	1.7	1.8	0.0	0.21	<0.001	0.08
Branched Chain	2.3	2.3	2.4	0.1	0.04	<0.001	0.08
Rumen pH							
h < 6.0 ⁵	10.1	10.9	10.8	1.6	0.84	-	-
h < 5.8	6.1	7.2	6.9	1.4	0.68	-	-
h < 5.6	3.1	4.3	4	1	0.48	-	-

¹ The control treatment (Con) was fed at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h.

² Isobutyrate and isovalerate.

³ A:P ratio is the ratio of acetate to propionate.

⁴ StDev is the LS mean standard deviation of pH.

⁵ LS mean of time in h that pH is below 6.0, 5.8, and 5.6.

^{LH/C} Preplanned contrasts of HL compared to LH or HL compared to C (at least $P < 0.05$).

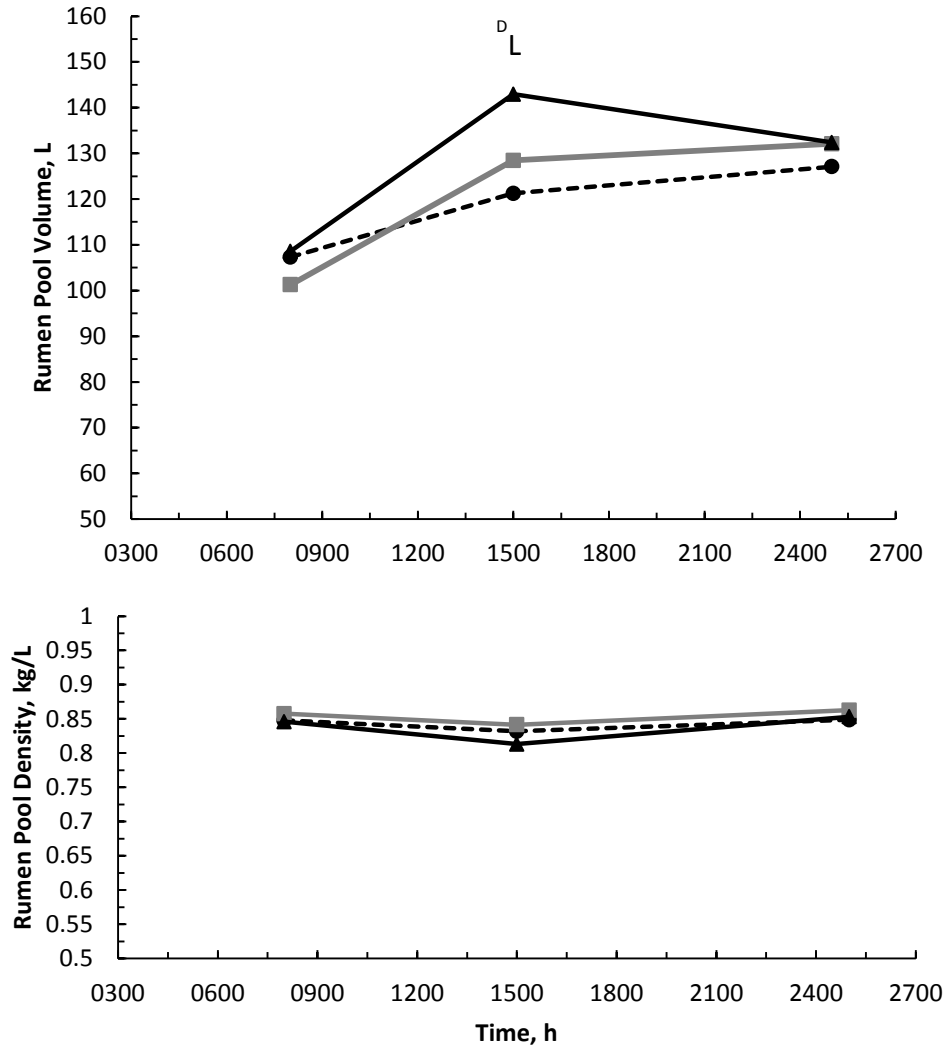


Figure 4.2 Temporal Pattern of Rumen Pool Volume and Density

Temporal pattern of rumen pool volume and density in cows fed one of the three feeding regimens. The control treatment (dotted line,) was fed at 0900 h, the high/low treatment (HL; solid gray line) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH; solid black line) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. Rumen fluid was sampled every 3 h. Top panel: Rumen empty volume (Treatment $P = 0.02$, Time $P < 0.001$, Treatment x Time $P = 0.09$) and bottom panel: Rumen empty density, there were not statistical differences at any time point (Treatment $P = 0.28$, Time $P = 0.16$, Treatment x Time $P = 0.96$). Cows fed Con diet (dotted line), HL diet regimen (solid gray line), and LH diet regimen (solid black line). Preplanned contrasts tested the effect of treatment at sampling time (L denotes the contrast of HL and LH, C denotes the contrast between HL and Con, and the superscripts denotes significant at each level; ^A $P < 0.001$, ^B $P < 0.001$, ^C $P < 0.05$, ^D $P < 0.1$).

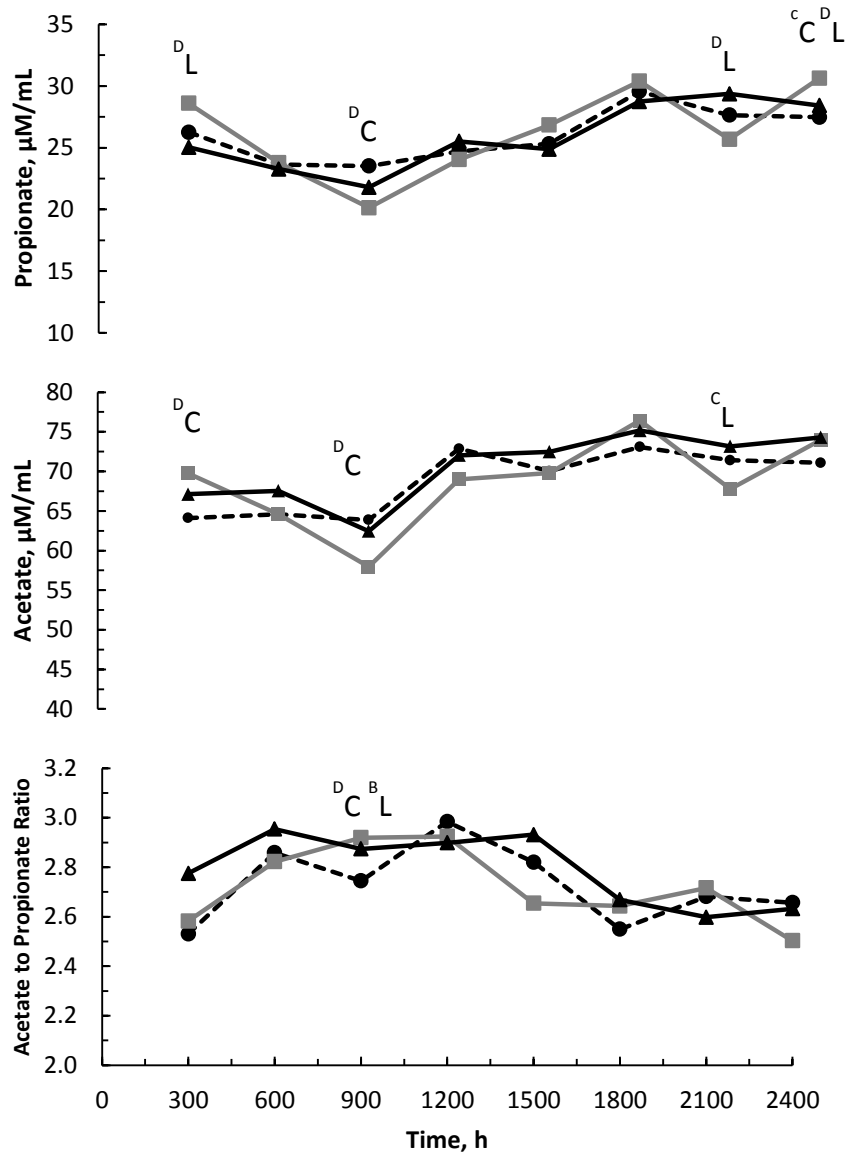


Figure 4.3 Temporal Pattern of Ruminal Concentrations of Acetate, Propionate, and the Acetate to Propionate Ratio

Temporal pattern of ruminal acetate, propionate, and the A:P ratio production in cows fed one of the three feeding regimens. The control treatment (dotted line,) was fed at 0900 h, the high/low treatment (HL; solid gray line) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH; solid black line) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. Rumen fluid was sampled every 3 h. Top panel: propionate concentration (Treatment $P = 0.82$, Time $P < 0.001$, Treatment x Time $P = 0.16$), middle panel acetate concentration (Treatment $P = 0.19$, Time $P < 0.001$, Treatment x Time $P = 0.16$), bottom panel: acetate to propionate ratio (Treatment $P = 0.17$, Time $P < 0.001$, Treatment x Time $P = 0.34$). Preplanned contrasts tested the effect of treatment at sampling time (L denotes the contrast of HL and LH, C denotes the contrast between HL and Con, and the superscripts denotes significant at each level; ^A $P < 0.001$, ^B $P < 0.001$, ^C $P < 0.05$, ^D $P < 0.1$)

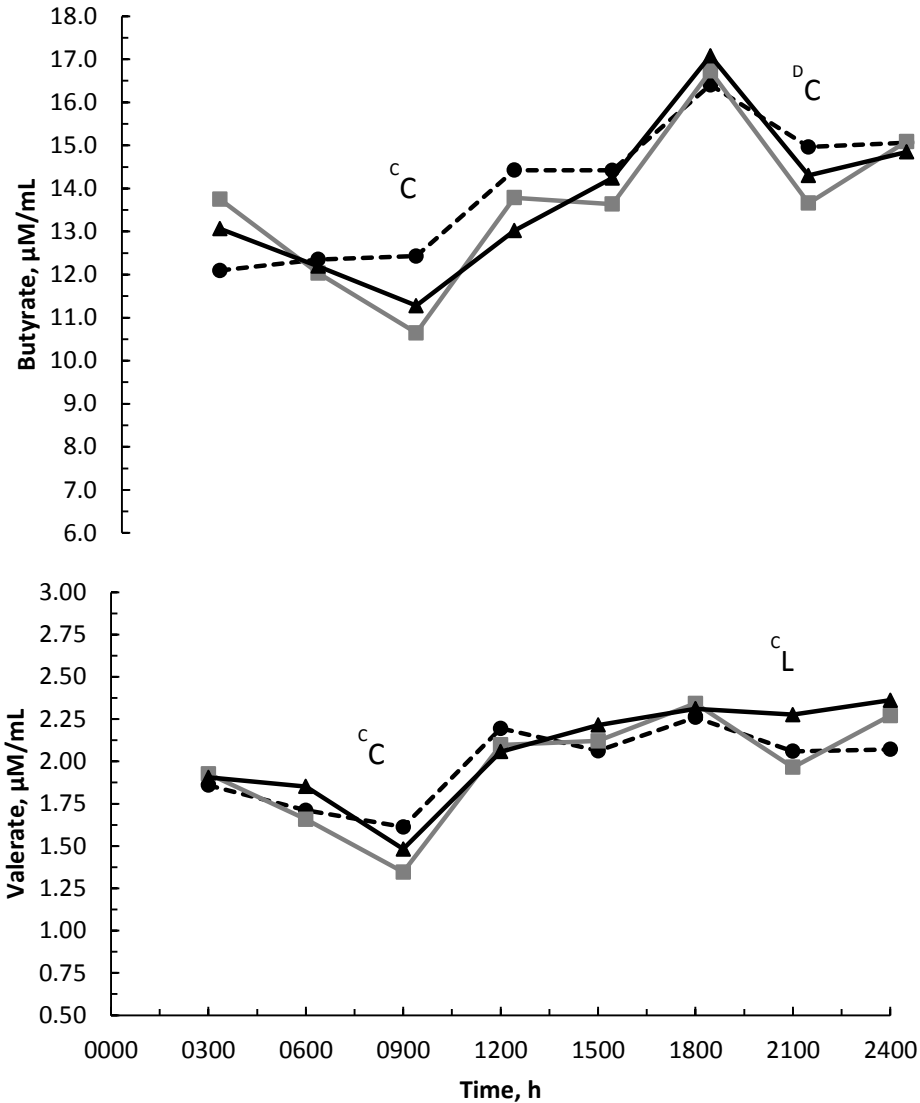


Figure 4.4 Temporal Pattern of Ruminal Butyrate and Valerate Concentrations

Temporal pattern of ruminal butyrate and valerate production in cows fed one of the three feeding regimens. The control treatment (dotted line,) was fed at 0900 h, the high/low treatment (HL; solid gray line) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH; solid black line) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. Rumen fluid was sampled every 3 h. Top panel: Butyrate concentrations (Treatment $P = 0.63$, Time $P < 0.001$, Treatment x Time $P = 0.37$), and bottom panel: valerate concentrations (Treatment $P = 0.17$, Time $P < 0.001$, Treatment x Time $P < 0.07$). Preplanned contrasts tested the effect of treatment at sampling time (L denotes the contrast of HL and LH, C denotes the contrast between HL and Con, and the superscripts denotes significant at each level; $^A P < 0.001$, $^B P < 0.001$, $^C P < 0.05$, $^D P < 0.1$).

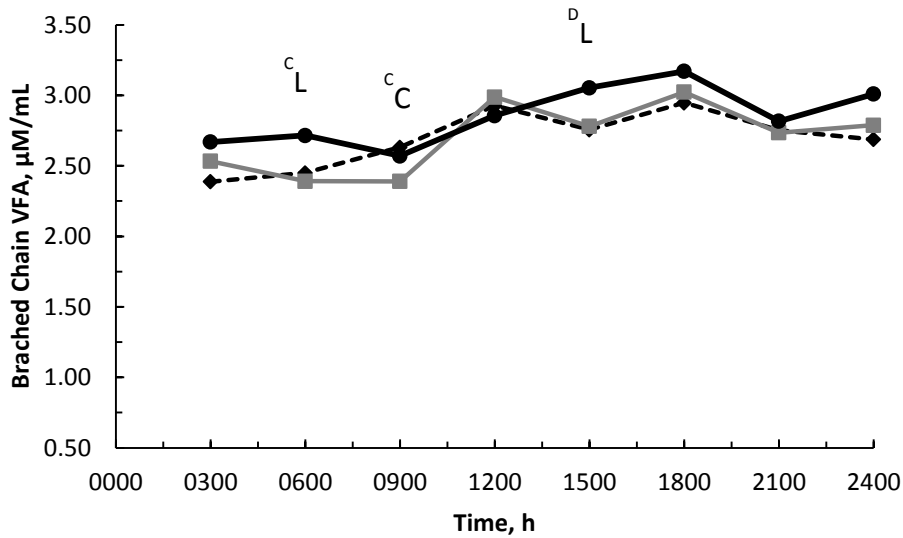


Figure 4.5 Temporal Pattern of Ruminal the Branched Chained VFA Concentrations

Temporal pattern of ruminal branched chain VFA production in cows fed one of the three feeding regimens. The control treatment (dotted line,) was fed at 0900 h, the high/low treatment (HL; solid gray line) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH; solid black line) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. Ruminal branched chain, isobutyrate and isovalerate concentration (Treatment $P = 0.05$, Time $P < 0.001$, Treatment x Time $P = 0.30$). Preplanned contrasts tested the effect of treatment at sampling time (L denotes the contrast of HL and LH, C denotes the contrast between HL and Con, and the superscripts denotes significant at each level; ^A $P < 0.001$, ^B $P < 0.001$, ^C $P < 0.05$, ^D $P < 0.1$)

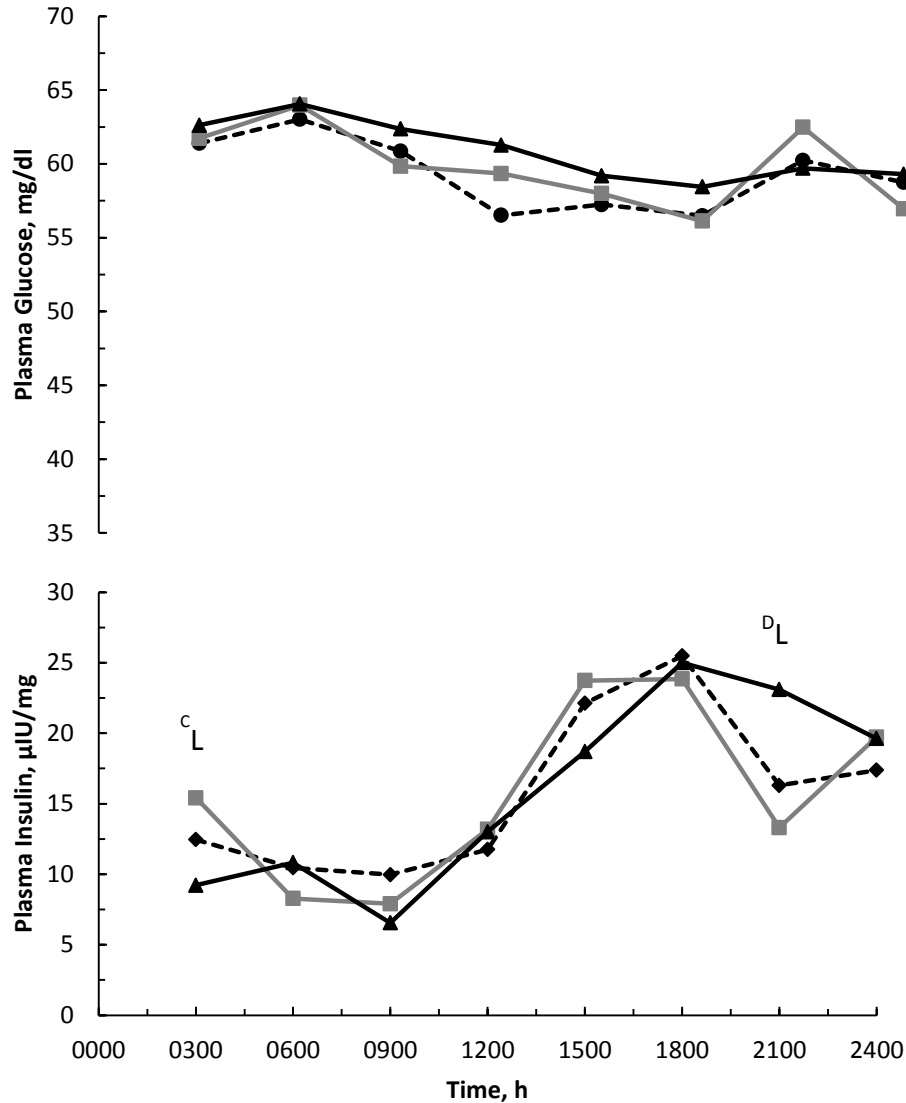


Figure 4.6 Temporal Pattern of Plasma Glucose and Insulin

Temporal pattern of plasma glucose and insulin in cows fed one of the three feeding regimens. The control treatment (dotted line,) was fed at 0900 h, the high/low treatment (HL; solid gray line) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH; solid black line) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. Blood was taken every 3 h. Top panel: Plasma glucose (Treatment $P = 0.14$, Time $P < 0.001$, Treatment x Time $P = 0.65$) and bottom panel: Plasma insulin (Treatment $P = 0.99$, Time $P < 0.001$, Treatment x Time $P = 0.58$). Preplanned contrasts tested the effect of treatment at each sampling time (L denotes the contrast of HL and LH, C denotes the contrast between HL and Con, and the superscripts denotes significant at each level; ^A $P < 0.001$, ^B $P < 0.001$, ^C $P < 0.05$, ^D $P < 0.1$)

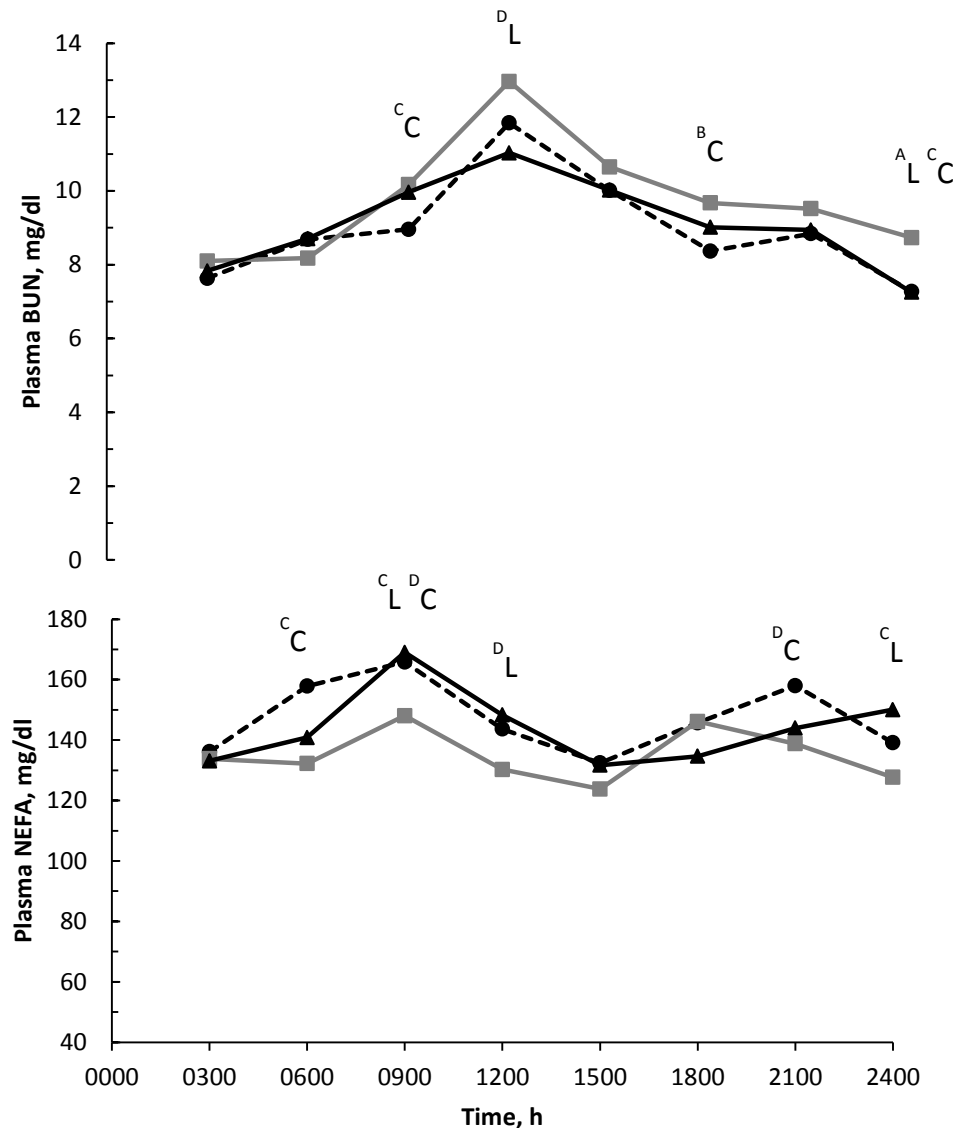


Figure 4.7 Temporal Pattern of Plasma Blood Urea Nitrogen (BUN) and Non-Esterified Fatty Acids (NEFA)

Temporal pattern of plasma BUN and NEFA in cows fed one of the three feeding regimens. The control treatment (dotted line,) was fed at 0900 h, the high/low treatment (HL; solid gray line) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH; solid black line) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h. Blood was taken every 3 h. Top panel: plasma BUN (Treatment $P < 0.01$, Time $P < 0.001$, Treatment x Time $P = 0.05$) and bottom panel: plasma NEFA (Treatment $P < 0.01$, Time $P < 0.001$, Treatment x Time $P = 0.32$). Preplanned contrasts tested the effect of treatment at each sampling time (L denotes the contrast of HL and LH, C denotes the contrast between HL and Con, and the superscripts denotes significant at each level; ^A $P < 0.001$, ^B $P < 0.001$, ^C $P < 0.05$, ^D $P < 0.1$).

Table 4.8 The effect of feeding a single TMR (Con) or feeding two TMR that differ in their forage to concentrate ratio at different times of the day on daily concentrations of blood metabolites.

	Treatments				<i>P</i> -values		
	Con ¹	HL	LH	SEM	Trt	Time	Trt*Time
Insulin, μ IU/mg	15.7	15.7	15.8	3.47	1.00	< 0.001	0.58
Glucose, mg/dL	59.3	59.8	60.9	1.73	0.14	< 0.001	0.65
NEFA, mg/dL	147.3	135.1 ^{LH/C}	144.0	10.80	< 0.01	< 0.001	0.32
BUN, mg/dL	9.0	9.8 ^{LH/C}	9.1	0.73	< 0.01	< 0.001	0.05

¹The control treatment (Con) was fed at 0900 h, the high/low treatment (HL) was fed H at 70% of daily offering at 0900 h and L at 30% of daily offering at 2200 h, and the low/high (LH) treatment was fed L at 30% of daily offering at 0900 h and H at 70% of daily offering at 1300 h.

Chapter 5

Future Direction and Conclusion

Milking cows four times a day while feeding either once or in four equal meals confirmed that there is a pattern to milk synthesis and it can be influenced by the timing of feed intake (Chapter 3). The change in phase of body temperature between 2x milking and 4x milking may also allude to a milking time and milking frequency affect circadian rhythms and possibly the circadian pattern of milk synthesis. This has direct implications for the dairy industry with regards to the timing of management procedures, especially feeding and milking. There may be specific times over the day that are optimal to provide fresh feed, and it may be more important to make the decision based on the pattern of milk synthesis than convenience. For example, if it is more beneficial for cows to be fed at 1800 h and milked at noon and midnight, the cost to the farm would be the same but an increase in milk and milk component yield could increase profit.

Although the expression of the circadian clock genes was not observed in this experiment it is expected that removing four, smaller feedings would decrease the amplitude of the core circadian genes in metabolic tissues such as the liver and adipose tissue. The circadian genes would then influence the amplitudes of the downstream genes including metabolic enzymes. This may minimize the fluctuations between nutrient storage and nutrient mobilization which should provide a consistent amount of nutrients available for milk synthesis. This should also affect the proposed clock in the mammary gland. The 4x fed cows displayed a more even pattern of milk synthesis over the day which suggests that the amplitude of clock is dampened. Further work is required to characterize the regulation of the mammary oscillator by absorbed nutrients. Additionally, other issues may arise due to the possible dampened oscillator similar to issues that arise in arrhythmic animal models.

The next step to understanding the effects of timing of feed intake and milking would be to uncouple them over the day. There are a couple ways to accomplish this. First, cows could be fed at 0600 h, 1800 h, and every six hours in four equal meals while milking four times a day. This should introduce a negative control, and allow observation of the response similar to Chapter 3 was due to stabilizing feed consumption or the effect of the timing of feed intake. Secondly, different the interaction of milking and feeding schedules could be tested to determine the effects of the combined timing of fresh feed and milking. Two of treatments would be milking at 0600 h and 1800 h while feeding at either 1200 or 2400 h. The other two treatments would be milking at 1200 h and 2400 h and fed at either 0600 or 1800 h.

Key insight was provided into feeding multiple rations over the day in Chapter 4. Although the data analysis for this experiment has not been completed, it suggests that feeding two rations differing in starch concentrations during key time points over the day may stabilize the rumen and increase efficiency. Further analyzing of rumen digesta composition and rumen nutrient pool sizes will give better insight into what is happening in the rumen over time, but the VFA and rumen empty volume data indicate a distinct pattern over the day. The production and DMI data indicate that the feeding regimens did not affect production, but decreased DMI indicating improving efficiency in the HL group. However, the HL group had slightly lower milk fat, which may indicate that the timing of feed offering was not optimal.

One concern to stabilizing rumen fermentation is that it might abolish any and all rhythms associated with feeding behavior and/or milk synthesis. An asynchronous environment could have drastic consequences as seen in experiments where the SCNs are ablated in the mouse. Ablating the SCN causes the behaviors of mice to become arrhythmic. An arrhythmic mouse has lost all activity patterns and does not respond light/dark or scheduled feeding (Marchant and Mistlberger, 1997). By synchronizing the ration to the needs of the rumen environment at particular times over the day, a crucial, yet unknown, pattern may be abolished. Greater issues

may evolve like the inability of the cow to metabolically prepare for an influx of nutrients from the gastrointestinal tract.

Circadian gene expression was not investigated because of the invasiveness of sample collection. It is expected that feeding multiple, different rations over the day will have an effect on gene expression in metabolically important tissues. One of the rations may take a higher priority in entraining the circadian system. This may be based on a nutrient deficiency or the timing of feeding. The dominant ration would act as a stronger zeitgeber and entrain behavior and metabolism to this feeding. If this occurs we would expect to see a phase shift between the treatments.

To move on from this experiment, a repeat of the experimental setup should be done. The rations needed to be more different in their NDF content. The high fiber and control diet were too similar. Next, the feeding times needed to be based on feeding behavior data, which we didn't have at the time. Further analysis of feeding behavior will also provide a better time to feed the different parts of feeding regimen. In addition to feeding behavior, a short experiment should be conducted to understand how much time the rumen requires to reflect the diets. This could be accomplished by having 3-6 rumen cannulated cows by which are fed a distinct diet high in starch content. Immediately after the diet is given, start taking samples every 30 minutes for 24 h. This should give us a better idea of how the rumen changes over the day given a specific diet. Some disturbance would occur because of the frequent sampling, but it could be minimized by sampling the cows in hour intervals but half the cows 30 minutes after the other half. This would give samples every 30 minutes. After this is accomplished, the next step would to choose the best feeding regimen and then test the interaction with milking times. As described above, milking at 1200 and 2400 h vs. 0600 and 1800 h will allow insight into an interaction with feeding a single TMR vs. multiple TMR rations over the day.

Another aspect to understanding feeding behavior would be to look at hunger and satiety hormones. Examining blood concentrations of hunger-satiety hormones such as leptin, neuropeptide y, and cholecystokinin could provide some cause and effect relationships to feeding bouts. It would be easy to overlay graphs of these plasma hormones over the day onto the feeding behavior data to see if any relationships develop. Many hormones have been shown circadian in nature (see Chapter 2), but little to none have been characterized in the bovine, and none have been analyzed in relation to feeding behavior data.

In the experiments conducted, we have discovered new information of importance to the dairy industry. First and most important, feeding smaller quantities multiple times over the day increased component milk fat concentration and yield. For farms where milk fat is below their goal, feeding multiple times a day to spread intake across the day may increase milk fat by stabilizing rumen fermentation. This should be relatively easy to accomplish and does not require purchasing additional feed ingredients. Feeding four times a day may not be required to increase milk fat, but when feeding less frequently the timing and amount of feed offered would be key to stabilizing intake over the day. There is some evidence to suggest feeding multiple rations in a specific feeding regimen can increase production efficiency, but the timing of these feeding regimens and nutrient concentrations needs to be further examined before specific recommendations can be made.

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Appendix A- List of Abbreviations

ADF – acid detergent fiber

BUN – blood urea nitrogen

CBT- core body temperature (measured through intravaginal probe)

CCG – clock controlled gene

Cir – circadian rhythm

Cry - cryptochrome

DIM – days in milk

DMI – dry matter intake

ECM – energy corrected milked $[(0.092*MY+0.0563*protein\%+0.192)*MY]$

FAA – food anticipatory activity

FCM – fat corrected milk $[(0.4255*MY)+(16.425*((fat\%/100)*MY/2.2))]$

FEO- food entrainable oscillator

KO – knock out

MI – milking interval

NDF – neutral detergent fiber

NeCM – net energy corrected milk (also referred to as ECM)

NEFA – non-esterified fatty acids

Per - period

SCN – suprachiasmatic nucleus

T2DM – Type Two Diabetes Mellitus

Ultra – ultradian rhythm

VFA – volatile fatty acids

WT – wild type

Appendix B - Definitions

Clock- a timing mechanism that is composed of molecular oscillators, e.g. a circadian clock contains oscillators that have a 24 h cycle

Coupling/uncoupling – the alignment of the phases of multiple clocks so that they oscillate with the same period/ the misalignment of the periods of multiple clocks so they oscillate with different phases

Cryptochrome 1, 2 - the negative elements in the primary loop of the molecular mechanism to produce circadian rhythms

Entrainment – the synchronization or alignment of the internal biological clock rhythm, including its phase and period, to external time cues, such as the natural dark/light cycle.

Oscillator – a system of components that interact to produce a rhythm with a definable period length (Bell-Pedersen et al., 2005)

Phase – an instantaneous reference point to determine where in time the rhythm is oscillating; it's usually in reference to when the zenith or nadir of the cycle falls with respect to the solar day

Period – the time it takes a oscillator to finish one full cycle; for circadian periods it takes 24 h even in the absence of the light/dark cycle

Period 1, 2, 3 – the negative elements in the primary loop of the molecular mechanism to produce circadian rhythms

Rhythm – a pattern that repeats itself at specific times over a period of time; a circadian rhythm occurs every 24 h

Master Clock – also known as the suprachiasmatic nucleus and is located right behind the optic chiasm in the hypothalamus; it sets the phase for the peripheral tissue clocks

Peripheral Clocks – also known as slave clocks; any tissue that houses a clock; the period and phase are usually set by the master clock

Ultradian – a cycle that completes multiple revolutions in less than 24 h

Zeitgeber – an external stimulus that entrains a circadian clock so that a certain point in the rhythm stably hits a certain point in the external environment; e.g. like the onset of sleep occurs during the dark phase of the light/dark cycle

Appendix C

Chapter 3 – Extra Figures

Table C.1 Treatment means for plasma metabolites for cows fed once a day (1x Fed) or in four equal meals every six hours (4x Fed).

	Treatment		SEM	<i>P</i> -values		
	1x Fed ¹	4x Fed		Trt	Time	Trt*Time
BUN (mg/dL)	11.06	11.12	0.48	0.92	< 0.001	0.02
NEFA (mg/dL)	143.88	146.32	7.04	0.81	< 0.001	0.29
Glucose (mg/dL)	60.28	59.32	0.69	0.33	< 0.001	< 0.01
Insulin (μIU/dL)	13.93	11.55	2.31	0.06	< 0.001	< 0.001

¹LS means for the treatment of cows fed once a day (1x Fed) or in four equal meals every six hours (4x Fed)

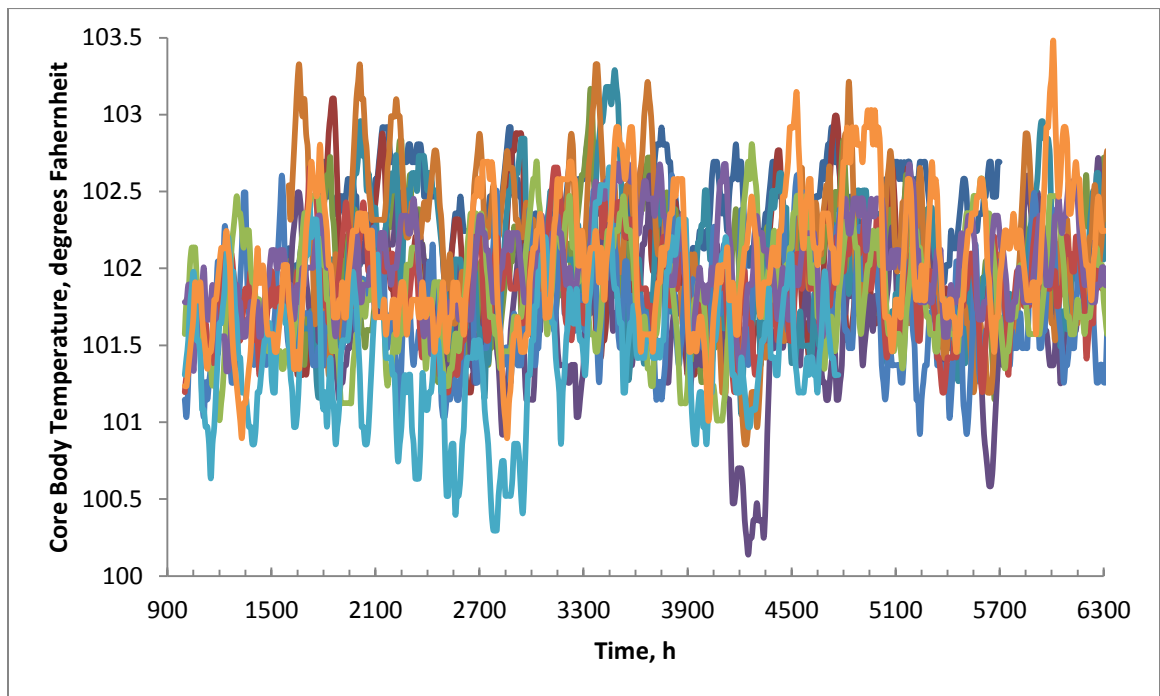
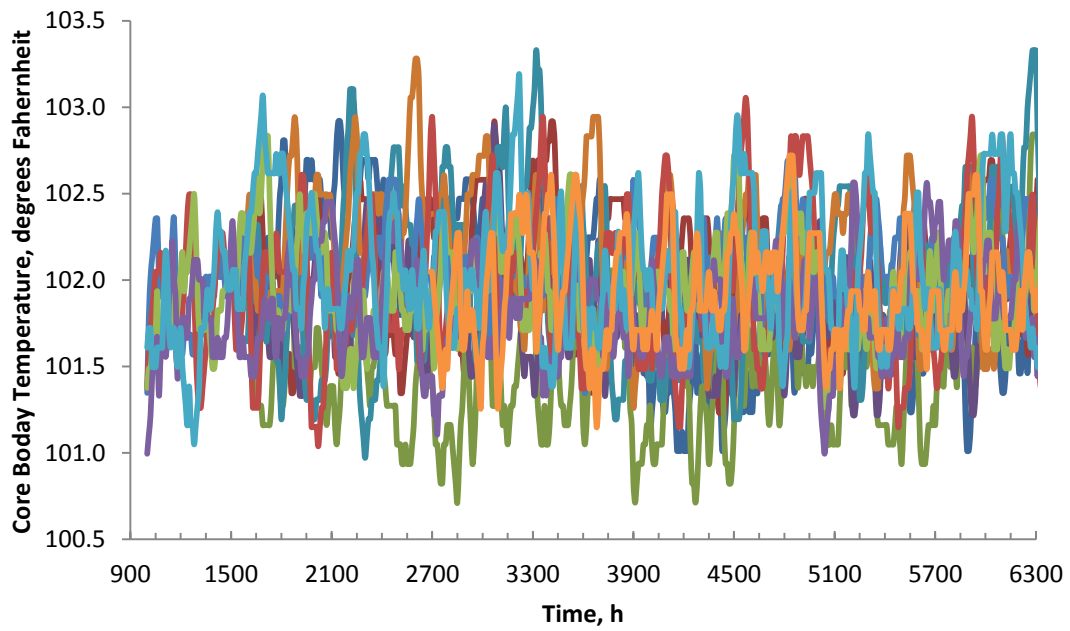


Figure C.1 Raw Data of Core Body Temperature

Temporal pattern of core body temperature taken every ten minutes over three days in nine cows. Cows were milked every 6 h. Top Panel: Temperature for cows fed every six h in four equal meals starting at 1000 h and going for 53 h from 1000 h (2 days). Bottom Panel: CBT for cows fed once per day starting at 1000 h and going for 53 h from 1000 h (2 days).

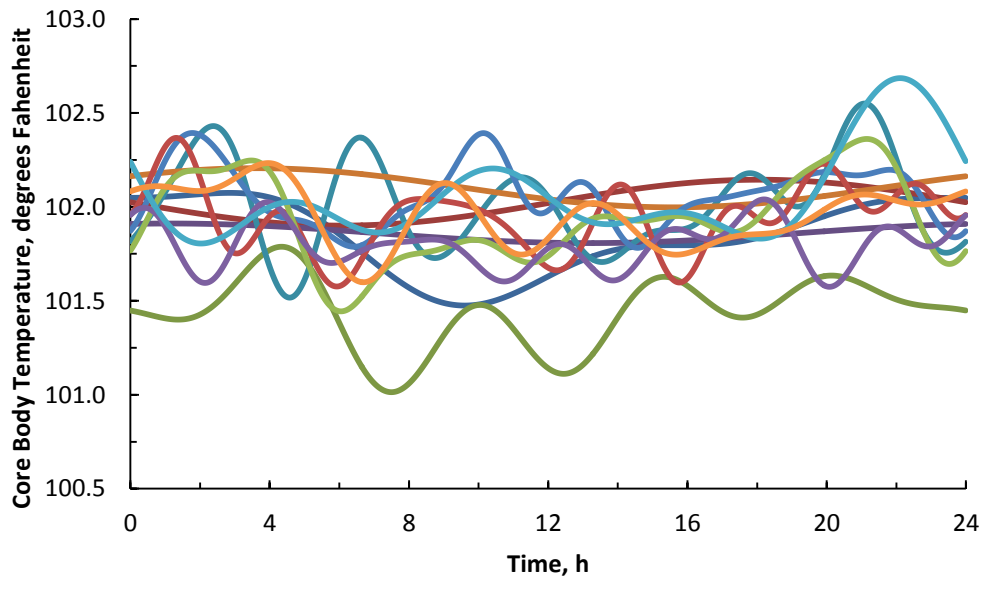
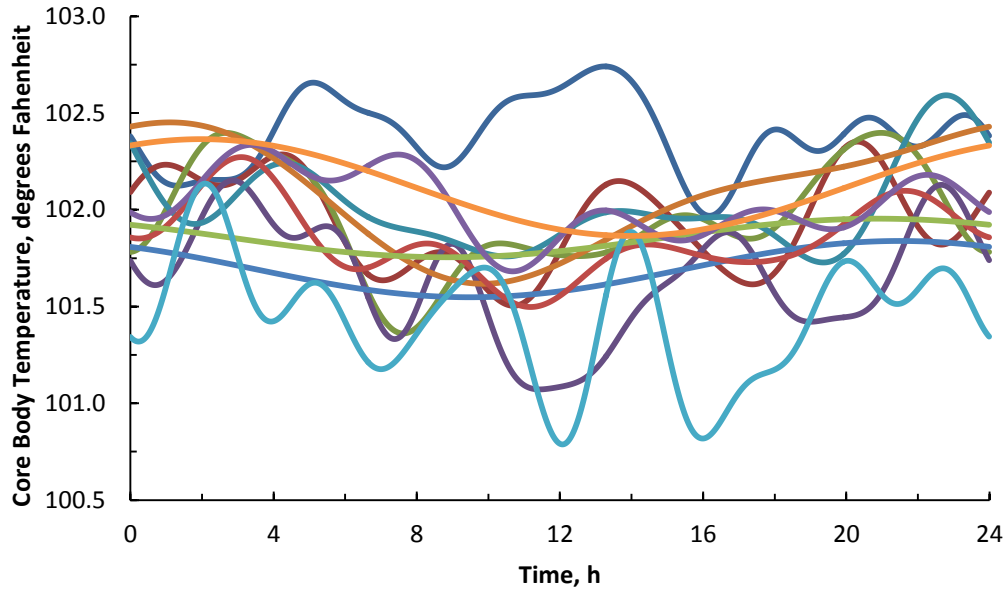


Figure C.2 Core Body Temperature Fitted Curves

Temporal pattern of fitted sine wave curves for core body temperature taken every ten minutes over three days in nine cows. Cows were milked every 6 h. Panel A: Temperature for cows fed once per day. Panel B: Temperature for cows fed in four equal meals every six h.

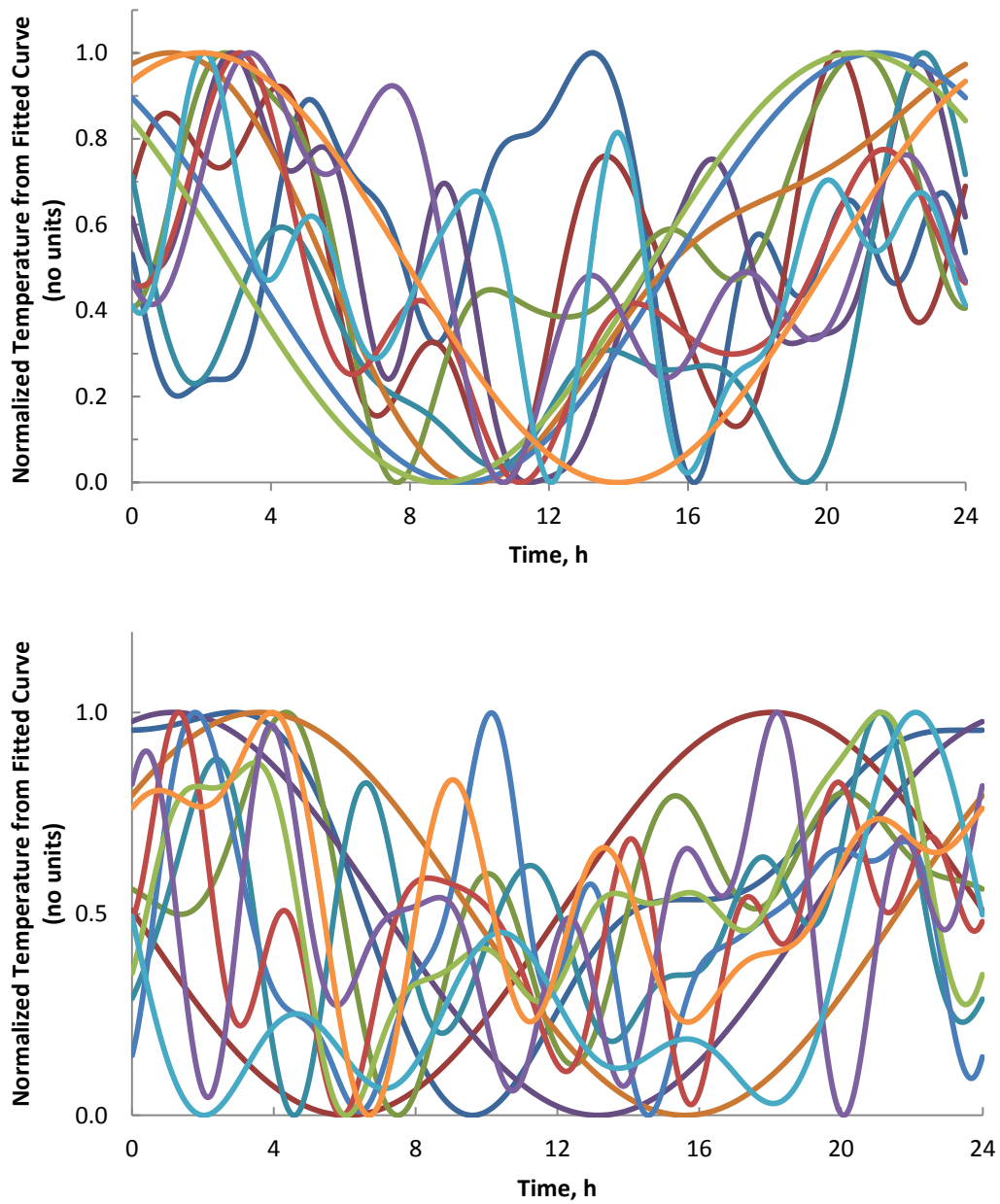


Figure C.3 Core Body Temperature Normalized Fitted Curves

Temporal pattern of normalized fitted sine wave curves for core body temperature taken every ten minutes over three days in nine cows. Cows were milked every 6 h. Top Panel: Temperature for cows fed once per day (Phase at 1400 h). Panel B: Temperature for cows fed in four equal meals every six h (Phase at 1030 h; $P < 0.05$).

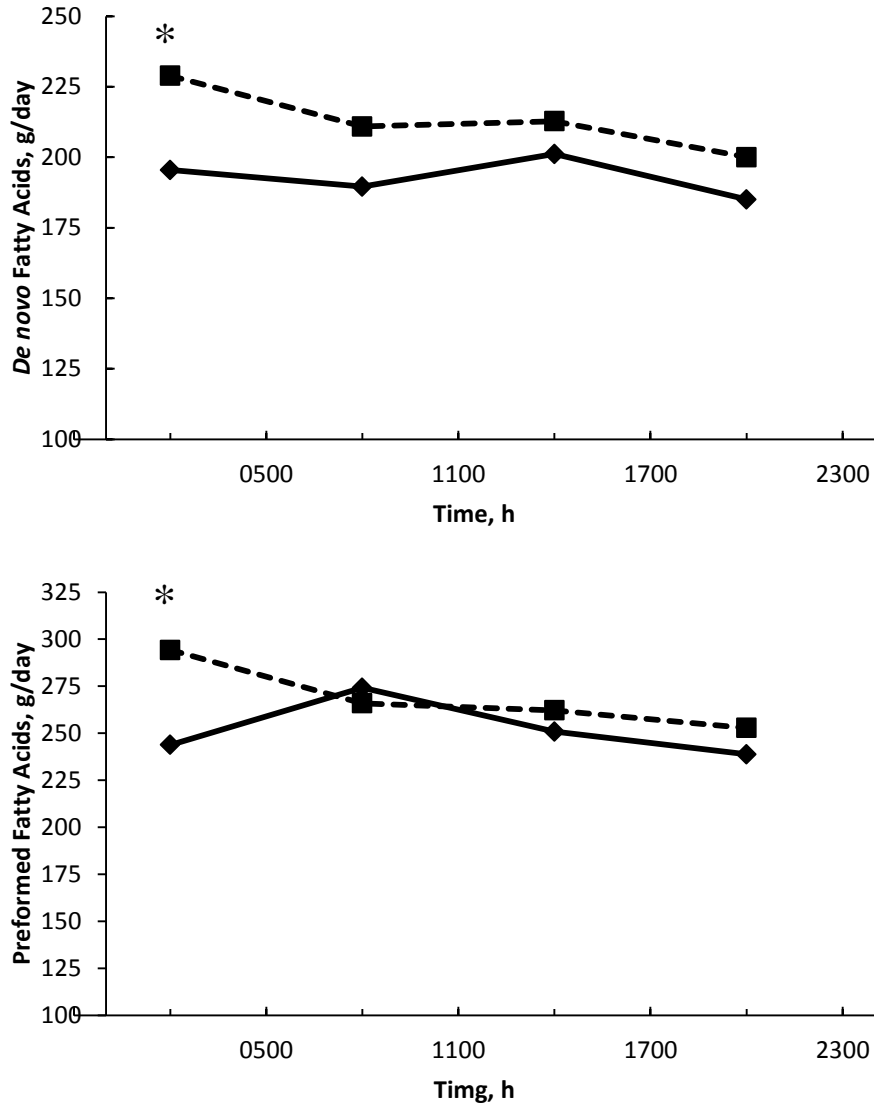


Figure C.4 Temporal Pattern in *de novo* and Preformed Fatty Acids

Temporal pattern of milk fatty acid yields in cows fed once per day or in four equal meals every 6 h. Cows were milked every 6 h. Top Panel: *De novo* Fatty Acids (Treatment $P = 0.05$, Time $P = 0.20$, Treatment x Time $P = 0.74$), and Bottom Panel: Preformed Fatty Acids (Treatment $P = 0.21$, Time $P = 0.33$, Treatment x Time $P = 0.17$). Cows fed once a day (solid line) or in equal meals every six hours (dashed line). Planned contrasts tested the effect of treatment at each milking. ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

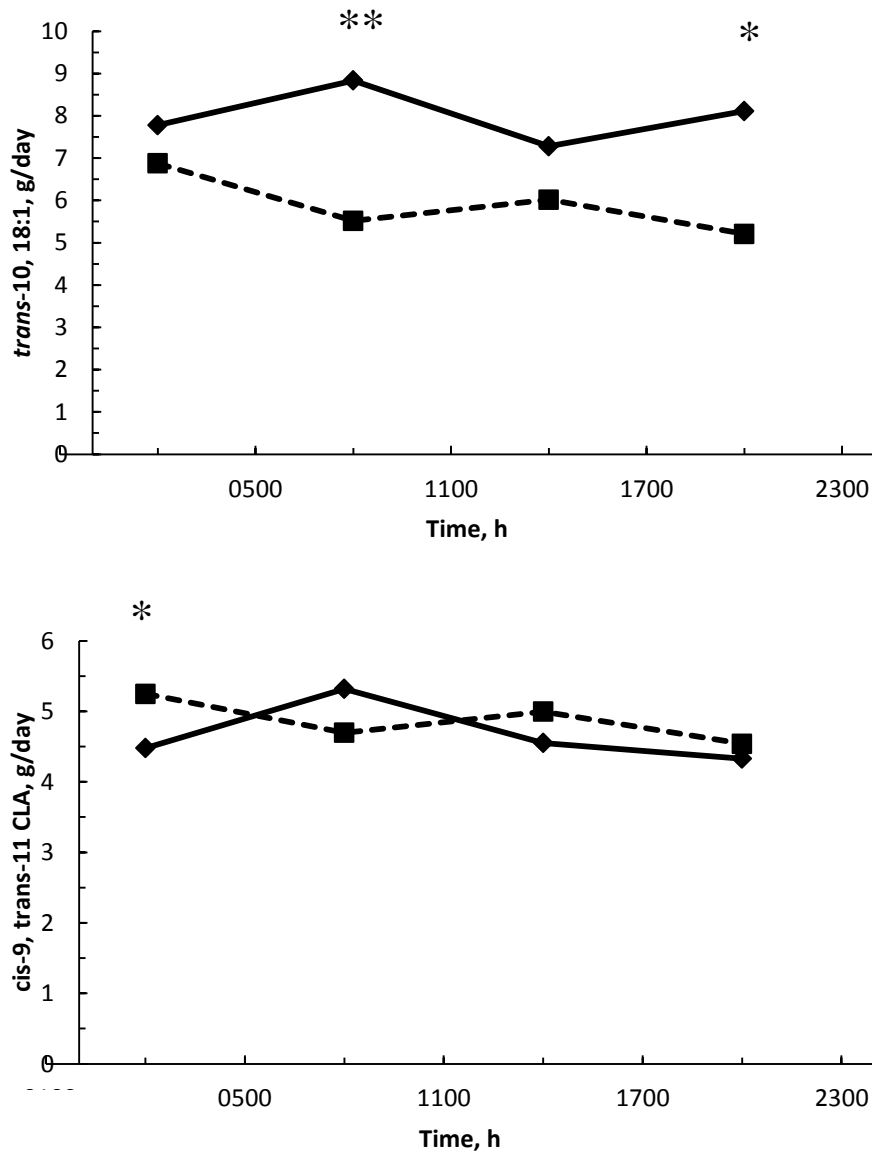


Figure C.5 Temporal Patterns in Trans Fatty Acid Isomers

Temporal pattern of milk fatty acid yields in cows fed once per day or in four equal meals every 6 h. Cows were milked every 6 h. Top Panel: *Trans*-10, 18:1 (Treatment x Time $P = 0.02$), and Bottom Panel B: *cis*-9, *trans*-11 CLA (Treatment x Time $P = 0.03$). Cows fed once a day (solid line) or in equal meals every six hours (dashed line). Preplanned contrasts tested the effect of treatment at each milking. († $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

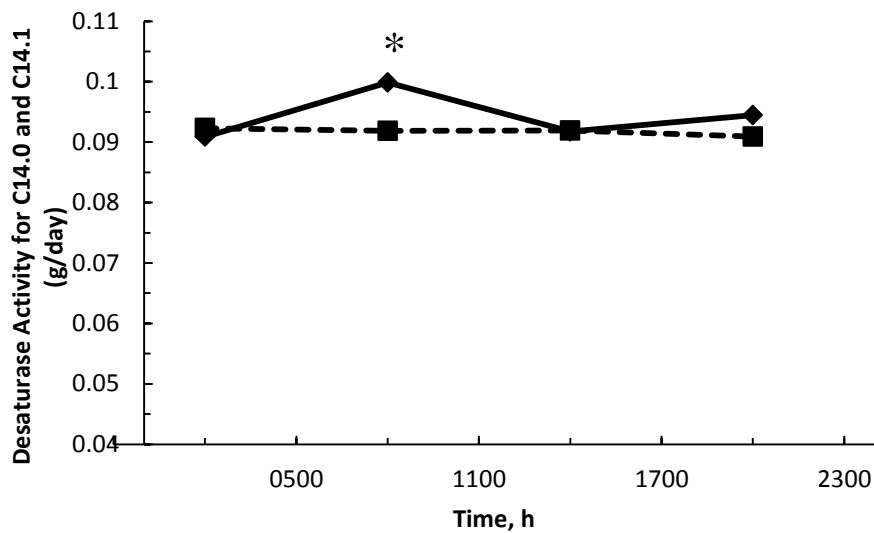
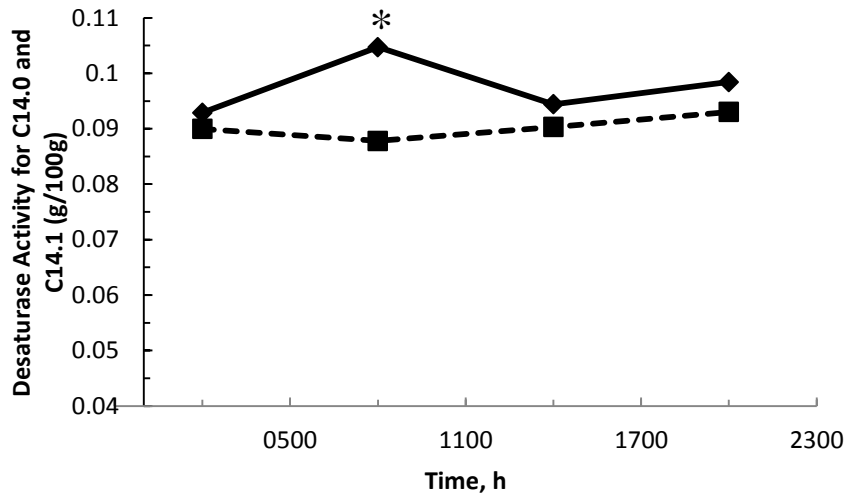


Figure C.6 Desaturase Activity for C14.0 and C14.1

Temporal pattern of milk fatty acid desaturase activity in cows fed once per day or in four equal meals every 6 h. Cows were milked every 6 h. Desaturase activity for c14.0 and c14.1 Top Panel: Percent of total fatty acids (g/100g) (Treatment x Time $P = 0.09$). Bottom Panel: yields (g/day) (Treatment x Time $P = 0.04$). Cows fed once a day (solid line) or in equal meals every six hours (dashed line). Preplanned contrasts tested the effect of treatment at each milking. ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

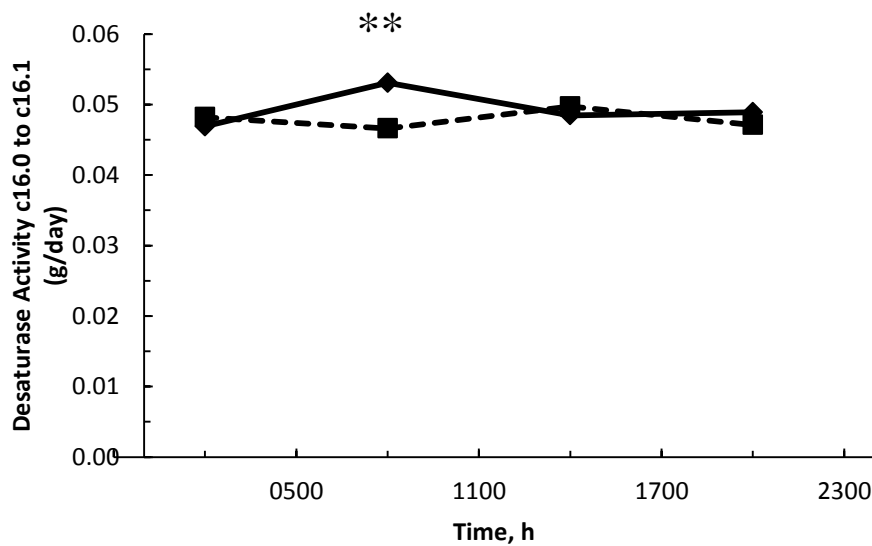
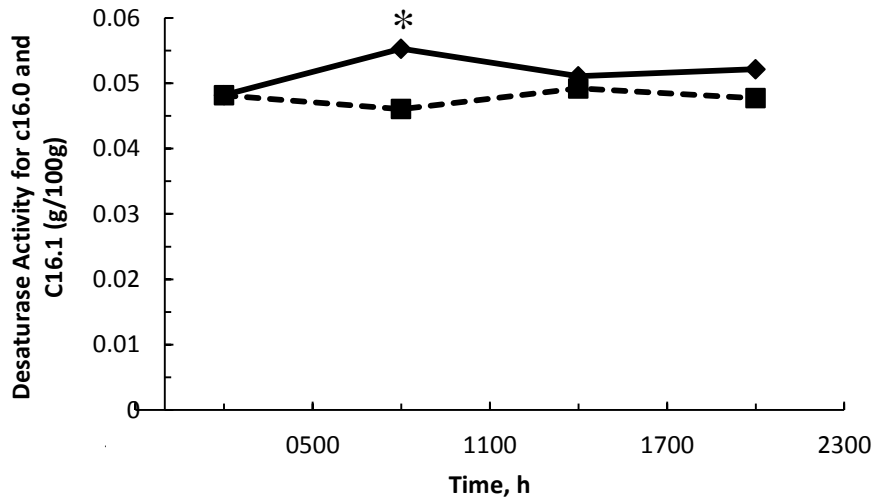


Figure C.7 Desaturase Activity for C16.0 and C16.1

Temporal pattern of milk fatty acid desaturase activity in cows fed once per day or in four equal meals every 6 h. Cows were milked every 6 h. Desaturase activity for c16.0 and c16.1 Top Panel: Percent of total fatty acids (Treatment x Time $P = 0.003$). Bottom Panel: Fatty acid yields (g/day) (Treatment x Time $P = 0.002$). Cows fed once a day (solid line) or in equal meals every six hours (dashed line). Preplanned contrasts tested the effect of treatment at each milking. ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$).

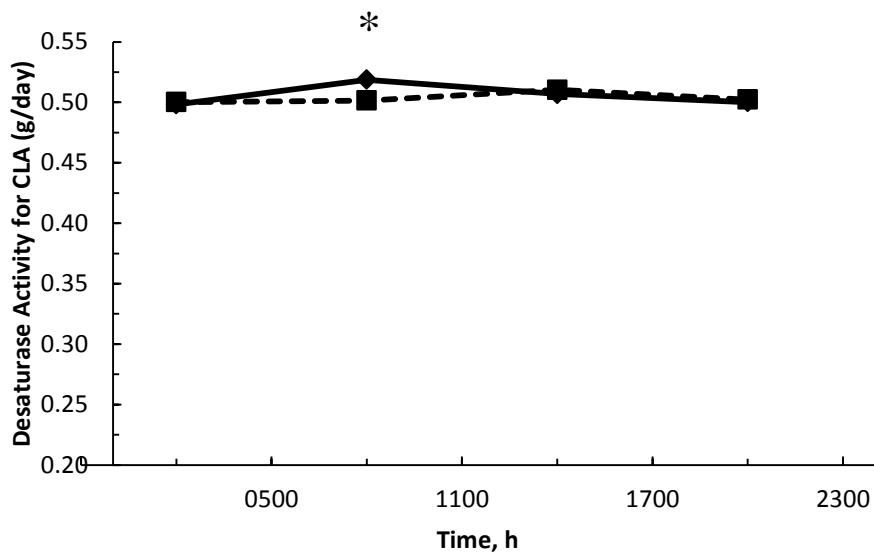
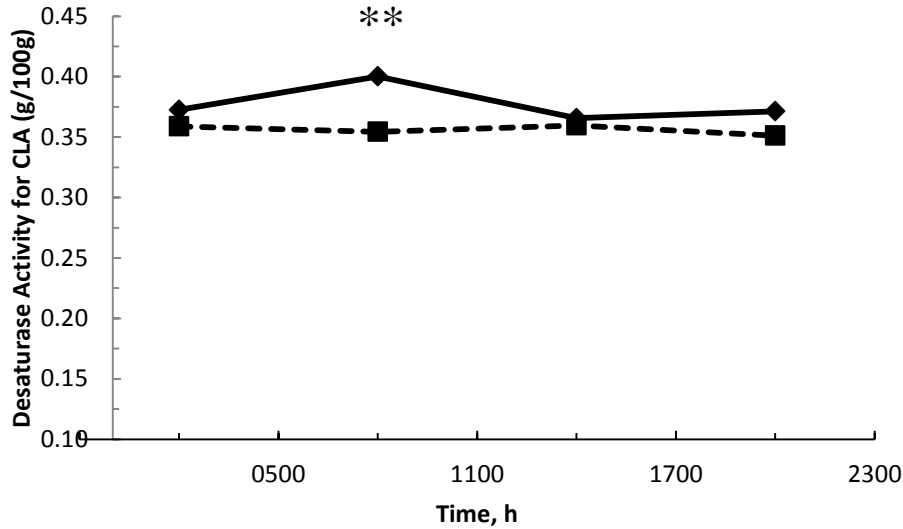


Figure C.8 Desaturase Activity for *trans*-12, C18.1 and *cis*-9, *trans*-11, CLA

Temporal pattern of milk fatty acid desaturase activity in cows fed once per day or in four equal meals every 6 h. Cows were milked every 6 h. Desaturase activity for *trans*-12, c18.1 and *cis*-9, *trans*-11, CLA Top panel: Percent of total fatty acids (g/100g) (Treatment x Time $P < 0.001$). Bottom panel: Fatty acid yields (g/day) (Treatment x Time $P = 0.07$). Cows fed once a day (solid line) or in equal meals every six hours (dashed line). Preplanned contrasts tested the effect of treatment at each milking. ($\dagger P < 0.10$, $* P < 0.05$, $** P < 0.01$, and $*** P < 0.001$)