The Pennsylvania State University
The Graduate School
Department of Animal Science

NUTRITIONAL AND ENVIRONMENTAL FACTORS REGULATING
BIOLOGICAL RHYTHMS OF MILK SYNTHESIS IN DAIRY CATTLE

A Dissertation in
Animal Science

by
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ABSTRACT

Dairy cows exhibit well-characterized daily patterns of feed intake and milk synthesis. These patterns may represent circadian rhythms, endogenous repeating cycles of approximately 24 h that govern most physiological processes. Circadian rhythms are controlled by a set of ‘clock’ transcription factors that oscillate over 24 h and govern gene expression across the day.

There is an increasing body of evidence demonstrating a role of nutrient intake in modifying circadian rhythms in peripheral tissues. However, this effect has not been well-characterized in dairy cattle. In addition to a daily rhythms of milk synthesis, dairy cows display seasonal changes of milk production that are not well understood. The objective of this dissertation was to examine the factors affecting daily and annual rhythms of milk synthesis. Specifically, we wanted to examine the effects of nutrient intake on the daily rhythms of milk synthesis, and the relationships between cow-level and environmental factors and the annual rhythms of milk production.

To accomplish these objectives, 6 experiments were conducted, with the first 4 examining the role of nutrient intake on the daily rhythms of milk synthesis, and the final 2 characterizing factors influencing annual rhythms of milk production. First, the effect of time-restricted feeding on the daily patterns of milk synthesis and plasma hormones and metabolites was examined in an experiment where feed was temporally restricted to cows for 16 h/d either during the day (0700 to 2300 h) or at night (1900 to 1100 h). This experiment demonstrated that night restricted feeding shifted the peak of milk yield from morning to evening, while shifting the peak of milk fat and protein concentration from evening to morning. Moreover, the daily rhythms of plasma glucose, nonesterified fatty acids, plasma urea nitrogen, and insulin were shifted about 8 h by night-restricted feeding. The objective of the second experiment was to investigate if the changes in daily patterns of milk synthesis during time-restricted feeding were caused by changes in the molecular circadian clock of the mammary gland. This experiment compared a control
group with feed available continuously to cows under night-restricted feeding (feed available for 16 h/d from 2000 h to 1200 h). Results demonstrated that night-restricted feeding altered the daily rhythms of expression of clock genes circadian locomoter output cycles kaput, cryptochrome 1 and REV-ERBa, suggesting that nutrients entrain the molecular circadian clock of the mammary gland. The final two experiments examined the role of specific nutrients on the daily rhythms of milk synthesis. In one experiment, a high C18:1 oil was abomasally infused either 24 h/d or for 8 h during the day (0900 to 1700 h) or the night (from 2100 to 0500). Day-infusion increased the amplitudes of milk and milk fat and protein yield while reducing the amplitudes of milk fat and protein concentration. However, treatment had little influence on the phase of the rhythms, suggesting that the timing of fatty acid infusion did not entrain the mammary clock. In the other experiment, sodium caseinate solution was abomasally infused either 24 h/d or for 8 h during the day (0900 to 1700 h) or the night (from 2100 to 0500). Night-infusion induced a daily rhythm of milk yield, but no rhythm was present during continuous or daytime infusion. Infusion of sodium caseinate during the day reduced the amplitude of fat concentration and increased the amplitude of protein concentration, while night-infusion decreased the amplitude of protein concentration. Treatment had little effect on the time at peak of the rhythm, suggesting that the mammary circadian clock was not entrained to the time of protein infusion. Furthermore, night-infusion shifted the peak of the nonesterified fatty acid rhythm from morning to evening.

The final 2 experiments characterized factors influencing the annual rhythms of milk synthesis. First, annual rhythms of milk fat and protein concentration were compared among regions of the United States using monthly averages of Federal Milk Marketing Orders. The annual rhythms had greater amplitudes in northern regions compared to southern regions suggesting that they are related to photoperiod. In the same report, the effect of cow-level factors on annual rhythms of milk, fat, and protein yield, and milk fat and protein concentration were examined using data from 11 dairy herds in Pennsylvania. Rhythms were consistent regardless of
herd, lactation number, and genotype, suggesting that they are highly conserved among individual cows. Finally, annual rhythms of milk, fat and protein yield and milk fat and protein concentration were studied using dataset obtained from Dairy Records Management Systems and, relationships between annual rhythms of production and daylength, change in daylength, and environmental temperature were determined. Annual rhythms differed between northern and southern states, with northern states having greater amplitudes of milk fat and protein concentration and southern states having greater amplitudes of milk yield. Moreover, the rhythms of milk fat and protein concentration were highly correlated with absolute daylength, while the rhythm of milk yield was highly correlated with the change in daylength, suggesting that they are controlled through two separate oscillatory mechanisms.

In conclusion, the daily rhythms of milk synthesis are modified by the time of feeding and this effect appears mediated by the molecular circadian clock. The timing of both fatty acids and protein affect the robustness of milk synthesis rhythms, without large effects on their time of peak. Finally, yearly patterns of milk production appear to constitute an annual rhythm, with the rhythms of fat and protein concentration being entrained by daylength and the rhythm of milk yield being entrained by the change in daylength.

**Keywords:** Biological rhythm, milk synthesis, dairy cattle, food entrainment
# TABLE OF CONTENTS

List of Figures .................................................................................................................. xi
List of Tables ...................................................................................................................... xiii
List of Supplemental Figures ............................................................................................. xiv
List of Supplemental Tables ............................................................................................... xv
List of Abbreviations .......................................................................................................... xvi
Acknowledgements .......................................................................................................... xix
Chapter 1 Introduction ....................................................................................................... 1
Chapter 2 Literature Review ............................................................................................... 5
  Biological Rhythms ........................................................................................................... 5
  Molecular Structure of Biological Clocks ........................................................................ 7
    Transcriptional-Translational Feedback Loops ............................................................ 7
    Post-Transcriptional Modifications .............................................................................. 9
    Post-Translational Modifications ............................................................................. 10
  Epigenetics and the Clock ............................................................................................... 11
Entrainment of Biological Clocks ........................................................................................ 11
  Light Entrainment .......................................................................................................... 12
  Food Entrainment .......................................................................................................... 13
  Temperature Entrainment ............................................................................................. 15
Circadian Rhythms and Nutrient Metabolism ..................................................................... 16
  Cellular Energy and Redox Status and the Circadian Clock .......................................... 16
  Glucose Metabolism and the Clock ............................................................................. 18
  Lipid Metabolism and the Clock ............................................................................... 20
  Circadian Rhythms in Microorganisms ...................................................................... 22
Annual Rhythms .................................................................................................................. 25
  Annual Rhythms and Reproduction ........................................................................... 26
Seasonal Changes in Feeding Behavior and Metabolism .................................................. 28
Seasonal Changes in Immunity ......................................................................................... 31
Epigenetic Control of Seasonality .................................................................................... 34
Chapter 5 The effects of time of fatty acid infusion on the daily rhythms of milk synthesis and plasma hormones and metabolites in dairy cows

ABSTRACT

INTRODUCTION

MATERIALS & METHODS

Animals and Treatments

Milk Sampling and Analysis

Plasma Sampling and Analysis

Body Temperature Analysis

Statistical Analysis

RESULTS AND DISCUSSION

Milk and Milk Components Synthesis

Daily Yields and Daily Rhythms of Milk Fatty Acids

Daily Rhythms of Plasma Metabolites

Daily Rhythm of Body Temperature

CONCLUSIONS

FIGURES

SUPPLEMENTAL FIGURES

SUPPLEMENTAL TABLES
Chapter 6 The effect of timing of protein infusion on the daily rhythms of milk production and plasma hormones and metabolites ................................................................. 119
   ABSTRACT .................................................................................................................... 120
   INTRODUCTION .......................................................................................................... 122
   MATERIALS & METHODS .......................................................................................... 124
      Animals and Treatments ......................................................................................... 124
      Milk Sampling and Analysis .................................................................................... 125
      Plasma Sampling and Analysis ............................................................................... 125
      Statistical Analysis ................................................................................................. 126
   RESULTS AND DISCUSSION .................................................................................... 127
      Daily Milk Yield and Milk Components .................................................................. 127
      Daily Rhythms of Milk Yield and Milk Components ............................................... 128
      Daily Yields and Daily Rhythms of Milk Fatty Acids ............................................ 130
      Daily Rhythms of Plasma Metabolites .................................................................... 131
   CONCLUSIONS .......................................................................................................... 135
   FIGURES ...................................................................................................................... 136
   SUPPLEMENTAL FIGURES ....................................................................................... 142
   SUPPLEMENTAL TABLES .......................................................................................... 143

Chapter 7 Annual rhythms of milk and milk fat and protein production in dairy cattle in the United States .......................................................... 145
   INTRODUCTION .......................................................................................................... 148
   MATERIALS & METHODS .......................................................................................... 149
      USDA Milk Market Data ........................................................................................... 149
      Cow-Level Data ....................................................................................................... 150
   RESULTS ...................................................................................................................... 151
      USDA Milk Market Data ........................................................................................... 151
      Cow-Level Data ....................................................................................................... 153
   DISCUSSION ................................................................................................................ 156
   CONCLUSIONS .......................................................................................................... 163
   FIGURES ...................................................................................................................... 164
   TABLES ......................................................................................................................... 169
   SUPPLEMENTAL FIGURES ....................................................................................... 170
Chapter 8 Annual rhythms of milk synthesis in dairy herds in four regions of the United States and their relationships to environmental predictors. ........................................ 175

ABSTRACT .................................................................................................................. 176

INTRODUCTION ........................................................................................................ 178

MATERIALS & METHODS .......................................................................................... 179

Data Collection ........................................................................................................... 179

Cosinor rhythmometry ............................................................................................... 180

Effect of breed on annual rhythms of production .................................................... 180

Comparison of annual rhythm with temperature ..................................................... 181

Relationships among environmental variables and milk production responses ..... 181

RESULTS ...................................................................................................................... 182

Annual Rhythms within Selected States ................................................................. 182

Annual Rhythms among Breeds ............................................................................. 183

Comparison of Annual Rhythm and Temperature Models .................................. 185

Relationships among Environmental Variables and Milk Production .............. 186

Regression Equations to Adjust for Seasonal Changes in Production ............... 187

DISCUSSION ................................................................................................................ 187

CONCLUSIONS .......................................................................................................... 193

FIGURES ..................................................................................................................... 195

TABLES ....................................................................................................................... 198

SUPPLEMENTAL TABLES .......................................................................................... 201

Chapter 9 Integrative Discussion ......................................................................... 205

References ................................................................................................................... 212
LIST OF FIGURES

Figure 2-1. Parameters used in the quantification of biological rhythms. .......................... 36
Figure 2-2. Structure of the mammalian transcriptional circadian clock. ............................. 37
Figure 2-3. Physiological mechanisms governing seasonal reproduction. ............................ 38
Figure 3-1. Effect of day- versus night-restricted feeding on the daily pattern of feed intake.................................................................................................................. 55
Figure 3-2. Effect of day- versus night-restricted feeding on daily rhythms of milk yield and milk components. .......................................................................................................................... 56
Figure 3-3. Effect of day- versus night-restricted feeding on the daily production and daily pattern of milk fatty acids. ........................................................................................................ 57
Figure 3-4. Effect of day- versus night-restricted feeding on daily rhythms of plasma hormones and metabolites .............................................................................................................. 58
Figure 3-5. Effect of day- versus night-restricted feeding on the circadian rhythm of body temperature in dairy cows. .................................................................................................................. 59
Figure 4-1. Schedule of feeding, lighting and sampling during the experiment ................. 82
Figure 4-2. Effect of night-restricted feeding on average daily feed intake and milk synthesis................................................................................................................................. 83
Figure 4-3. Effect of night-restricted feeding on milk fatty acid yields by origin .......... 84
Figure 4-4. Effect of night-restricted feeding on the daily patterns of expression of molecular clock genes in the mammary gland. .................................................................................. 85
Figure 4-5. Effect of night-restricted feeding on the daily patterns of plasma metabolites. ................................................................................................................................................ 86
Figure 5-1. Schedule of feeding, lighting and sampling during the experiment ............ 108
Figure 5-2. Effect of time of fatty acid infusion on dry matter intake. ............................ 109
Figure 5-3. Effect of time of fatty acid infusion on daily milk, fat and protein yield and fat and protein concentration. ................................................................................................................. 110
Figure 5-4. The effects of time of fatty acid infusion on the daily rhythms of milk synthesis........................................................................................................................................ 111
Figure 5-5. Effect of time of fatty acid infusion on the daily yields of fatty acids ....... 112
Figure 5-6. Effect of time of fatty acid infusion on daily rhythms of fatty acids by source. ........................................................................................................................................ 113
Figure 5-7. The effects of time of fatty acid infusion on daily rhythms of plasma metabolites .................................................................................................................................. 114
Figure 5-8. The effect of the time of fatty acid infusion on the daily rhythms of core body temperature. .................................................................................................................. 115
Figure 6-1. Schedule of feeding, lighting and sampling during the experiment .......... 136
Figure 6-2. Effect of time of protein infusion on dry matter intake. ............................. 137
Figure 6-3. Effect of time of protein infusion on daily milk, fat and protein yield and fat and protein concentration. ........................................................................................................ 138
Figure 6-4. The effects of time of protein infusion on daily rhythms of milk synthesis. ............................................................................................................................................... 139

Figure 6-5. Effect of time of protein infusion on daily yields and daily rhythms of milk
FA by source. ........................................................................................................................................... 140

Figure 6-6. The effects of time of protein infusion on daily rhythms of plasma
metabolites. .................................................................................................................................................. 141

Figure 7-1. Map of United States Federal Milk Marketing Orders. .............................. 141

Figure 7-2. Annual rhythms of fat concentration in selected Federal Milk Market orders.
................................................................................................................................................................. 164

Figure 7-3. Annual rhythms of protein concentration in selected Federal Milk Market
orders.......................................................................................................................................................... 165

Figure 7-4. The effect of parity on annual rhythms of milk yield and composition..... 166

Figure 7-5. The effect of DGAT1 K232A polymorphism on annual rhythms of milk and
milk fat concentration and yield. .................................................................................................................. 167

Figure 8-1. Annual rhythms of milk, fat and protein yield and fat and protein
concentration in Holstein dairy herds in Florida (FL), Minnesota (MN), Pennsylvania
(PA), and Texas (TX). ................................................................................................................................ 167

Figure 8-2. Annual rhythms of milk and milk fat and protein yield and milk fat and
protein concentration by breed................................................................................................................... 168

Figure 8-3. Relationship among environmental predictors and annual rhythms of milk
and milk fat and protein yield and milk fat and protein concentration by breed. ............ 169
LIST OF TABLES

Table 3-1. Effect of day versus night-restricted feeding on total daily DMI, milk yield, and milk composition.......................................................... 60
Table 4-1. Bovine primers used to quantify mammary gene expression with RT-qPCR. 87
Table 7-1. The acrophase and amplitude of a cosine function with a 12-month period fit to the regional monthly averages of milk fat and protein concentration. ................. 169
Table 8-1. Comparison between models testing the fit of a cosine function versus maximum temperature. ................................................................. 198
Table 8-2. Equations for cosine regression equations for milk and milk fat and protein yield and milk fat and protein concentration for Florida (FL), Minnesota (MN), Pennsylvania (PA), and Texas (TX). ................................................................. 199
Table 8-3. A summary of milk yield and milk fat and protein concentration responses in experiments testing the effects of heat stress in environmental chambers. ............... 200
LIST OF SUPPLEMENTAL FIGURES

Supplemental Figure S3-1. Effect of the day versus night-restricted feeding on feeding behavior................................................................. 61
Supplemental Figure S5-1. Effect of time of fatty acid infusion on milk urea nitrogen concentration................................................................................................................................. 116
Supplemental Figure S 6-1. Effect of time of protein infusion on lactose yield........ 142
Supplemental Figure S7-1. Annual rhythms of fat concentration in Federal Milk Market orders................................................................................................................................. 170
Supplemental Figure S7-2. Annual rhythms of protein concentration in Federal Milk Market orders................................................................. 171
Supplemental Figure S7-3. Variability in acrophase and amplitude of daily rhythms of milk, fat and protein yield among 11 herds in Pennsylvania................................. 172
Supplemental Figure S7-4. Variability in acrophase and amplitude of daily rhythms of milk fat and protein concentration among 11 herds in Pennsylvania......................... 173
Supplemental Figure S7-5. Repeatability of annual rhythms across years............. 174
LIST OF SUPPLEMENTAL TABLES

Supplemental Table S3-1. Diet and nutrient composition of the experimental diet...... 62
Supplemental Table S3-2. Effect of day- versus night-restricted feeding on total daily and daily rhythms of individual milk fatty acids. ............................................................. 63
Supplemental Table S4-1. Diet and nutrient composition of experimental diet......... 88
Supplemental Table S4-2. Effect of night-restricted feeding on daily yields of individual milk fatty acids................................................................. 89
Supplemental Table S5-1. Diet and nutrient composition of the experimental diet..... 117
Supplemental Table S5-2. Fatty acid profile of oil used for abomasal infusion of fatty acids. .............................................................................................................. 118
Supplemental Table S6-1. Diet and nutrient composition of the experimental diet.... 143
Supplemental Table S6-2. Amino acid profile of sodium caseinate. ......................... 144
Supplemental Table S8-1. Adjustment factors to correct for annual rhythms of milk production in Florida.............................................................................................. 201
Supplemental Table S8-2. Adjustment factors to correct for annual rhythms of milk production in Minnesota................................................................. 202
Supplemental Table S8-3. Adjustment factors to correct for annual rhythms of milk production in Pennsylvania.................................................................................. 203
Supplemental Table S8-4. Adjustment factors to correct for annual rhythms of milk production in Texas......................................................................................... 204
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-NAT</td>
<td>Arylalkylamine N-acetyl transferase</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACTB</td>
<td>Beta-actin</td>
</tr>
<tr>
<td>ADF</td>
<td>Acid detergent fiber</td>
</tr>
<tr>
<td>AICAR</td>
<td>AMPK-inhibitors 5-aminoimidazole-4-carboxamide ribonucleoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>(AMP)-activated protein kinase</td>
</tr>
<tr>
<td>AMS</td>
<td>Agricultural Marketing Service</td>
</tr>
<tr>
<td>B2M</td>
<td>Beta-2 microglobulin</td>
</tr>
<tr>
<td>BCFA</td>
<td>Branched-chain fatty acid</td>
</tr>
<tr>
<td>BH</td>
<td>Biohydrogenation</td>
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<td>BHBA</td>
<td>β-hydroxybutyrate</td>
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<td>BMAL1</td>
<td>Brain and muscle ARNT-like 1</td>
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<tr>
<td>BVD</td>
<td>Bovine viral diarrhea</td>
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<tr>
<td>CCG</td>
<td>Clock controlled gene</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Circadian locomotor output cycles kaput</td>
</tr>
<tr>
<td>CIRP</td>
<td>Cold-inducible RNA-binding proteins</td>
</tr>
<tr>
<td>CK1ε</td>
<td>Casein kinase 1ε</td>
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<tr>
<td>CP</td>
<td>Crude protein</td>
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<tr>
<td>CPT</td>
<td>Carnitine palmitoyltransferase</td>
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<tr>
<td>CRY</td>
<td>Cryptochrome</td>
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<td>CYP7A1</td>
<td>7-alpha-hydroxylase</td>
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<td>D-box binding protein</td>
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<td>DGAT1</td>
<td>Diacylglycerol O-acyltransferase 1</td>
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<td>DHIA</td>
<td>Dairy Herd Information Association</td>
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<tr>
<td>DIO</td>
<td>Deiodinase</td>
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<td>DM</td>
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<td>Dorsomedial hypothalamus</td>
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<td>DMI</td>
<td>Dry matter intake</td>
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<td>DRMS</td>
<td>Dairy Records Management Systems</td>
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<td>E4BP4</td>
<td>E4- promoter binding protein</td>
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<td>Ether extract</td>
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<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAA</td>
<td>Food anticipatory activity</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FBXL3</td>
<td>F-box/LRR-repeat protein 3</td>
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<tr>
<td>FEO</td>
<td>Food-entrainable oscillator</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FMMO</td>
<td>Federal milk marketing order</td>
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FOXO1  Forkhead box protein O1  
GIP  Gastric inhibitory peptide  
GLP1  Glucagon-like peptide 1  
GRE  Glucocorticoid-response element  
HAT  Histone acetyltransferase  
HDAC  Histone deacetylase  
HLF  Hepatic leukemia factor  
HMG-CoA  3-hydroxy-3-methyl-glutaryl-Coenzyme A  
HSF1  Heat-shock factor 1  
IGF-1  Insulin-like growth factor  
ipRGC  Intrinsically photosensitive retinal ganglion cell  
KLF15  Krüppel-like factor 15  
LSM  Least square means  
MEL  Melatonin  
miRNA  MicroRNA  
mTOR  Mechanistic target of rapamycin  
MTP  Microsomal triglyceride transfer protein  
MUN  Milk urea nitrogen  
NAMPT  Nicotinamide phosphoribosyltransferase  
NDF  Neutral detergent fiber  
NE  Norepinephrine  
NF-κB  Nuclear factor-κB  
NK  Natural killer  
NPAS4  Neuronal PAS domain protein 4  
OBCFA  Odd- and branched-chain fatty acids  
OCFA  Odd-chain fatty acid  
OTU  Operational taxonomic unit  
PBMC  Polymorphonuclear blood monocyte  
PER  Period  
PGC-1α  Proliferator-activated receptor gamma cofactor 1α  
PPAR  Peroxisome proliferator-activated receptor  
PPRE  Peroxisome proliferator-activated receptors response element  
PUN  Plasma urea nitrogen  
psbAI  Photosystem II protein  
PT  Pars tuberalis  
PTB  Polypyrrolidine tract-binding protein  
PVN  Paraventricular nucleus  
qPCR  Quantitative polymerase chain reaction  
RAPTOR  Regulatory-associated protein of mTOR  
ROR  Retinoic acid-related orphan receptor  
RORE  Retinoic acid-related orphan receptor binding element  
RPS9  Ribosomal protein 9
<table>
<thead>
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<tr>
<td>S14</td>
<td>Spot 14</td>
</tr>
<tr>
<td>SCC</td>
<td>Somatic cell count</td>
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<tr>
<td>SCD</td>
<td>Stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SDPP</td>
<td>Short day photoperiod</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
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<tr>
<td>SHP</td>
<td>Small heterodimer partner</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtulin 1</td>
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<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
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<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Thyroxine</td>
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<tr>
<td>t10</td>
<td>trans-10 C18:1</td>
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<td>t11</td>
<td>trans-11 C18:1</td>
</tr>
<tr>
<td>TEF</td>
<td>Thyrotrophic embryonic factor</td>
</tr>
<tr>
<td>TMR</td>
<td>Total mixed ration</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
</tr>
<tr>
<td>TTFL</td>
<td>Transcription translation feedback loop</td>
</tr>
<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acid</td>
</tr>
<tr>
<td>VMH</td>
<td>Ventromedial hypothalamus</td>
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</table>
ACKNOWLEDGEMENTS

I have frequently joked that all the friends and family members that have provided love and support throughout my Ph.D. program and should be listed as co-authors on all of my manuscripts because the research truly could not have been performed without them. In the sincerest sense, this is true. I have been extremely blessed to have an incredible support system around me over the past 4 years, and there is no conceivable way I could have finished my Ph.D. without them.

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Chapter 1

Introduction

Milk production from dairy cows provides an important source of nutrients for human diets. As ruminants, dairy cows convert fibrous plant material into high-quality protein, making them integral to sustainable food production. Moreover, the dairy industry is an important piece of the United States agricultural economy and accounts for approximately 10% of total agricultural sales according to the USDA Economic Research Service. Globally, the per capita demand for dairy products has increased a total of 9% since 1970, and is expected to rise another 20% from now until 2030 as economic development continues throughout the world (FAO, 2012). The dairy industry, along with all of animal agriculture, has been receiving increased scrutiny regarding potential negative environmental impacts. There has been public pressure to reduce greenhouse gas emissions from livestock species in order to slow the effects of global climate change. Additionally, over the past 4 years, the U.S. dairy industry has experienced a period of unprecedentedly low milk prices, making margins for dairy producers low to nonexistent. Together, these demands have created imminent desire for creative solutions to improve the efficiency of milk production.

One potential approach to improve the efficiency of dairy cattle is by developing a better understanding of the biological rhythms governing milk synthesis. Biological rhythms are endogenous cycles that govern the behavior and physiology of nearly all organisms and allow them to predict changes in the external environment before they occur. These rhythms occur across multiple time scales ranging from a year to less than a day. Of these, the most commonly described are circadian rhythms, which are approximately 24 h in length, and circannual rhythms,
which are approximately 1 year. Both circadian and circannual rhythms are controlled at a cellular level through a host of proteins comprising a ‘molecular clock’. In recent years, there has been an increasing body of literature demonstrating an intimate relationship between these biological rhythms and metabolism in laboratory models and humans. Diseases such as obesity and type II diabetes have been associated with disruption of circadian rhythms and nutritional approaches such as time-restricted feeding appear to have potential benefits for metabolic health and reduced aging (Kessler and Pivovarova-Ramich, 2019). However, there is still a dearth of research examining the regulation of these biological rhythms in livestock species.

While still poorly understood, there is compelling evidence to suggest that biological rhythms are important for milk synthesis in dairy cows. Dairy cows exhibit differences in milk yield and composition across the day, with milk yield being greater in the morning than evening and milk fat and protein concentration being greater in the evening (Gilbert et al., 1972). Initial research has suggested that these patterns represent a daily rhythm and that this rhythm is responsive to changes in feeding time (Rottman et al., 2014). Additionally, several hormones and metabolites follow a diurnal pattern in dairy cows, suggesting that the metabolism of other tissues is also controlled by circadian rhythms (Giannetto and Piccione, 2009). Milk yield also follows a yearly pattern, with increases in milk and milk components during the winter and decreases in the summer (Wood, 1976). While these rhythms appear to be important, little is known about their regulation. Developing a better understanding of these biological rhythms may allow for development of nutritional and management strategies to improve the efficiency of dairy production.

The overall objective of this dissertation was to examine the relationship factors regulating the biological rhythms of milk synthesis. Specifically, we investigated the effects of nutrient intake on the daily rhythms of milk synthesis, as well as the relationships among cow- and environmental factors and the annual rhythms of milk production. We expect that total food
intake and individual macronutrients will entrain the daily rhythms of milk synthesis by modifying the circadian clock of the mammary gland. Moreover, we anticipate that an annual rhythm governs yearly changes in milk production, and that this rhythm is consistent among years, herds, and individual cows, but is responsive to changes in photoperiod.

First, a review of the literature providing a background of the basic molecular mechanisms governing circadian and circannual rhythms, as well as their relationships to food intake and metabolism is presented (Chapter 2). The aim of the first experiment (Chapter 3) was to examine the effects of the time of feed intake on the daily rhythms of milk synthesis by restricting the time of feed availability to either the day or night. We expected that the daily rhythms of milk synthesis would be shifted by the time of feed restriction. To investigate if changes in the rhythms of milk synthesis due to the time of feed intake were mediated by the molecular circadian clock, a follow-up experiment examining the role of time-restricted feeding on the daily patterns of clock gene expression in the mammary gland was conducted (Chapter 4). We expected that expression of clock genes would be rhythmic and that the rhythms would be shifted by night-restricted feeding. The next two experiments focused on the importance of individual nutrients for entrainment of the daily rhythms of milk synthesis. Chapter 5 examined the role of the timing of fatty acid infusion and Chapter 6 examined the role of the timing of protein infusion over the day on the daily rhythm of milk synthesis. We expected that the time of fat infusion would alter the daily rhythms of milk and milk fat synthesis, while the time of protein infusion would alter the daily rhythms of milk and milk protein synthesis.

Chapters 7 and 8 focus on the factors contributing to annual rhythms of milk synthesis. The analysis reported in Chapter 7, which has been published in the Journal of Dairy Science, examines the effects of geographic region on annual rhythm of fat and protein concentration using data from U.S. milk markets, as well as the factors such as lactation number and genotype. We expected that lactation number and genotype will not affect annual rhythms, but that they will
be affected by geographic region. This experiment was followed by another analysis using a large dataset from Dairy Records Management Systems to determine better elucidate the effects of region of the U.S. on the annual rhythms of milk, fat and protein yield and fat and protein concentration, as well as examine potential environmental signals entraining these rhythms (Chapter 8). Based on the results of the previous experiment, we expected that geographic region would affect the annual rhythms, and these rhythms would be highly correlated with the change in photoperiod. Finally, an integrative discussion describing the potential implications, limitations of these experiments, and future directions is provided in Chapter 9.
Chapter 2

Literature Review

Biological Rhythms

The ability of organisms to perceive time and coordinate behavior and metabolism bestows a tremendous evolutionary advantage, allowing them to maximize energetic resources and avoid harmful circumstances. Reliably predicting food availability and predator activity, even by mere minutes, can be the difference between survival and death for many species. Furthermore, the ability for an organism to monitor time of day allows them to temporally segregate incompatible physiological processes and maximize energetic efficiency by preventing futile cycles of metabolism. To facilitate the coordination between the temporal changes in the external environment, and their own intrinsic metabolism, animals have developed inherent biological rhythms. These rhythms persist in the absence of external cues and are responsive to entrainment, or resetting environmental signals.

The most well-studied biological rhythms are circadian rhythms, which are repeating biological cycles of approximately 24 h. The term circadian was coined in 1959 by Franz Halberg and is derived from the Latin terms circa, meaning ‘about’; and diēm, meaning ‘day’ (Halberg et al., 1959). However, descriptions of daily rhythms had been made much earlier. Androsthenes, a scribe for Alexander the Great discovered daily patterns of tamarind (Tamarindus indica) leaf movements in the 4th century B.C. (Moore-Ede, 1982). These first recorded observations indicating that daily rhythms may be endogenous were made Jean-Jacques d’Ortous de Mairan in 1729. He noted that the flowering plant Mimosa pudica, which goes
through cycles of open leaves during the day and closed leaves overnight, continued to follow this pattern even when plants were placed in constant darkness (de Mairan, 1729). This discovery was revisited nearly 100 years later by Alphonse de Candolle who observed that the rhythm of plant movement also persisted under constant light, and that the period of leaf movement was slightly shorter than 24 h (De Candolle, 1832).

While these initial discoveries were important to developing the idea of endogenous daily rhythms, the field of chronobiology – the study of biological rhythms - was not established until the middle of the 20th century. Three scientists, Erwin Bünning, Jürgen Aschoff and Colin Pittendrigh are credited for developing the foundational principles of biological rhythms. Together they established that biological rhythms are endogenous, meaning that they are self-sustained through physiological mechanisms in the animal, that they are free-running, meaning that they can persist without external stimuli, and that they are entrainable, meaning that they are adaptable to changes in the environment (Daan, 2000). At the same time, biological rhythms on time scales greater or less than 24 h were proposed. Aschoff (1955) first suggested that seasonal changes in behavior such as hibernation or migration may be due to an endogenous ‘circannual’ rhythm. This proposition was later confirmed in Willow Warblers (Phylloscopus trochilus), which exhibited seasonal migratory behavior when placed in constant conditions (Gwinner, 1968). Moreover, great steps in the quantification of biological rhythms were made during this time. Biological rhythms are characterized by their period length, which is the amount of time to complete one cycle (e.g. 24 h, 12 m), acrophase, which is the time of the rhythm’s peak, and amplitude, or the distance from mean to peak (Figure 2-1).

A dogmatic shift in the understanding of biological rhythms was initiated by the now classic experiment performed by Konopka and Benzer (1971), who used mutagenesis to demonstrate that the length of a free-running circadian rhythm was controlled at the level of the gene. Later, an entire host of genes were discovered to make up what is now known as the
‘biological clock’, a network of transcription factors that differentially regulate gene expression across the 24 h day (Huang, 2018). These same transcription factors were later discovered to be responsible for the generation of circannual rhythms (Lincoln, 2019).

Molecular Structure of Biological Clocks

Circadian rhythms are generated in mammals through a hierarchical network of independent cellular clocks that communicate through neural and humoral signals. Every individual cell possesses their own molecular clock that is regulated by intracellular signaling cascades. A region of ~20,000 neurons in the hypothalamus known as the suprachiasmatic nucleus (SCN) is commonly acknowledged as the master pacemaker that is principally responsible for entrainment of rhythms in peripheral tissues (Partch et al., 2014). In addition to receiving inputs from the SCN, peripheral tissues relay rhythmic information to each other. For example, melatonin produced by the pineal gland and glucocorticoids produced by the adrenal cortex are important entraining signals for the clock of the pancreas, liver, and mammary gland (Mohawk et al., 2012). Peripheral tissues can also be entrained by other cues, primarily feed intake (Stokkan et al., 2001). Uncoupling of central SCN rhythms from rhythms of feed intake is proposed as a mechanism for the development of the metabolic disturbances caused by circadian maladies such as shift-work disorder chronic jet lag (Vetter and Scheer, 2017).

Transcriptional-Translational Feedback Loops

At its most basic level, the molecular circadian clock operates through interconnected transcriptional-translational feedback loops employing an array of ‘clock’ transcription factors (Figure 2-2). In mammals, the core negative feedback loop includes brain and muscle ARNT-like 1 (BMAL1), circadian locomotor output cycles kaput (CLOCK), period (PER) 1, 2 and 3, and cryptochrome (CRY) 1 and 2. BMAL1 and CLOCK form heterodimers that bind to specific DNA
sites called E-boxes [canonically CANNTG] in the promoter region of clock-controlled genes. Additionally, the BMAL1-CLOCK complex enhances the expression of PER and CRY proteins, which themselves dimerize and feedback on the BMAL1 and CLOCK genes to repress their expression (Takahashi, 2017). This cycle of activation and repression generates a rhythm of clock gene expression that is completed in approximately 24 h, which leads to 24 h rhythms of clock-controlled genes.

An ancillary feedback loop consisting of the orphan nuclear receptors REV-ERBα and β and RAR-related orphan receptor (ROR) α, β, and γ adds additional level of regulation to stabilize the core clock and provide secondary outputs for regulation of clock-controlled genes. Both receptors bind to retinoic acid-related orphan receptor binding elements (ROREs) [(G/A)GGTCA], with the ROR enhancing transcription and REV-ERB repressing transcription (Takahashi, 2017). Both BMAL1 and CLOCK contain ROREs and competitive binding by REV-ERB and ROR leads to either repression or activation of these transcription factors (Guillaumond et al., 2005). A third level of clock regulation utilizes the transcription factors D-box binding protein (DBP), thyrotrophic embryonic factor (TEF), hepatic leukemia factor (HLF) and E4-promoter binding protein (E4BP4, also known as NFIL3). The expression of DBP, TEF, and HLF is enhanced by the binding of the BMAL1-CLOCK complex to E-Box sequences, and themselves act as transcription enhancers by binding to D-Box promoter elements. Alternatively, E4BP4 expression is activated by REV-ERBα, and represses expression of D-Box element-containing genes through interference with DBP, TEF, and HLF binding (Papazyan et al., 2016). The transcription of clock-controlled genes is therefore coordinated through regulation by a wide host of transcription factors that act on either E-Box, RORE, or D-Box elements to influence their expression.
Post-Transcriptional Modifications

Beyond the transcriptional-translational feedback loops, a number of post-transcriptional modifications add an additional layer of regulation to affect both the rhythms of clock gene expression and their outputs. The importance of these modifications is becoming increasingly clear, with Reddy et al. (2006) demonstrating that nearly half of the rhythmic proteins present in the liver lacked corresponding rhythms in mRNA expression. Transcriptional termination is an important point of regulation for mRNA expression because it is required before transcriptional initiation can proceed. In addition to its role in E-box mediated transcriptional repression, PER represses transcriptional initiation through rhythmically binding to several RNA helicases, which make up the transcriptional termination complex for certain genes (Lim and Allada, 2013). Recent evidence suggests that alternative splicing of pre-mRNA is also regulated in a circadian manner (McGlincy et al., 2012). Microarray data demonstrated that 62 of 227 measured splicing factors displayed rhythmic gene expression (Grosso et al., 2008). Furthermore, Preußner et al. (2017) illustrated that circadian rhythms of body temperature entrain cellular rhythms of alternate splicing in the cerebellum and liver with the effect via heat-sensitive SR proteins.

The stability of mRNA is also influenced by circadian rhythms through silencing by RNA-binding proteins and microRNA (miRNA). Period 2 and CRY1 stability is decreased by RNA-binding proteins polypyrimidine tract-binding protein (PTB) and AU-rich element RNA-binding protein 1. Deletions of these proteins result in increased amplitudes of the circadian mRNA expression (Green, 2018). MicroRNA are small (~22 nt) non-coding RNA molecules that interact with the 3’ untranslated region of mRNA to repress its translation. The light inducible miRNA miR-132 augments the rhythms of PER1 expression in murine SCN neurons (Cheng et al., 2007). In the same study, miR-219-1 was identified as clock-controlled miRNA whose rhythm is ablated in CRY1/CRY2 double knockout mice. Later research implicated miR-219 as a link between shift-work disorder and increased breast cancer. Night shift workers have greater
methylation of the miR-219 promoter, which downregulates its expression and attenuates its anticarcinogenic activity (Shi et al., 2013). The miRNA 192/194 cluster was discovered to inhibit translation of all three PER genes, and overexpression and knockdown of these miRNAs leads to shortening and lengthening of the circadian period, respectively (Nagel et al., 2009). Furthermore, miR-122 is regulated in a circadian manner in the liver and causes rhythmic degradation of several genes involved in cholesterol and lipid metabolism (Mehta and Cheng, 2013).

**Post-Translational Modifications**

Post-translational modifications are also vital to the generation of 24-h rhythms within cells. Modifications to clock proteins introduce a delay between the activating and repressing arms of the transcriptional-translational feedback loop, ensuring that the cycle is maintained at a period of ~24 h. Without this delay, the cycle of activation and repression would last just a few hours (Gallego and Virshup, 2007). The most well-established example of post-translational regulation of circadian period length in mammals occurs through casein kinase 1ε-mediated phosphorylation. Casein kinase 1ε (CK1ε) decreases the stability of PER through phosphorylation, leading to its degradation via the ubiquitin-mediated proteosomal pathway (Vielhaber et al., 2000).

The importance of phosphorylation in the establishment of cell-autonomous rhythms is perpetuated by presence of circadian rhythms in cells that are completely devoid of rhythmic gene expression. Prokaryotic cyanobacteria can maintain 24-h cycles of growth, nitrogen fixation, and photosynthesis with a molecular clock based only on rhythmic phosphorylation of KaiA, KaiB, and KaiC proteins (Takai et al., 2006). Moreover, circadian oscillations of peroxiredoxin oxidation occur in differentiated red blood cells which contain no nucleus or DNA, suggesting that these oscillations are maintained through exclusively post-translational mechanisms (O’Neill and Reddy, 2011). Phosphoproteomic analysis is further uncovering the indispensable role of
phosphorylation in generation of circadian rhythms. Robles et al. (2017) determined using mass spectrometry that 25% (> 20,000 phosphosites) of the murine hepatic phosphoproteome are follow a 24 h rhythmic pattern, with phosphorylation cycles having markedly greater amplitudes than the rhythms of transcript or protein abundance.

**Epigenetics and the Clock**

Epigenetic modifications, such as DNA methylation and histone acetylation/deacetylation have a bidirectional link with the circadian clock. In the liver of mice, acetylation of the H3 histone follows a circadian rhythm, causing chromatin remodeling near the promoter regions of *Per1*, *Per2*, and *Cry1*, permitting their expression (Etchegaray et al., 2003). The circadian transcription factor CLOCK is itself a histone acetyltransferase (HAT), and its function is potentiated by its heterodimer BMAL (Doi et al., 2006). REV-ERBα, a transcription factor of the ancillary molecular clock, helps recruit the histone deacetylase 3 (HDAC3) to specific sites on histone 3, inhibiting the transcription of target genes (Yin et al., 2007). Azzi et al. (2014) demonstrated that DNA methylation patterns in the SCN of mice were associated with changes in the light-dark cycle.

**Entrainment of Biological Clocks**

Entrainment is the process of synchronizing endogenous biological clocks with external environmental rhythms. Environmental cues, called *Zeitgebers* (German for “time giver”), confer information about the environmental time to circadian oscillators to synchronize an organism’s internal clock (Aschoff, 1963). All organisms have endogenous rhythms that differ slightly from 24 h which allow them to be responsive to entrainment signals from the environment. When zeitgebers are absent, the rhythm is said to be freerunning, meaning that it expresses its endogenous period length. The length of the freerunning period, also called tau (τ), differs between species and even individuals. Humans, for example, have an average freerunning period
of 24.2 h (Czeisler et al., 1999). Entrainment of circadian clocks can occur through several cues, including the light/dark cycle, nutrient intake, and body temperature.

**Light Entrainment**

Light is the most prominent zeitgeber known to entrain the central clock of the SCN. Light is detected by the eye and transmitted to the SCN through two separate pathways. Rod and cone cells are the primary visual photoreceptors in the retina. After sensing light, they signal the SCN via retinal ganglion cells, setting its circadian clock (Drouyer et al., 2007). However, light entrainment can occur completely independent of rod and cone cells. Foster et al. (1991) demonstrated that mice possessing the rd/rd mutation, which causes degeneration of rod and cone cells and complete blindness, still entrained to the light-dark cycle, suggesting that an additional photoreceptor was involved in circadian entrainment. It was later discovered that the non-visual photopigment melanopsin can entrain the master clock through excitation of intrinsically photosensitive retinal ganglion cells (ipRGCs) in the eye (Panda et al., 2002). Exposure of ipRGCs to light causes photoisomerization of the melanopsin protein, leading to a G-protein signaling cascade which causes cell depolarization and increases the nerve firing rate (Hattar et al., 2002). The ipRGCs directly innervate the SCN via the retinohypothalamic tract, conveying light signals directly to the master clock. Panda et al. (2003) later confirmed the independence of circadian oscillation and rod and cone cells using rd/rd mice, as well as mice lacking melanopsin (Opn4−/−) and mice with both mutations. They found that photoentrainment occurred in mouse strains with single deletions, but did not occur in the absence of both receptor systems. In addition to establishing two separate pathways of circadian entrainment this study submitted that unlike birds, reptiles, fish and invertebrates which can detect light extra-ocularly in the pineal gland or deep-brain photoreceptors, mammals require ocular photoreceptors for photoentrainment.
**Food Entrainment**

Circadian rhythms of mammals can be entrained by nutrient intake. Early observations of rat behavior revealed an increase in locomotor activity in the 2 to 4 h prior to feeding (Richter, 1922). These patterns of food anticipatory activity (FAA) were later shown to persist for up to 3 to 4 days after complete food removal (Stephan, 2002). While the light dark cycle is the predominant zeitgeber for entraining behavioral rhythms, circadian rhythms of FAA can overtake them during conditions of restricted food availability. Stokkan et al. (2001) detected a phase shift of *Per1* expression corresponding to feeding time in the liver and lung of mice, but not the SCN when food was restricted to the inactive period of the day. The amount of entrainment is proportional to the energy content of the feed, feeding a highly palatable, high-energy feed at a specific time each day is sufficient to entrain FAA, even if food availability is not restricted (Mistlberger, 1994). Additionally, food entrainment can maintain circadian behavioral rhythms when signaling from the central clock is absent. Krieger et al. (1977) observed that rats with lesions of their SCN still exhibited entrainment of daily locomotor activity, core body temperature, and blood glucocorticoid concentration rhythms to once per day feeding. These discoveries suggest that food entrainment is controlled by an independent oscillator separate from the SCN.

While there has been evidence substantiating the presence of food-entrainable circadian rhythms, the search for the so-called *food-entrainable oscillator* (FEO) has proven onerous. Initially, the adrenal gland was targeted as the source of food-entrainable oscillations because of the role of glucocorticoids in food anticipation, but adrenalectomy failed to affect the circadian rhythms of FAA (Stephan, 2002). Several regions of the central nervous system have been explored for their potential role as an FEO. Ablation of the ventromedial hypothalamus (VMH), a region involved in satiety signaling and thermoregulation, eliminated the entrainment of body temperature and glucocorticoids to meal time in some early short-term experiments, but this
effect did not occur when recovery of greater than 14 weeks was allowed following surgery (Davidson, 2009). More recent experiments have failed to show correlations between VMH lesions and FAA (Gooley et al., 2006). The paraventricular nucleus (PVN) is a region of the brain involved in appetite regulation and stress. Lesioning of the PVN failed to reduce food-anticipatory food-bin approach behavior, although general locomotion was reduced (Mistlberger and Rusak, 1988).

The dorsomedial hypothalamus (DMH) has been highly implicated as the location of an FEO, but results have been inconsistent. It was initially explored as a candidate because of its previously discovered roles in feeding behavior regulation, stress response, and involvement in the circadian rhythms of cortisol release (Chou et al., 2003). Gooley et al. (2006) observed that food restriction synchronizes daily rhythms of DMH neuronal firing to the time of feed delivery. Additionally, rhythmic expression of Per1 and Per2 is induced in the DMH during time-restricted feeding (Mieda et al., 2006). However, Landry et al. (2006) failed to impair daily rhythms of FAA upon partial or complete lesioning of the DMH. These results were later corroborated by Landry et al. (2007) and Moriya et al. (2009). More recent evidence suggests that the effect of DMH lesions on FAA is time-of-day dependent. Landry et al. (2011) demonstrated that DMH-lesioned rats had less robust, but still present, rhythms of FAA when fed during the day, but that FAA was unaffected when they were fed at night. The inability to isolate the FEO to a specific region of the brain suggests that food-entrained rhythms may actually be generated by a network of separate oscillators that communicate with each other to maintain food-anticipatory rhythms of behavior, body temperature, and glucocorticoid signaling.

Communication from the viscera to the brain has been investigated as a mechanism for entrainment by nutrient intake. However, ablation of both vagal and non-vagal afferents nerves emanating from the viscera did not alter FAA (Davidson, 2009). Ghrelin is a peptide hormone produced by the oxyntic cells of the stomach that is directly responsible for hunger signaling. The
expression of *Per1* and *Per2* in oxyntic cells is in antiphase with ghrelin secretion, and the rhythms of their expression are altered by feeding time (LeSauter et al., 2009). Knockout of the ghrelin receptor greatly reduces food-anticipatory locomotor activity in mice, suggesting that ghrelin is important for this behavior (Blum et al., 2009). Research performed in goldfish (*Carassius auratus*) demonstrated that ghrelin treatment elevated hepatic and hypothalamic *Per1*, 2 and 3 expression by over 2-fold and that treatment with a ghrelin antagonist completely abolished daily rhythms of FAA (Nisembaum et al., 2014).

**Temperature Entrainment**

Environmental temperature is a common entraining cue for poikilotherms, who experience dramatic changes in physiology when external temperature changes. Additionally, Some plants and algae can entrain to just 1 to 2°C differences in temperature across the day (Rensing and Ruoff, 2002). In mammals, the circadian rhythms of body temperature can entrain biological clocks in peripheral tissues. Brown et al. (2002) ascertained that rhythms of *Per2* expression could be entrained in rat fibroblast cells by temperature cycles that oscillated by 4°C. Saini et al. (2012) later revealed similar results in human and mice fibroblast, but revealed that as little as a 1°C change in temperature is sufficient for entrainment. These authors also revealed that temperature entrainment was dependent on the protein heat-shock factor 1 (HSF1). In addition to HSF1, cold-inducible RNA-binding proteins (CIRPs) link body temperature and the circadian clock. Cycles of body temperature affect the regulation of polyadenylation sites by CIRP, thus affecting the stability of target RNAs (Ki et al., 2015). While peripheral tissues appear responsive to temperature, the SCN possesses mechanisms to resist feedback of body temperature on the central clock. Buhr et al. (2010) discovered that communication between the dorsomedial and ventrolateral regions of the SCN via L-type calcium channels prevents temperature from resetting the SCN clock.
Circadian Rhythms and Nutrient Metabolism

As mentioned above, feeding behavior is a strong clue for circadian entrainment. In addition to entrainment of whole-body rhythms by meal delivery or high-calorie feed supplementation, specific nutrients can directly affect the molecular clock within individual cells. Cellular energy status, redox status, and direct action by metabolically important hormones can directly impact the circadian clock (Peek et al., 2012). Furthermore, the molecular clock exerts a reciprocal relationship on cellular metabolism. By temporally segregating biochemically incompatible processes, the circadian clock prevents futile cycling and maximized energetic resources within the cell. Moreover, the efficiency of nutrient utilization is optimized by the circadian clock because it coordinates the proper metabolic machinery with the time of nutrient availability.

Cellular Energy and Redox Status and the Circadian Clock

Cellular energy status can entrain circadian rhythms through adenosine monophosphate (AMP)-activated protein kinase (AMPK). During states of prolonged fasting and ATP depletion, AMPK is activated. Activated AMPK can then affect the period length of the cellular circadian clock by two separate pathways leading to CRY or PER degradation. First, AMPK phosphorylates CK1ε at serine 389, which causes an increase in its activity leading to increased degradation of PER2 and shortening of the circadian period (Jordan and Lamia, 2013). Furthermore, AMPK can directly phosphorylate CRY1 and CRY2, which then interact with F-box/LRR-repeat protein 3 (FBXL3), leading to their polyubiquitination and proteosomal degradation (Lamia et al., 2009). Treatment with the AMPK antagonists, AMPK-inhibitors 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) or metformin, has been shown to cause a phase shift in the circadian clock of the liver, suggesting that AMPK may play a role in
entrainment (Jordan and Lamia, 2013). In addition to its effects on circadian clock proteins, AMPK appears to be responsible for circadian regulation of its target proteins. Both acetyl-coA carboxylase (ACC), the rate-limiting enzyme for fatty acid biosynthesis, and regulatory-associated protein of mTOR (RAPTOR), a protein involved in signaling cascade of insulin to mechanistic target of rapamycin (mTOR), display 24 h rhythms of AMPK-mediated phosphorylation (Davies et al., 1992; Lamia et al., 2009).

Cellular redox state is dependent on the ratio of the oxidized and reduced forms of the cofactor nicotinamide adenine dinucleotide (NAD+/NADH). The molecular clock directly impacts redox status through regulation of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD+ salvage pathway (Peek et al., 2012). The CLOCK:BMAL1 complex binds to E-boxes in the NAMPT promoter, upregulating its expression and causing NAMPT and NAD+ to oscillate in a diurnal manner (Ramsey et al., 2009). The diurnal pattern of NAD+ also likely affects the circadian cycling of other metabolically important proteins though its effect on sirtulin 1 (SIRT1). Sirtulin 1 is an NAD+-dependent deacetylase that regulates the expression of a wide array of metabolically important transcription factors, including peroxisome proliferator-activated receptor gamma cofactor 1α (PGC-1α, involved in mitochondrial biogenesis and respiration), sterol regulatory element-binding protein 1c (SREBP-1c; involved in lipid synthesis and glucose metabolism), and forkhead box protein O1 (FOXO1; involved in insulin signaling) (Peek et al., 2012). Nakahata et al. (2009) confirmed that SIRT1 activity follows a circadian pattern and that selective inhibition of NAMPT can abolish this rhythm. Furthermore, SIRT1 can also feedback to inhibit the BMAL1-CLOCK complex through deacetylation of BMAL1, preventing activation of clock-controlled genes (Tong et al., 2015).

The cellular redox state or peroxiredoxin proteins has also been shown to itself be a circadian oscillator (Peek et al., 2012). Peroxiredoxins are proteins that both serve as antioxidants which scavenge hydrogen peroxide (H2O2) and whose regulation is a component of H2O2-
mediated signal transduction (Rhee et al., 2005). Therefore, the oxidation status of peroxiredoxins can affect the transcription and regulation of other target genes and proteins. O’Neill et al. (2011) discovered that peroxiredoxins can maintain self-sustained circadian oscillations, independent of transcription. They later discovered that this mechanism allows red blood cells, which lack a nucleus, to express circadian rhythms (O’Neill and Reddy, 2011). The oxidation rhythms of peroxiredoxins is out of phase with the rhythms of Bmal1 mRNA, and this phase relationship differs among tissues, suggesting the peroxiredoxins may represent an entirely different oscillator than the TTFL (Edgar et al., 2012).

**Glucose Metabolism and the Clock**

Glucose metabolism in mammals is highly influenced by the circadian system. Plasma glucose and insulin concentrations exhibit 24 h rhythms in a variety of species including humans (Van Cauter et al., 1991), mice (Sadacca et al., 2011), rats (Bellinger et al., 1975), sheep (McMillen et al., 1987), and cattle (Rottman et al., 2014). Furthermore, liver glycogen concentration follows an entrainable circadian rhythm (Halberg et al., 1960). Both Bmal1 and Clock are essential for maintenance of these rhythms, and global knockout of either gene abolishes the daily oscillations of plasma glucose concentration and hepatic gluconeogenesis (Rudic et al., 2004). In addition to absolute glucose and insulin concentrations, insulin-stimulated glucose uptake is controlled by circadian rhythms. In the medical field, knowledge that glucose tolerance in humans varies across the day has existed since the late 1960’s (Bowen and Reeves, 1967). Van Cauter et al. (1997) determined that 2 h post-infusion blood glucose concentrations are typically 30 to 50 mg/dL greater when glucose infusion is performed the afternoon compared to in the morning. The insulin-secreting islet β cells of the pancreas express 24 h rhythms of molecular clock genes in mice, and when these rhythms are abolished by tissue-specific Bmal1 knockout, subjects exhibited severe glucose tolerance and insufficient glucose-stimulated insulin production (Sadacca et al., 2011).
Besides its effects on glucose metabolism, the circadian clock appears to affect other aspects of carbohydrate metabolism. Thaiss et al. (2016) determined using metabolomic analysis that the conversion of sucrose to lactate was rhythmic.

Circadian rhythms of glucose metabolism may also be controlled through rhythms of incretin release. Incretins, which include glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) are released from the enteroendocrine cells of the gut in response to feed intake and enhance insulin release from the pancreas. Like insulin, the responsiveness of incretins to feed intake shows robust circadian rhythms in humans and model organisms (Reimann and Reddy, 2014). L cells, responsible for the production of GLP-1, show 24 h oscillations of *Bmal1* and *Per2 in vitro* (Reimann and Reddy, 2014). Gil-Lozano et al. (2014) illustrated that GLP-1 secretion in response to various secretagogues followed a diurnal pattern. A less well-characterized incretin, oxyntomodulin, appears to play an important role in food entrainment. Landgraf et al. (2015) detected that oxyntomodulin entrains the rhythms of *Per1* and *Per2* expression via binding to the glucocorticoid receptor and that knockout of this protein blocks food entrainment of the liver. Therefore, it is a promising candidate for the link between food intake and the molecular clock.

Another link between circadian clocks and glucose metabolism is glucocorticoid signaling. Glucocorticoids are a class of steroid hormones produced by the adrenal cortex that have a somewhat antagonistic role to insulin, causing greater hepatic gluconeogenesis, reduced glucose uptake, and increased lipid mobilization (Kuo et al., 2015). They are increased during fasting, but are also greatly elevated in response to stress. In addition to their role in stress and metabolism, glucocorticoids are a major output of the core circadian clock. Glucocorticoids follow a consistent daily rhythm that peaks at the start of the active phase in both nocturnal and diurnal animals (Dickmeis, 2009). This rhythm is modulated directly by the SCN, which innervates the adrenal gland and affects its release in a circadian manner (Mohawk et al., 2012).
Glucocorticoids directly entrain circadian rhythms in peripheral tissues. This mechanism is frequently exploited when studying circadian rhythms *in vitro*, when the synthetic glucocorticoid dexamethasone is used to synchronize cultured cells (Balsalobre et al., 2000). The promoter regions of *Bmal1, Cry1, Per1*, and *Per2* contain glucocorticoid-response elements (GREs) that regulate the circadian clock in peripheral tissues. (Mohawk et al., 2012). Furthermore, treatment with glucocorticoids decrease the rate of entrainment of peripheral clocks to the time of food intake (Minh et al., 2001). So et al. (2009) discovered that deletion of the GRE in *Per2* protects individuals from insulin resistance, demonstrating the intimate link between the glucocorticoid rhythm and glucose metabolism.

**Lipid Metabolism and the Clock**

Several aspects of lipid metabolism are controlled by the molecular clock. Carnitine palmitoyltransferase (CPT) 1 and 2 control fatty acid β-oxidation by limiting the rate of fatty acyl entry through the inner and outer mitochondrial membranes, respectively. The protein abundance of both CPT1 and CPT2 follows circadian rhythms with CPT1 peaking at 17 h post-light exposure and CPT2 peaking at 6 h post-light exposure (Neufeld-Cohen et al., 2016). Acetyl-CoA carboxylase, the enzyme that catalyzes the rate-limiting reaction of *de novo* lipogenesis, also follows a diurnal pattern, likely due to regulation by AMPK (Gnocchi et al., 2015). The master lipid regulator sterol regulatory element-binding transcription factor 1 (SREBP1) also displays circadian rhythms of activity, which appear to be directly driven by REV-ERBα (Le Martelot et al., 2009). Recent research has suggested that circadian rhythms of both SREBP1 and another lipogenic factor, thyroid hormone-responsive Spot 14 (S14), are entrained by the time of feed intake in the mammary gland of mice (Ma et al., 2013).

Lipid trafficking from the intestine also appears to be regulated in a circadian manner. Intestinal mRNA and protein expression and activity of microsomal triglyceride transfer protein
(MTP), an enzyme involved in the assembly of chylomicrons, follows a daily pattern with greatest expression at 2400 h. (Pan and Hussain, 2007). This daily pattern is regulated directly by CLOCK through its effects on the transcription factor called “small heterodimer partner (SHP)”, which enhances expression of MTP (Pan et al., 2010). Pan and Hussain (2007) revealed that absorption of \(^3\)H-labeled triolein cholesterol followed a daily pattern with greater absorption at 2400 h than 1200 h. Another potential link between the circadian clock and lipid transport is the protein nocturnin. Nocturnin controls gene expression by deadenylation of the poly(A) tail of mRNA which leads to its degradation, and its expression is activated by BMAL/CLOCK (Stubblefield, 2012). Global knockout of the nocturnin gene results in reduced export of chylomicrons out of enterocytes (Douris et al., 2011).

Bile acid metabolism and cholesterol synthesis are also intimately linked with the circadian clock. 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) reductase, the rate limiting enzyme for the synthesis of cholesterol, exhibits a diurnal pattern of expression leading to a circadian rhythm of cholesterol synthesis (Shefer et al., 1972). Additionally the rate-limiting enzyme in bile acid synthesis, 7-alpha-hydroxylase (CYP7A1) follows a circadian rhythm (Chiang, 2009). This rhythm is directly regulated by REV-ERB\(\alpha\), which binds to D-box elements on the CYP7A1 promoter and enhances its transcription. Circadian rhythms of bile acid synthesis allow for greater amounts of bile acids to enter the small intestine and emulsify dietary lipids during the active phase when food intake is more likely to occur. Knockout of the circadian clock has detrimental effects on bile acid homeostasis. Ma et al. (2009) discovered that \(Per1^{-/-}\ Per2^{-/-}\ double knockout mice lost their circadian rhythms of bile acid synthesis, leading to a greater synthesis and over accumulation of bile acids in the liver.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that act as lipid sensors and master regulators of lipid and glucose metabolism. The three primary isoforms include PPAR\(\alpha\), PPAR\(\gamma\), and PPAR\(\delta\), which have different binding affinities to specific fatty acids and are
distributed at different expression levels across tissues. All three PPAR isoforms, along with peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), display circadian rhythms of expression (Chen and Yang, 2014). An E-Box element has been identified in the promoter of PPARα in mice, suggesting direct regulation by BMAL/CLOCK (Oishi et al., 2005). The rhythms of PPARα are likely responsible for diurnal regulation of SREBP1 and fatty acid synthase (FAS) and HMG-CoA because it activates expression of these genes (Chen and Yang, 2014). Nuclear translocation of PPARγ in bone-marrow stromal cells is stimulated by the circadian polyadenylase nocturnin (Kawai et al., 2010). Furthermore, liver PPARδ is expression is regulated in a circadian manner by miR-122, a cyclic microRNA that is activated by REV-ERBα (Gatfield et al., 2009). The relationship between PPARs and the clock is bidirectional. The promoter regions of Bmal1 and Rev-erba possess PPAR response elements (PPREs) and are activated upon PPAR binding (Ribas-Latre and Eckel-Mahan, 2016). Through this mechanism, lipids feed back and affect the molecular clock.

**Circadian Rhythms in Microorganisms**

A growing body of evidence has suggested an important role for circadian rhythms in modulating growth and metabolism of microorganisms. The idea that microorganisms are capable of maintaining biological rhythms is relatively new. Prior to the 1980’s prokaryotes were considered too “simplistic” and too short-living to express circadian rhythms. Grobelaar et al. (1986) first observed endogenous circadian rhythms of nitrogen fixation in the cyanobacteria. These findings were later validated by Kondo et al. (1993), who used a luciferase reporter to demonstrate that the cyanobacterial species *Synechococcus* sp. PCC7942 exhibits daily rhythms of photosystem II protein (*psbA1*) gene expression under constant conditions. Their results challenged the previous dogma that bacteria do not possess rhythms by demonstrating that *S. elongatus* possess the three criteria necessary for circadian rhythmicity: entrainment by environmental stimuli,
maintenance of approximately 24 h cycles after removal of external cues, and maintenance of rhythms under a wide range of environmental temperatures. Since the initial confirmation of rhythms in cyanobacteria, over 100 different clock mutant strains of bacteria have been identified with circadian periods ranging from 16 to 60 h (Kondo et al., 1994).

The structure of the cyanobacterial clock differs considerably from the molecular clock of eukaryotes. Unlike the core eukaryotic clock, the core cyanobacterial clock operates exclusively through a post-translational oscillator centered around the protein KaiC (Swan et al., 2018). Along with KaiC, two members of the same gene family, KaiA and KaiB, are responsible for generating cycles of phosphorylation and dephosphorylation. Binding of KaiA to unphosphorylated KaiC during the day promotes a conformational change that leads to autophosphorylation of KaiC using cellular ATP as a phosphorus donor. The phosphorylation of KaiC leads to the recruitment of KaiB, which creates a separate conformational state that inhibits KaiA binding (Kageyama et al., 2006). The lack of KaiA binding decreases the ATPase activity of KaiC and causes autodephosphorylation. As phosphorylation state decreases, KaiB disassociates with KaiC, allowing KaiA to bind again and the cycle to repeat. The KaiABC oscillator activates a signaling cascade that activates the response regulator RpaA which regulates the transcription of circadian-associated genes (Swan et al., 2018). Entrainment of the cyanobacterial clock occurs both through sensing the ratio of ATP to ADP within the organism, as well as through the presence of oxidized quinone metabolites which act as electron shuttles during photosynthesis (Williams et al., 2002; Ivleva et al., 2006). Interestingly, the growth cycle of cyanobacteria actually lasts only about 6 h, but the phase of the circadian clocks is passed onto daughter cells to maintain their rhythms (Schibler and Sassone-Corsi, 2002).

Besides *Synechococcus*, several other bacterial species exhibit circadian rhythmicity. Circadian rhythms of bioluminescence have been exhibited in the purple photosynthetic bacterial species *Rhodobacter sphaeroides* (Min et al., 2005). *Rhodopseudomonas palustris* TIE-1 show
self-sustained circadian rhythmicity of nitrogen-fixation that is entrained by oxygen (Ma, 2011). Cycling patterns of anaerobic fermentation have been observed in continuous cultures of *Clostridium acetobutylicum*, exhibiting 24-h cycles of acetate, propionate and butyrate, ethanol, and biomass concentrations (Yerushalmi and Volesky, 1989). Additionally, transcriptomic analysis uncovered 24-h temporal dynamics of metabolic gene expression in six oceanic bacterioplankton (Ottesen et al., 2014).

Microorganisms may play an important role in modulating circadian rhythms in the host. The intestinal microbiome and the host alimentary system have a bi-directional relationship through which perturbations in either system can affect the circadian entrainment of the other. Estimates suggest that 15 to 17% of the operational taxonomic units (OTU; proxy for species) and 23% of the functional pathways of the colonic microbiome undergo daily rhythms (Thaiss et al., 2014; Zarrinpar et al., 2014). These rhythms function to affect microbial metabolism, including growth, energy metabolism, and amino acid metabolism, as well as regulate the spatial organization of bacteria through affecting chemotaxis and mucosal adherence (Liang and FitzGerald, 2017). Inhibition of the gut microbiome, either in germ-free models or through antibiotic treatment, leads to damping of the circadian rhythms in the gut epithelium and liver (Leone et al., 2015; Thaiss et al., 2016). Reciprocally, *Per1/2* double knockout mice failed to demonstrate circadian rhythms of microbial movement and mucosal attachment (Thaiss et al., 2016). The signaling mechanisms by which the host and microbiome communicate temporal information are still being elucidated. Short chain fatty acids, particularly butyrate, produced by hindgut fermentation follow daily rhythms, and induce cyclicity in colonic epithelial cells, suggesting that they may be an important entrainment signal derived from the microbiome (Leone et al., 2015). Murakami et al. (2016) discovered using a fecal transplant model that PPARγ signaling may be responsible for microbiome-entrainment of the circadian clock.
Humoral signals from the host also act directly to entrain the rhythms of the microbiome. Paulose et al. (2016) discovered that the gastrointestinal bacterium *Enterobacter aerogenes*, which displays daily patterns of motility, is responsive to treatment by exogenous melatonin. While it has not yet been examined, cortisol may also be a mechanism whereby the host can entrain the microbiome. As previously discussed, glucocorticoids follows a circadian rhythm that is directly entrained by the master clock of the SCN. Feeding exogenous cortisol has been shown to modulate the gut microbiome of pigs to promote the growth of coliform bacteria and suppress *Salmonella* (Petrosus et al., 2018). Alverdy and Aoys (1991) implied that treatment with glucocorticoids can cause increased bacterial adherence to the mucosal wall of the hindgut. Salivary cortisol concentration follows a 24 h rhythm in humans (Katz and Shannon, 1969). Glucocorticoid signaling through saliva may be particularly impactful for ruminants, because saliva enters the rumen immediately after secretion and can directly impact the microbial community.

**Annual Rhythms**

In addition to circadian rhythms, many organisms have evolved a circannual timing system to coordinate physiology across the year. This system uses environmental signals, primarily the photoperiod, or length of daylight, to synchronize the organism’s physiology to the seasonal changes in climate. Seasonal temperature changes occur due to the 23.5° angle of the earth’s axis which causes the amount of solar radiation reaching different hemispheres of the earth’s surface to change across the year (Foster and Kreitzman, 2009a). These changes in temperature are furthered by *albedo*, the degree of sunlight reflectance from the earth’s surface back into space. Albedo is greatest near the earth’s poles because ice reflects the greatest amount of sunlight, while water reflects less solar energy than land (Sellers et al., 1997). Other annual changes such as jet streams,
ocean currents, tidal cycles, and cycles of rainfall create additional seasonal climates for organisms to anticipate (Namias, 1976).

Many animals exhibit circannual rhythms regulating numerous physiological processes including growth, reproduction, metabolism, and behavior. For example, migratory birds display fattening and nocturnal activity (zugunrhue) coinciding with spring and fall migration. These behaviors persist under constant light:dark (L:D) cycles, and can be modified by altering photoperiod (Gwinner, 1996). Many mammals including horses, sheep, and hamsters exhibit endogenous yearly rhythms of fertility (Gerlach and Aurich, 2000). Moreover, many mammals and birds conserve energy in the winter through hibernation or through entering a state of winter torpor (Geiser and Ruf, 1995).

**Annual Rhythms and Reproduction**

Many animals in seasonal environments have evolved to reproduce during a certain time of the year. There is a clear advantage to birthing young during a time of high feed availability or low predator abundance. In herbivorous mammals, births are typically timed to occur in the spring when forage is readily available, providing the mother with ample calories to nurse her young (Foster and Kreitzman, 2009b). Sheep (*Ovis aries*) are a classic example of seasonal breeders. Domesticated ewes exhibit estrus and ovulation only during autumn and early winter resulting in parturition in mid-spring (Robinson, 1951; Shelton and Morrow, 1965). Rams increase the secretion of testosterone and production of sperm during the period of July through December, corresponding to the time of estrus in the females (Lincoln, 1976; Dacheux et al., 1981).

Photoperiod is the major driver of the seasonality in sheep, but it is modulated by many other factors such as nutritional status, previous lambing date and lactation (Lincoln, 1998). Photoperiodic information is first sensed by the retina and transmitted to the suprachiasmatic nuclei (SCN) of the hypothalamus by the photopigment melanopsin via photosensitive retinal ganglion
(Foster and Kreitzman, 2009b). Daylight increases the oscillation of the SCN (VanderLeest et al., 2007). During periods of low SCN activity (i.e. dark photoperiod), norepinephrine signals the release of melatonin from the pineal gland via efferent nerve signaling (Simonneaux and Ribelayga, 2003). The binding of norepinepherine causes an influx of calcium into pineal cells, activating arylalkylamine N-acetyl transferase (AA-NAT), the rate limiting enzyme in melatonin production (Klein et al., 1983).

The pituitary gland is responsible for integrating photoperiodic information with reproductive signals. Thantrophi cells in the pars tuberalis (PT) of the anterior pituitary gland are rich in melatonin receptors (Morgan et al., 1989). These melatonin receptors are species-specific, and are expressed at comparable levels throughout the year (Morgan et al., 1994). Briefly, melatonin regulates the molecular clock of the PT by inducing *Cry1* expression and repressing *Per1* expression (Jilg et al., 2005; Johnston et al., 2006). This causes the relationship between abundance of *Per1* and *Cry1* to expand and contract throughout the day, with *Cry1* expression reaching a maximum at dusk and *Per1* peaking at dawn. The diurnal relationship of *Per1* and *Cry1* is an example of an internal coincidence model that creates a yearly timekeeping mechanism implicated in driving seasonal hormonal changes.

The seasonal effect of photoperiod modulates reproduction via thyroid hormone production. Triiodothyronine (T3), the active form of thyroid hormone, affects the pulsatile release of GnRH from the hypothalamus, subsequently affecting the release of LH and FSH from the anterior pituitary gland (Misztal et al., 2002). The activity of thyroid hormone is dependent on the synthesis of T3 from its inactive prohormone thyroxine (T4). The enzymatic conversion of T4 to T3 is dependent on the production of the enzyme thyroxine deiodinase 2 (DIO2), which cleaves an iodine moiety from the outer ring of T4 (Watanabe et al., 2004). A second deiodinase, DIO3, can inactivate T3 by cleaving a second iodine molecule, to synthesize diiodothyronine (T2). Photoperiod-dependent melatonin release regulates the expression of both DIO2 and DIO3, as well
as the release of thyroid stimulating hormone (TSHB), which is responsible for the initial production of $T_4$ (Yoshimura, 2013). The production of DIO2 from specialized tanycyte cells in the ependyma is increased, leading to greater $T_3$ production (Dardente, 2015). Alternatively, DIO3 production is increased during short-day photoperiods, leading to inactivation of $T_3$ (Barrett et al., 2007). Both mechanisms affect the season-dependent release of gonadotrophins, leading to the annual rhythm of fertility (Figure 2-3).

Kisspeptin, a hypothalamic peptide that controls the release of GnRH via estrogen feedback, may also play an intermediate role in modulating seasonal breeding pattern of sheep. Short-day photoperiod downregulated kisspeptin gene expression, and subsequently reducing negative feedback on GnRH secretion from the hypothalamus (Clarke et al., 2009; Castro, 2016). In male hamsters, treatment with exogenous kisspeptin was able to restore reproductive function during short-day lighting (Bailey et al., 2017). This effect appears to be melatonin-dependent because the photoperiodic effect does not occur after pineal ablation (Revel et al., 2006). Bartzen-Sprauer et al. (2014) demonstrated that in addition to kisspeptin, its co-activators neurokinin B and dynorphin are also inhibited during short-day lighting, potentially helping to direct the seasonality of reproduction. While the relationship between photoperiodic melatonin signaling and kisspeptin production is clear, the mechanism governing this relationship has yet to be discovered.

**Seasonal Changes in Feeding Behavior and Metabolism**

In the winter, reduced sunlight and cold temperatures lead to dormancy in most plants. As a result, the availability of feed for herbivorous mammals is reduced. To avoid winter starvation, these animals must alter their metabolism to prepare for food shortage. In modern livestock farming systems, issues related to winter feed availability have been resolved through feed storage systems such as ensiling forages, hay production, and the drying of cereal grains. However, the seasonal rhythm of forage production is still relevant for rangeland animals, and many of the physiological
mechanisms that animals have adapted to cope with seasonal feed availability may still exist. Furthermore, the seasonal changes in plant growth can have tremendous consequences on the nutrient composition of feed harvested at different times of the year. In the northern United States, digestibility of most legumes declines with the high-temperatures of mid-summer because of increased lignification of stalks, causing second and third cuttings of alfalfa to be less digestible than alfalfa harvested at first cutting (Van Soest, 1994). Understanding how yearly changes affect feeding behavior and metabolism in domestic ruminants may provide management strategies to improve animal efficiency.

In many wild animals, food intake and body weight exhibit dramatic fluctuations throughout the year. The body weight of golden-mantled ground squirrels (Callospermophilus lateralis) peaks from November to February and then declines to a nadir in June and July (Zucker and Boshes, 1982). Pallid bats (Antrozous pallidus) have substantially greater body weight from October to December than from April to September (Beasley et al., 1984). These seasonal changes in feed intake appear to also be present in domesticated livestock. Recently, Ueda et al. (2016) reported that total rumen dry matter and fiber pool size were greater in the autumn than in the spring in dairy cows, with a reciprocal relationship of volatile fatty acid concentration within rumen fluid. Alternatively, grazing sheep have been shown to increase feed intake in the spring (Milne et al., 1978). While these changes may be related to changes in quality of pasture across the year, evidence suggests that annual patterns of feed intake occur in non-pasture raised animals. Feedlot beef cattle fed stored forage also have greater feed intake in the fall than in the spring (Mujibi et al., 2010). Together, these results may indicate that cattle have an annual rhythm of feed intake.

Leptin may be one factor responsible for the seasonal changes in feed intake. Leptin is a hormone produced by adipose tissue that acts as a satiety signal. Leptin secretion of male woodchucks (Marmota monax) peaks in the spring, causing a concurrent minima of yearly feed intake (Concannon et al., 2001). Sheep exhibit seasonal changes in leptin, with greater leptin
concentrations occurring in August compared to January (Henry et al., 2010). Rousseau et al. (2003) discovered that the effect of leptin on adiposity in Siberian hamsters (*Phodopus sungorus*) is affected by photoperiod, with long-day housed hamsters being more resistant to lipolysis-inducing effects of leptin. However, administering exogenous melatonin appears to have no effect on the annual rhythms of plasma leptin concentrations, suggesting that the photoperiodic effects on leptin concentration may be melatonin-independent (Mustonen et al., 2005). Despite the intriguing breadth of evidence correlating seasonal feed intake to leptin secretion, mechanisms governing this relationship have not been explored.

Metabolic function is also under the influence of seasonality. An extreme example of the seasonal role in metabolic rate occurs in animals living in polar climates. Many arctic animals adapt to cold temperature by restricting blood flow to their extremities to maintain a higher core body temperature. In species such as arctic wolves, foxes, muskrats, and reindeer this can mean that the temperature of their feet can drop to near 0°C (Brix et al., 1990). Caribou maintain blood fluidity in their cold extremities by increasing the concentration of fatty acids, especially unsaturated fatty acids, in bone marrow (Meng et al., 1969). These changes are also often accompanied by a decrease in metabolic rate and activity to conserve body fat reserves (Scholander et al., 1950). Many non-arctic animals also exhibit seasonal changes in metabolic rate. The wild Przewalski horse (*Equus ferus przewalskii*) displays an annual rhythm of basal metabolic rate with a peak occurring in August (Arnold et al., 2006), and the North American moose (*Alces alces*) exhibits a 1.4-fold increase in resting heat production during the summer compared to the winter (Regelin et al., 1985).

There has been a large amount of research examining the effect of photoperiod on milk production dairy cattle. It has been known for several decades that a long light period increases dry matter intake, milk production, and weight gain of dairy cattle (Peters et al., 1978, 1981). Dahl et al. (1997) observed that exposing dairy cows to a long day photoperiod (18 h light: 6 h dark) increased milk yield and concentrations of circulating IGF-1 compared to the natural winter
photoperiod of < 13 h of light. On the other hand, exposure to a short-day photoperiod in non-lactating cows during the dry period before calving improved milk production in the subsequent lactation (Miller et al., 2000). These production responses are likely due to the photoperiodic effects on prolactin signaling. Crawford et al. (2015) demonstrated that the positive effects of long-day lighting during lactation could be mimicked by the addition of exogenous prolactin, suggesting that it is the primary driver of the effects of photoperiod on milk synthesis.

Despite many species demonstrating photoperiod-driven seasonal rhythms in feed intake and metabolism in mammals, there have been few studies determining if these rhythms can be maintained under constant conditions. Brinklow and Loudon, (1990) established that Red Deer (Cervus elaphus) exhibit seasonal rhythms of feed intake even when placed in constant 18h light:6 h dark cycles and fed ad libitum. Conceivably, a circannual rhythm of feed intake and metabolism could exist and be driven by endogenous yearly rhythms of prolactin. Prolactin plays a role in feed intake as well as the initiation and maintenance of lactation in dairy cows (Bauman and Currie, 1980; Teyssot et al., 1981; Lawrence et al., 2000). Prolactin is released from the pars distalis (PD) of the anterior pituitary in response to melatonin. An inherent yearly rhythm of prolactin concentration is maintained in a constant photoperiod (Martinet et al., 1992). The signal that directs seasonal prolactin release based on photoperiodic information has yet to be identified, but a hypothetical ‘tuberalin’ protein is theorized to stimulate the release of prolactin (Foster and Kreitzman, 2009b). Dupré et al. (2010) identified the tachykinins Substance P and Neurokinin A as potential ‘tuberalin’ proteins. The Tac1 gene, which encodes both proteins was shown to increase expression during long-day photoperiods, and both are secretagogues for prolactin.

Seasonal Changes in Immunity

Activation of the immune system is a highly energy-demanding process. It is estimated that infection can lead to a 50% increase in basal metabolic rate (Lochmiller and Deerenberg, 2000).
Therefore, the animal must balance energy efficiency with the ability to mount an appropriate immune response. Many infectious agents follow distinct yearly patterns. Seasonal variables such as temperature, humidity, and sunlight, as well as host behavior, such as social interaction, all affect the survivability of pathogens (Grassly et al., 2006). Influenza is one of the most well-known examples of a seasonal disease in humans, with outbreaks occurring most frequently in the late fall and winter of temperate climates (Tamerius et al., 2013). In pigs pneumonia infections are most likely to occur during late winter and spring (Cowart et al., 1992). In the winter and spring, bovine viral diarrhea (BVD) incidence is the greatest (Radostits and Littlejohns, 1988). The yearly pattern of disease exposure creates predictable patterns that may be exploited by the immune system of animals. By keeping the immune system ‘primed’ to respond to the pathogens that are most likely to be encountered, it can most efficiently allocate resources without expending unnecessary energy.

Photoperiod can alter the growth and function of many immune cells. The number of circulating leukocytes, B cells, T cells, and natural killer (NK) cells are increased by short day photoperiod (SDPP) in Siberian hamsters (Bilbo et al., 2002). Furthermore, SDPP increases the ability of NK cells to produce cytotoxicity via oxidative burst (Yellon et al., 1999). Non-lactating dairy cattle increase neutrophil chemotaxis and lymphocyte proliferation in response to SDPP (Auchtung et al., 2004). Additionally, season of the year can affect the functionality of specific immune cells. Mann et al. (2000) observed a greater T_s1: T_s2 ratio in Rhesus macaques (Macaca mulatta) in the summer versus winter. Alternatively, the response of polymorphonuclear blood monocytes (PBMCs) to IL-2 stimulation was greater in the winter than summer.

One mechanism of photoperiodic changes in immune function may be melatonin signaling in immune cells. Many immune cells including B cells, T cells, and PBMCs have membrane and nuclear melatonin receptors (Pozo et al., 1997, 2004). The binding of melatonin to these receptors can have multiple effects on the immune system. Treatment with exogenous melatonin increases the systemic expression of IL-1α, IL-1β, IL-5, IL-6, and IL-10 in endotoxemia-challenged mice.
(Effenberger-Neidnicht et al., 2014). Removal of endogenous melatonin signaling via a pinealectomy decreased the ability for splenocytes to react to antigen stimulation, and caused a near depletion of lymphocytes (Maestroni et al., 1986). Melatonin itself can function as an antioxidant by scavenging free radicals to ameliorate oxidative stress (Mayo et al., 2003).

The seasonal change in vitamin D may be another possible explanation for the effects of photoperiod on the immune system. Vitamin D receptors (VDR) are present in nearly all immune cells including macrophages, dendritic cells, neutrophils, T cells, and B cells (Baeke et al., 2010). Photoperiod affects the function of vitamin D through sunlight-mediated vitamin D activation. Briefly, ultraviolet radiation from the sun catalyzes the conversion of 7-dehydrocholesterol to cholecalciferol (Vitamin D$_3$) in the epidermal layer of the skin. After two successive hydroxylation steps in the liver and kidney, respectively, the active form of vitamin D (1,25-dihydroxyvitamin D$_3$) is available for binding to the VDR (Horst et al., 2005). The role of sunlight in activation implies a strong photoperiodic effect on vitamin D metabolism. Indeed, 1,25-dihydroxyvitamin D$_3$ shows a strong yearly rhythm with plasma concentration peaking in the summer (Stumpf and Privette, 1991).

While roles of melatonin and vitamin D may imply that yearly changes in immune function are a result of strictly exogenous photoperiod, there is evidence to suggest an underlying endogenous circannual rhythm of immune function. Brock detected a circannual rhythm in the proliferation of B and T cells in C57BL/6 mice held in a constant photoperiod (12h light: 12h dark) and temperature (22.5°C) (Brock, 1983). A similar experiment conducted in dogs under the same photoperiod length also saw a circannual rhythm of total leukocytes and lymphocytes (Sothern et al., 1993). These experiments provide some credence to the idea of an inherent circannual rhythm of immune function, however, no mechanism has been described.
Epigenetic Control of Seasonality

Emerging evidence suggest that epigenetic modifications are important for the maintenance and synchronization of circannual rhythms. Stevenson and Prendergast (2013) demonstrated that SDPP increased the amount of DNA methylation at the promoter region of the dio3 gene in the hypothalamus of Siberian hamsters. This led to decreased expression of diodinase 3, and greater inactivation of thyroid hormone. The authors concluded that hypermethylation at the dio3 promoter helps precipitate seasonal reproduction by inhibition of reproductive activity during summer months (Stevenson and Prendergast, 2013). Interestingly, DNA methyltransferase and global methylation were actually lower in hamsters under SDPP, likely indicating a myriad of other epigenetic changes are under photoperiodic control in the hypothalamus. The same authors observed that the same mechanism of dio3 methylation can occur in a cell-autonomous manner within immune cells. The increased methylation of deiodinase 3 caused an accumulation of T3 within lymphocytes under SDPP (Stevenson et al., 2014). Furthermore, supplementing with exogenous T3 caused lymphocytes to exhibit greater sensitivity to inflammation.

Stoney et al. (2016) provided additional evidence that epigenetic programming may play a role the seasonal changes of the immune system. They observed that expression of HDAC 4, 5, and 9 were found to be greater in mice under long-day (16h L: 8h D) versus short-day (8h L: 16h D) photoperiod (Stoney et al., 2017). These histone deacetylases were shown to reduce the expression of nuclear factor-κB (NF-κB), a master regulator of inflammation, during SDPP. Winter-related behaviors also appear to be under the influence of epigenetic changes. Alvarado et al. (2015) demonstrated that thirteen-lined ground squirrels (Ictidomys tridecemlineatus) exhibit a reduction in global DNA methylation within muscle and liver at the onset of winter torpor.

Stevenson and Lincoln, (2017) have proposed a model of epigenetic control of circannual rhythms, which postulates that circannual information is programmed into cells during early stem cell development. They believe that this mechanism forms an ‘epigenetic memory’ of circannual
information, which can be reprogrammed in adult cells if seasonal inputs change. In insects, it appears that seasonal information can be passed transgenerationally. *Nasonia* wasps (*Nasonia vitripennis*) transmit seasonal signals of diapause from mother to an undifferentiated egg, which was recently discovered to be a result of photoperiod-dependent DNA methylation (Pegoraro et al., 2016). A similar mechanism appears to occur in *Sarcophaga* flies, which demonstrate the ability to transmit photoperiodic signals from mother to embryo during early larval development.

**Conclusions**

Across multiple time-scales, biological rhythms serve to coordinate physiology with the external environment. These biological rhythms are highly conserved across multiple organism, illustrating their importance. Nutrient metabolism, in particular, is highly influenced by both circadian and circannual rhythms. Moreover, nutrients themselves are important for entrainment of biological clocks. Desynchronization of the day-light cycle from the time of feed intake can lead to metabolic diseases such as obesity and type II diabetes in humans. Moreover, matching the time of feed intake with the timing of the biological clock in livestock species may serve as an opportunity to improve the efficiency of animal production.
**Figure 2-1.** Parameters used in the quantification of biological rhythms.

Period is the length of time to complete one cycle of the rhythm. Amplitude is the difference between peak and mean. Double amplitude is the difference between peak and trough. Acrophase is the time at peak. Bathyphase is the time at trough. Phase advance refers to shifting the rhythm curve so that the acrophase occurs earlier than it did previously. Phase advance refers to shifting the rhythm curve so that acrophase occurs later than it did previously.
**Figure 2-2.** Structure of the mammalian transcriptional circadian clock.

Key: BMAL1: Brain and muscles ARNT-like 1, CCG: Clock controlled gene, CK1ε: Casein kinase 1ε, CLOCK: circadian locomotor output cycles kaput, CRY: Cryptochrome, E-Box: Enhancer-box [canonically CANNTG], P: Phosphate group, PER: Period, RORα: Retinoic acid-related orphan receptor α, RORE: Retinoic acid-related orphan receptor response element. Adapted from (Green et al., 2008).
**Figure 2-3.** Physiological mechanisms governing seasonal reproduction.

**Key:** DIO2: diodinase 2, GnRH: gonadotropin releasing hormone, MEL: Melatonin, NE: norepinephrine, PT: Pars tuberalis, SCN: Suprachiasmatic nucleus, T3: Triiodothyronine, TSH: Thyroid stimulating hormone, UV: Ultraviolet.
Chapter 3

The effects of day-versus night-restricted feeding of dairy cows on the daily rhythms of feed intake, milk synthesis and plasma metabolites

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This chapter will be submitted as a manuscript to the Journal of Dairy Science.
ABSTRACT

The timing of feed intake can alter circadian rhythms of peripheral tissues. Dairy cows have well-recognized daily rhythm of milk synthesis, but the effect of feeding time on these rhythms is poorly characterized. The objective of this experiment was to determine if the time of feed intake modifies the daily patterns of milk synthesis, key plasma metabolites, and body temperature in dairy cows. Sixteen Holstein cows were randomly assigned to one of two treatment sequences in a cross-over design with 17 d periods. Treatments included day-restricted feeding (DRF) with feed available from 0700 to 2300 h and night-restricted feeding (NRF) with feed available from 1900 to 1100 h. To determine a rhythm of milk synthesis, cows were milked every 6 h on the last 7 d of each period and blood samples were collected to represent every 4 h over the day and analyzed of glucose, insulin, non-esterified fatty acids (NEFA), and plasma urea nitrogen (PUN). Body temperature was measured using indwelling vaginal temperature data logger. The daily rhythms of milk yield and composition were modified by timing of feed availability. Peak milk yield was shifted from morning in DRF to evening in NRF, while milk fat, protein, and lactose concentration peaked in the evening in DRF and the morning in NRF. Plasma glucose, insulin, NEFA, and PUN fit daily rhythms in all treatments. Night-feeding increased the amplitude of glucose, insulin, and NEFA rhythms and shifted the daily rhythms by 8 to 12 h ($P < 0.05$). Night feeding also phase delayed the rhythm of core body temperature and DRF increased its amplitude. Altering the time of feed availability shifts the daily rhythms of milk synthesis and plasma hormone and metabolite concentrations and body temperature, suggesting that these rhythms may be entrained by food intake.

**Keywords:** daily rhythm, milk synthesis, food entrainment, circadian
INTRODUCTION

Feeding behavior, milk synthesis, and plasma metabolites of dairy cows follow daily patterns that may be regulated by endogenous circadian rhythms (Giannetto and Piccione, 2009). Circadian rhythms are repeating cycles of about 24 h that govern many physiological functions and allow organisms to anticipate changes in their environment. They are regulated at both the systems level, through neural and humoral communication between tissues, and at the cellular level, through transcriptional-translational feedback loops of ‘clock’ transcription factors and 24-h protein phosphorylation cycles. In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and is entrained by light through visual and non-visual photoreceptors in the eye (Panda et al., 2002). However, food intake can entrain circadian rhythms of peripheral tissues such as liver and adipose tissue, independent of the SCN (Casey and Plaut, 2012).

Milk synthesis follows a daily rhythm in dairy cows with peak milk yield occurring in the morning, and milk fat and protein concentration peaking in the evening (Rottman et al., 2014). This adaptation may have evolved to provide neonates with energy-dense milk at night when environmental temperature is lower and nursing and foraging activity is minimal. Evidence for a role of the circadian system in lactation has been demonstrated in both rodents and humans, with over 7% of the human mammary transcriptome oscillating in a circadian manner (Maningat et al., 2009; Casey and Plaut, 2012).

Dairy cows also exhibit a crepuscular pattern of feed intake, consuming the majority of their feed during the daylight hours with large bouts of intake in the morning and evening (Albright, 1993). Furthermore, intake is stimulated by feed delivery and milking (DeVries et al., 2005). Although most commercial dairy farms feed a total mixed ration (TMR) which is meant to provide cows with a consistent concentration of nutrients across the day, the daily pattern of
intake and sorting of feed cause nutrient intake and absorption to fluctuate diurnally (Rottman et al., 2015). Moreover, the daily patterns of important peripheral hormones and metabolites such as glucose, urea, nonesterfied fatty acids (NEFA), and insulin can be altered by the time of feed intake (Nikkhah et al., 2008; Niu et al., 2014).

While there is evidence supporting a role of the mammary clock in lactation, little is known about the relationship between feeding and daily rhythms of milk synthesis. Rottman et al. (2014) observed that the amplitudes of milk fat and protein concentration are reduced by feeding cows 4x/d compared to 1x/d. However, the response of the daily pattern of milk synthesis to altering the timing of nutrient intake has not been examined. Time-restricted feeding is often employed when studying food entrainment because it creates more robust changes in daily rhythm compared to altering feeding time alone (Stokkan et al., 2001). The objectives of this study were to determine the effects of the time of time-restricted feeding on daily rhythms of milk synthesis and the daily rhythms of plasma hormones and metabolites and body temperature. Our hypothesis was that restricting feed availability to 16 h over the dark period would invert the daily rhythm of milk synthesis relative to feed available for 16 h during the light period and result in a commensurate change in the daily pattern of plasma metabolites, with smaller changes in the daily rhythm of body temperature.

**MATERIALS & METHODS**

**Animals and Treatments**

All experimental procedures were approved by the Pennsylvania State University Institutional Care and Use Committee. Sixteen multiparous Holstein cows (183 ± 103 d postpartum; mean ± SD) from the Pennsylvania State University Dairy Research and Teaching Center were randomly assigned to one of two treatments sequences (n = 8 cows per sequence) in
a cross-over design. Treatment periods were 17 d and included 10 d of treatment adaptation with 2x/d milking and 7 d of 4x/d milking. Animals were housed in individual tie-stalls and had \textit{ad libitum} access to water. Cows were maintained in a 19 h light and 5 h dark photoperiod with lights on from 0500 h to 0000 h. Treatments included (1) day-restricted feeding (DRF) with feed available for 16 h/d from 0700 to 2300 and (2) night-restricted feeding (NRF) with feed available for 16 h/d from 1900 to 1100 (\textbf{Figure 3-1A}). All cows were fed the same diet offered at 110\% of the previous day’s intake and intake was recorded daily. Feed was mixed once daily at 0700 and immediately delivered to the DRF group. The remaining feed was compressed into plastic barrels, covered with plastic, and stored at ambient temperature until feeding to NRF at 1900 h. Feed samples were collected on d 7 and 14 of each period, composited by period and analyzed for dry matter, crude protein, fiber, neutral detergent fiber, acid detergent fiber, and ash concentrations according to Rico and Harvatine (2013) (\textbf{Supplemental Table S3-1}). The experiment was conducted from February to March of 2016.

\textit{Milk Sampling and Analysis}

Cows were milked 4x/d (0500, 1100, 1700 and 2300 h) for the last 7 d of each period to observe the daily rhythm of milk synthesis. Milk collected at each time point represented milk synthesis during previous 6 h, and data were expressed as the midpoint of the previous milking interval (3 h prior to collection). Milk yield was measured at each milking using an integrated milk meter (AfiMilk MPC Milk Meter; Afimilk Agricultural Cooperative Ltd., Kibbutz Afikim, Israel) and corrected for the deviation of each individual stall according to Andreen et al. (2018). Milk was sampled at all milkings on the last 2 d of each period. One subsample was stored at 4°C with a preservative (Bronolab-WII; Advanced Instruments, Inc., Noorwood, MA) prior to analysis of fat and protein concentration by Fourier transform infrared spectroscopy (Fossomatic 4000 Milko-Scan and 400 Fossomatic, Foss Electric; Dairy One DHIA). A second subsample was
stored without preservative at 4°C and centrifuged at 2300 x g within 12 h. The resulting fat cakes were stored at -20°C and analyzed for concentrations of individual fatty acids (FA) according to Baldin et al. (2018).

**Feeding Behavior Observation and Analysis**

The daily pattern of feed intake was monitored in nine of the 16 cows using an automated feed observation system described by Rottman et al. (2015). Briefly, feed in hanging feed tubs was weighed and recorded every 10 s from d 8 to 17 of each period by an electronic load cell connected to a data acquisition system. To characterize feeding behavior, the number, length and size of meals as well as the intermeal interval, eating time, and eating rate were determined as described Niu et al. (2017). Hunger ratio was calculated as meal size divided by the previous intermeal interval and satiety ratio was determined as meal size divided by the ensuing intermeal interval. To characterize the rate of feed intake across the day, the data were smoothed by calculating a running average over 180 s, the rate of feed intake was the determined over 10 minute intervals before averaging across 2 h intervals as described by Rottman et al. (2015).

**Plasma Sampling and Analysis**

Blood was collected by venipuncture of a coccygeal vessel using potassium EDTA vacuum tubes (Greiner Bio-One North America, Inc., Monroe, NC). Samples were collected at six time points on d 15 to 17 of each period to represent every 4 h across the day (0300, 0700, 1100, 1500, 1900, and 2300 h). Blood was immediately placed on ice and centrifuged within 1 h at 2300 x g for 15 min at 4°C. Plasma was collected and stored at -20°C for analysis of glucose, NEFA, plasma urea nitrogen (PUN), and insulin concentrations according to Rottman et al. (2014). Briefly, plasma glucose concentration was analyzed using a glucose oxidase/peroxidase enzymatic colorimetric assay (No. P 7119, Sigma-Aldrich, St. Louis, MO), NEFA concentration
was measured with an acyl-CoA oxidase/peroxidase enzymatic colorimetric assay (NEFA-HR (2), Wako Diagnostics, Richmond, VA), PUN was assayed using a modified Berthelot methodology (Modified Enzymatic Urea Nitrogen Procedure No. 2050; Stanbio Laboratory, Boerne, TX), and insulin was determined using a porcine $^{125}$I-insulin radioimmunoassay kit with correction based on bovine insulin (PI-12K Porcine Insulin RIA, EMD Millipore, Billerica, MA).

**Body Temperature Recording**

Core body temperature was recorded every 10 min on d 12 to 17 of each period using an intravaginal temperature data logger. A miniature plastic-coated thermometer (iBCod; Alpha Mach Inc., Sainte-Julie, QC, Canada) was fastened to a progesterone-free CIDR vaginal implant (Zoetis, Inc., Parsippany-Troy Hills, NJ) and placed centrally in the vagina of the cows using an insertion tool. Raw data was averaged over 2 h intervals.

**Statistical Analysis**

All statistical analysis was performed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Daily DMI, milk production, and FA yields were analyzed using the fixed effects of treatment, period, and the interaction of treatment by period and the random effect of cow. Repeated measures analysis was performed on data observed multiple times per day to test the interaction of treatment and time of day on milk production, rate of feed intake, and plasma metabolites and hormones. The model included the fixed effect of treatment, period, and the interaction of treatment and period, the random effect of cow, and the repeated effect of time of day. The AR(1) or ARH(1) covariance structure was selected based on convergence criteria, and denominator degrees of freedom were adjusted using the Kenward-Roger method. Preplanned contrasts tested the effect of treatment at each time point.
Time course data for milk production, plasma hormones and metabolites, and body temperature were fit to the linear form of a cosine function using random regression in SAS 9.4. The model included the fixed effect of treatment, cosine terms, and the interaction of treatment with the cosine terms and the random effects of cow and period. A 12 h harmonic was also tested for the daily patterns of plasma hormones and metabolites and body temperature but was removed because it did not improve model fit according to Bayesian information criterion. The fit of the 24 h cosine was determined using a zero-amplitude test, an F-Test comparing a full model containing the linear form of the cosine function to a reduced linear model. The acrophase (time at peak) and the amplitude (difference between peak and mean) of the 24 h rhythm were calculated and significance determined as described by Niu et al. (2014). In all analyses, points with Studentized residuals outside of ± 3.5 were removed. Statistical significance was declared at $P < 0.05$ and trends acknowledged at $0.05 < P < 0.10$. High resolution figures were generated using an add-in for Microsoft Excel (Kraus, 2014).

**RESULTS**

*Eating Behavior*

Total daily dry matter intake was not affected by the time of feed restriction (Table 1). Both treatments consumed the greatest amount of feed in the first 2 h following feed delivery (Figure 3-1B). However, the NRF treatment had a higher rate of intake after feed delivery, consuming 21.6% (9.6 kg) of their feed per hour in the first two hours after feed delivery compared to 15.6% (5.9 kg) in the DRF group ($P < 0.0001$).

The time of feed availability had no effect on meal size, number of meal bouts per day, eating time/d, eating rate, or average intermeal interval (Supplemental Figure S3-1). However,
DRF increased average meal length 5.1 min/d \((P = 0.02)\) and increased hunger ratio 40\% \((P = 0.01)\), while the satiety ratio was not modified.

**Rhythm of Milk and Milk Components**

The timing of feed restriction did not alter daily yield of milk and milk fat and protein or daily average concentration of milk fat and protein (Table 1). However, milk yield fit a cosine function with a 24 h period in both DRF \((P = 0.04)\) and NRF \((P < 0.0001; \text{Figure 3-2A})\). The acrophase of milk yield was delayed 8.1 h by NRF \((P < 0.05)\), and the amplitude of the rhythm was 32\% greater in DRF than NRF.

Night-restricted feeding decreased total daily milk fat yield \((P = 0.03; \text{Table 1})\). Milk fat yield fit a daily rhythm with an amplitude of 16.5 g and an acrophase at 0703 in NRF \((P < 0.0001)\), but did not fit a 24 h rhythm in the DRF (Figure 3-2B). Milk fat concentration fit a 24 h rhythm in NRF \((P < 0.0001)\) and tended to fit a daily rhythm in DRF \((P = 0.06; \text{Figure 3-2C})\). There was an effect of treatment on the rhythm, as milk fat concentration was phase shifted, peaking at 1156 h in DRF compared to 0207 h in NRF \((P < 0.05)\) and the amplitude was increased by 64\% in NRF compared to DRF \((P < 0.05)\).

Milk protein yield fit a daily rhythm in both DRF and NRF \((P < 0.001; \text{Figure 3-2D})\) with the peak of the rhythm occurring at 0211 h in DRF and at 0738 in NRF \((P < 0.05)\). The amplitude of the daily rhythm of protein yield was 30.1 g in DRF and was decreased to 10.4 g by NRF \((P < 0.05)\). Both DRF and NRF also exhibited daily rhythm of milk protein concentration \((P < 0.02; \text{Figure 3-2E})\). Treatment modified the timing of the rhythm with DRF peaking at 1525 h and NRF peaking at 0029 h \((P < 0.05)\). The robustness of the rhythm was increased over two-fold by NRF, which had an amplitude of 0.11\% compared to 0.05\% in DRF \((P < 0.05)\).
Milk FA Yield and Profile

Compared to NRF, day-restricted feeding increased FA originating from de novo synthesis in the mammary gland by 5% ($\Sigma < 16C; P = 0.03$) and mixed source FA that originate both from de novo synthesis and preformed FA uptake from plasma by a 9% ($\Sigma 16C; P = 0.001$; Figure 3-3A). However, no difference in yield of preformed FA ($\Sigma >16C$) was observed between treatments.

Both de novo and mixed source FA fit a cosine function with a period of 24 h in both treatments, but preformed FA only fit a cosine function in DRF ($P < 0.05$; Figure 3-3D-F). The acrophase of the de novo FA rhythm was delayed 6.7 h by NRF (0108 vs 0750 h; $P < 0.05$) and DRF increased the amplitude of the rhythm by 38% ($P < 0.05$). The acrophase and amplitude of the mixed FA yield rhythms were also altered by treatments, with DRF peaking at 0032 h and NRF peaking at 0721 ($P < 0.05$), and NRF having a 34% greater amplitude than DRF ($P < 0.05$). Similar to the de novo FA rhythm, night-restricted feeding increased the robustness of the mixed FA yield rhythm, increasing its amplitude 34% compared DRF ($P < 0.05$). Of the 44 individual fatty acids quantified, 15 fit a daily rhythm in both DRF and NRF, 10 fit a rhythm in DRF only, 6 fit a rhythm in NRF only, and 13 did not fit a rhythm in either treatment (Supplemental Table S2). Fatty acids generally followed a similar daily pattern as their source grouping (de novo, mixed source, preformed).

To assess the potential cause of the reduced de novo fatty acid synthesis, milk fat concentrations of trans-10 C18:1 (t10) and trans-11 C18:1 (t11) were determined. trans-10 C18:1 is produced by the alternate biohydrogenation pathways and is elevated during biohydrogenation-induced milk fat depression and t11 is a product of the normal biohydrogenation pathway and is increased with slowing of the normal biohydrogenation pathway (Griinari et al., 1998). The concentrations of t10 and t11 were analyzed as a percent of 18 carbon FA, rather than as a percent of total FA to avoid bias of changes in de novo and mixed FA. Night-restricted feeding increased
by 16.3% \( (P = 0.02) \) and \( t_{11} \) by 32.8% \( (P = 0.001) \) compared to NRF (Figure 3-3B). Both \( t_{10} \) and \( t_{11} \) fit daily rhythms in both treatments \( (P < 0.0001; \text{Figure 3-3G&H}) \). Milk fat \( t_{10} \) was phase delayed 12.1 h by NRF, with NRF peaking at 1038 and DRF peaking at 2234 \( (P < 0.05) \). The amplitude of \( t_{10} \) was increased by NRF compared to DRF \((0.08\% \text{ vs. } 0.06\%; P < 0.05)\). Like \( t_{10} \), night-restricted feeding caused a complete inversion of the daily rhythms of \( t_{11} \) concentration, shifting the peak from 1816 in DRF to 0604 in NRF \( (P < 0.05) \). The amplitude of \( t_{11} \) was not affected treatment \( (P > 0.10) \).

**Plasma Hormones and Metabolites**

Plasma glucose fit a 24 h cosine in both the DRF \( (P < 0.03) \) and NRF \( (P < 0.001) \), with NRF increasing the amplitude of the rhythm by 149% \( (P < 0.05; \text{Figure 3-4A}) \). Night-restricted feeding also phase shifted plasma glucose, with the acrophase occurring 10.2 h later in DRF than NRF \( (P < 0.05) \).

Plasma insulin concentration fit a 24 h cosine function in both treatments \( (P < 0.001) \), with no difference in mean insulin concentration between DRF and NRF \( (P = 0.69; \text{Figure 3-4B}) \). Night-restricted feeding shifted the acrophase of insulin production 8.0 h and increased the amplitude 0.9 μIU/mL compared to DRF \( (P < 0.05) \).

Concomitant with the shift in plasma insulin rhythms, the rhythms of plasma NEFA concentration were dramatically altered by treatment (Figure 3-4C). Both treatments exhibited 24 h rhythms in plasma NEFA \( (P < 0.01) \), but the acrophase of the rhythms were markedly different, with DRF rhythms peaking at 0614 and NRF peaking at 1657 \( (P < 0.05) \). Additionally, NRF increased the amplitude of the NEFA rhythm 3.3 fold \( (P < 0.05) \). This increase in amplitude in the NRF treatment was due to a dramatic rise in NEFA concentration to 214 μEq/L at 1900 h, 6 hours into the fasting period.
Plasma urea nitrogen fit a 24 h rhythm in DRF \((P = 0.02)\) and tended to fit a rhythm in NRF \((P = 0.08)\). The rhythm of NRF was completely inverted with respect to the DRF, with DRF peaking at 0209 h and NRF peaking at 1420 \((P < 0.05)\). Moreover, the amplitude of the PUN rhythm was 20% greater in DRF than NRF \((P < 0.05)\).

**Body Temperature**

Body temperature fit a 24 h cosine function in both treatments \((P < 0.05)\) and was modified by timing of feed restriction (Figure 3-5). NRF delayed the phase of the rhythm 15.5 h \((P < 0.05)\) and increased the amplitude 75% compared to DRF \((P < 0.05)\).

**DISCUSSION**

The rate of feed intake was greater after food delivery in the NRF compared to DRF, which is consistent with previous results reporting that feed delivery 1x/d at night increases intake in the first 2 to 3 h after feeding compared to feed delivery in the morning (Nikkhah et al., 2008; Niu et al., 2014). Cows naturally exhibit a crepuscular pattern of intake, with highest intake occurring at dusk and at dawn and a decline in intake overnight, and TMR-fed cows normally have high intake at feed delivery and the late afternoon and early evening and low intake in the overnight period (DeVries and von Keyserlingk, 2005). In NRF, feed was withheld during the high-intake afternoon period of the day (1100 to 1900 h), whereas DRF cows were without feed during the low-intake night. The greater rate of intake immediately after feeding in NRF suggests greater hunger signaling presumably due to the circadian pattern of hunger and satiety. Other species display 24 h rhythms of hunger. Humans, for example, exhibit the greatest appetite in the evening, and lowest in the morning, independent of sleeping time or food intake (Scheer et al., 2013). Moreover, rats display circadian rhythms of meal size and meal frequency under constant
illuminated (Rosenwasser et al., 1981). Despite the difference in the daily pattern of feed intake, total dry matter intake did not differ between treatments because DRF compensated by increasing intake in the afternoon compared to the corresponding period in NRF (0100 to 0700 h).

Dairy cows generally have greatest milk yield in the morning and greater fat and protein concentration in the evening when fed ad libitum with feed delivered in the morning (Quist et al., 2008; Rottman et al., 2014). Our results confirmed these findings, but demonstrated that night-restricted feeding shifts this daily pattern 8 hours later in the day. The change in the rhythms of milk and milk component synthesis in response to altered feeding time is either due to a change in available substrate for milk synthesis or entrainment of the mammary gland’s circadian clock. In FVB mice, time-restricted feeding shifted the rhythms of clock genes in the mammary gland, and affects the rhythmic gene expression of transcription factors (SREBP1c, Spot 14) and enzymes (SCD1 and FASN) related to milk fat synthesis (Unpublished data). These results suggest that alterations in the mammary molecular clock may mediate the response of feeding time on rhythms of milk synthesis, but further research must be conducted to evaluate this effect.

The decrease in total milk fat yield and de novo and mixed fatty acids in NRF compared to DRF indicates that de novo fatty acid synthesis was reduced by night-restricted feeding. Concomitant with the decrease in apparent de novo fatty acid synthesis, the concentration of t10 C18:1 was elevated in NRF. Trans-10 C18:1 is highly correlated with reduced milk fat synthesis in dairy cows as it is associated with production of bioactive intermediates of the trans-10 pathway (Lock et al., 2007). However, t11 C18:1, the intermediate of normal ruminal biohydrogenation was also increased by NRF, which commonly occurs due to a slowing of the normal pathway in conjunction with the shift to the alternate BH pathway (Rico and Harvatine, 2013). The decrease in ruminal biohydrogenation in NRF may be related to the daily pattern of intake. The NRF group consumed a large percentage of their feed during the first 2 hours after delivery, while DRF had more stable intake across the day. Previous research demonstrated that
stabilizing feed intake through 4x/d feeding increased milk fat yield and decreased t10 C18:0 (Rottman et al., 2014). The changes in daily patterns of de novo, mixed source, and preformed FA closely reflected the daily pattern of milk yield. The daily pattern of t10 C18:1 appeared to be highly impacted by time of feed restriction, peaking at the start of the fasting period in both treatments. Trans-11 C18:1, meanwhile, peaked in the middle of the feeding period, 13 hours after feed delivery in both treatments.

The effect of time of feed restriction on daily rhythms of fatty acids may also be due to differences in entrainment of the mammary circadian clock. Previous research from other models suggests circadian regulation of lipid metabolism. In lactating rats, mammary lipogenesis and activity of acetyl-CoA carboxylase (ACC), the enzyme that catalyzes the rate-limiting step in FA synthesis, follow clear daily pattern with a nadir at 1500 h (Munday and Williamson, 1983). Furthermore, Hems et al. (1975) detected daily rhythms of FA synthesis in the livers of mice. Similarly, the current study indicates that FA synthesis follows a daily pattern in the mammary gland of cows. Several metabolic pathways linking the circadian clock to FA metabolism have been characterized, including the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors, which act as master regulators of lipid metabolism in multiple tissues and the cellular energy sensor AMP-activated protein kinase (AMPK) influences the period of the circadian clock while also increasing the expression of ACC (Jordan and Lamia, 2013; Chen and Yang, 2014). The results of this experiment provide additional support that de novo FA synthesis is controlled by the circadian rhythm of the mammary gland and suggest that this rhythm is modified by timing of nutrient absorption.

Similar to previous reports in dairy cattle (Giannetto and Piccione, 2009) and other species (Rudic et al., 2004), plasma glucose concentration fit a 24 h rhythm. Furthermore, both glucose and insulin concentrations were phase shifted by NRF compared to DRF. Circadian control over glucose metabolism in experimental models has been well-established. In mice, a
functioning circadian clock in pancreatic β-cells is required for maintenance of insulin sensitivity (Sadacca et al., 2011). The amplitude of NEFA concentration was greatly increased in NRF compared to DRF. This is likely a consequence of the daily pattern of hunger and satiety. Cattle typically increase feed consumption during the afternoon, suggesting greater hunger signaling during this time (Ray and Roubicek, 1971). The results of the current experiment suggest that fasting during the high-intake afternoon period of the day causes greater lipid mobilization than fasting during the low-intake overnight period. These results were similar to those reported in rats, which displayed a shift in the daily pattern of insulin release when they were fasted during the early part of their active period (Shimizu et al., 2018). Insulin is a potent inhibitor of hormone sensitive lipase, the enzyme responsible for causing lipid mobilization from adipose tissue (Locher et al., 2011). Peak NEFA concentration in both treatment groups coincided with the nadir of insulin release. Shostak et al. (2013) reported that lipolysis from white adipose tissue follows a daily pattern, and showed using ClockΔ19 and Bmal1−/− mice that these patterns were controlled by the molecular circadian clock. The current study provides evidence suggesting a similar mechanism may occur in dairy cows.

The shift in body temperature by the time of time-restricted feeding likely demonstrates a change in the central circadian rhythm. This result of the current study showed a more extreme shift in the rhythm of body temperature than previously observed by Niu et al. (2014), who reported a 3 hour phase delay after feeding at 2030 compared to 0830, without restricting the time of feed availability. Our results also agreed with research performed in mice which showed shifts in the body temperature rhythms when food was restricted to the inactive period (Damiola et al., 2000). The change in body temperature may also be responsive to feeding pattern due to changes in heat produced by rumen fermentation across the day. This may be a mechanism by which ruminants can directly modify body temperature in response to feeding. Shifts in the rhythm of body temperature may play a role in altering other daily rhythms within the animal because body
temperature has been shown to be capable of entraining peripheral circadian clocks in mammals (Brown et al., 2002).

In conclusion, timing of feed intake dramatically altered daily rhythms of milk synthesis, plasma hormones and metabolites, and body temperature. Modification of the phase of milk synthesis by temporal changes in absorption of nutrients indicates possible changes in the mammary molecular clock. Timing of nutrient intake also influences the central circadian clock, evidenced by the shift in the body temperature rhythm. The fasting response was more dramatic for cows on the NRF treatment, having a greater post-feeding feed consumption rate, and greater pre-meal NEFA concentrations, which is likely due to the circadian rhythm of food intake. This study supports the hypothesis that timing of nutrient absorption modified the daily rhythms of milk synthesis, likely due to entrainment of the mammary circadian clock.
**FIGURES**

**Figure 3-1.** Effect of day- versus night-restricted feeding on the daily pattern of feed intake.

Panels show: (A) Daily schedule of feed availability and milking time for day-restricted feeding (DRF; feed available for 16 h/d from 0700 to 2300) and night-restricted feeding (NRF; feed available for 16 h/d from 1900 to 1100) treatments and milking times. Cows were adapted to feeding schedules for 10 d prior to 7 d of 4x milking. (B) Effects of day versus night feed availability on the rate of feed intake (kg DM/h). Data are presented as the least square means with standard error bars for every 2 h period. Preplanned contrasts of the effect of treatment at each time point are shown (*P < 0.05).
Figure 3-2. Effect of day- versus night-restricted feeding on daily rhythms of milk yield and milk components.

Treatments were feed available for 16 h during the day [day-restricted feeding (DRF); feed from 0700 to 2300] or feed available for 16 during the night [night-restricted feeding (NRF); feed from 1900 to 1100]. Data are presented as LSM with SEM bars. Panels show the effect of day vs. night-restricted feeding on the daily pattern of A. milk yield (kg), B. milk fat yield (g), C. milk fat concentration (%), D. milk protein yield (g), E. milk fat concentration (%). 1Amplitude- difference between peak and mean. 2Acrophase-time at peak of the rhythm. 3P-value of the zero-amplitude test. The black and white bars above the x-axis display the light: dark cycle.
Figure 3-3. Effect of day- versus night-restricted feeding on the daily production and daily pattern of milk fatty acids.

Treatments were feed available for 16 h during the day [day-restricted feeding (DRF); feed from 0700 to 2300] or feed available for 16 during the night [night-restricted feeding (NRF); feed from 1900 to 1100]. Panels show effect of day vs. night-restricted feeding on (A) total daily yield of de novo (Σ <16C FA), mixed (Σ 16C FA), and preformed (Σ >16C FA) FA (g/d), (B) daily average milk fat concentration of trans-10 C18:1 (t10) trans-11 C18:1 (t11; % of total C18 FA), (C) daily patterns of milk denovo FA yield (g/d), (D) daily patterns of milk t10 concentration (% of total C18 FA), (E) daily patterns of milk mixed source FA yields (g/d), (F) daily patterns of milk t11 concentration (% of total C18 FA), (G) daily patterns of milk preformed FA yields (g/d). 1Amplitude- difference between peak and mean. 2Acrophase-time at peak of the rhythm. 3P-value of the zero-amplitude test. Data are presented as LSM with SEM bars. The black and white bars above the x-axis display the light: dark cycle.
Figure 3-4. Effect of day- versus night-restricted feeding on daily rhythms of plasma hormones and metabolites.

Treatments were feed available for 16 h during the day [day-restricted feeding (DRF); feed from 0700 to 2300] or feed available for 16 during the night [night-restricted feeding (NRF); feed from 1900 to 1100]. Panels show effect of day vs. night-restricted feeding on (A) daily patterns of plasma glucose concentration (mg/dL), (B) daily patterns of plasma insulin concentration (μIU/mL), (C) daily patterns of plasma NEFA concentration (μEq/L), (D) daily patterns of plasma urea nitrogen concentration (mg/dL), (E) daily patterns of milk mixed source FA yields (g/d), (F) daily patterns of milk t11 concentration (% of total C18 FA), (G) daily patterns of milk preformed FA yields (g/d). Data are presented as LSM with SEM bars. 1Amplitude-difference between peak and mean. 2Acrophase-time at peak of the rhythm. 3P-value of the zero-amplitude test. The black and white bars above the x-axis display the light: dark cycle.
Figure 3-5. Effect of day- versus night- restricted feeding on the circadian rhythm of body temperature in dairy cows.

Treatments were feed available for 16 h during the day [day-restricted feeding (DRF); feed from 0700 to 2300] or feed available for 16 during the night [night-restricted feeding (NRF); feed from 1900 to 1100]. Data presented as 2 h means and SEM bars of body temperature collected every 10 min by a vaginal temperature data logger. 

<table>
<thead>
<tr>
<th>Trt</th>
<th>Mean</th>
<th>Amp¹</th>
<th>Acro²</th>
<th>P-value³</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRF</td>
<td>38.7</td>
<td>0.04b</td>
<td>0154a</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>NRF</td>
<td>38.7</td>
<td>0.07a</td>
<td>1724b</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

¹Amplitude- difference between peak and mean. ²Acrophase-time at peak of the rhythm. ³P-value of the zero-amplitude test. The black and white bars above the x-axis display the light: dark cycle.
**TABLES**

**Table 3-1.** Effect of day versus night-restricted feeding on total daily DMI, milk yield, and milk composition.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>SEM$^2$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI, kg/d</td>
<td>DRF</td>
<td>1.15</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>1.34</td>
<td>0.68</td>
</tr>
<tr>
<td>Milk yield, kg</td>
<td>DRF</td>
<td>33.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>33.2</td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>DRF</td>
<td>3.54</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>3.45</td>
<td></td>
</tr>
<tr>
<td>Fat Concentration, %</td>
<td>DRF</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Fat Yield, g/d</td>
<td>DRF</td>
<td>1125</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>1061</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>DRF</td>
<td>3.28</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td>Protein Concentration, %</td>
<td>DRF</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Protein Yield, g/d</td>
<td>DRF</td>
<td>1059</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>NRF</td>
<td>1010</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Treatments were feed available for 16 h during the day [day-restricted feeding (DRF); feed from 0700 to 2300] or feed available for 16 during the night [night-restricted feeding (NRF); feed from 1900 to 1100.
Supplemental Figure S3-1. Effect of the day versus night-restricted feeding on feeding behavior.

Treatments were feed available for 16 h during the day [day-restricted feeding (DRF); feed from 0700 to 2300] or feed available for 16 during the night [night-restricted feeding (NRF); feed from 1900 to 1100]. Data are presented as LSM with SEM bars. Panels show effect of day vs. night-restricted feeding on A. average meal size (kg) and average number of meal bouts/d, B. Meal length and intermeal interval (min), C. eating time (min/d) and eating rate (kg/min), D. hunger and satiety ratios. Hunger ratio=meal size/preceding intermeal interval. Satiety ratio=meal size/post-meal interval.
SUPPLEMENTAL TABLES

Supplemental Table S3-1. Diet and nutrient composition of the experimental diet.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients, % of DM</td>
<td></td>
</tr>
<tr>
<td>Corn silage(^1)</td>
<td>40.3</td>
</tr>
<tr>
<td>Alfalfa haylage(^2)</td>
<td>19.3</td>
</tr>
<tr>
<td>Canola meal</td>
<td>13.2</td>
</tr>
<tr>
<td>Ground corn</td>
<td>12.6</td>
</tr>
<tr>
<td>Roasted soybeans</td>
<td>4.6</td>
</tr>
<tr>
<td>Molasses</td>
<td>3.8</td>
</tr>
<tr>
<td>Grass hay/straw(^3)</td>
<td>3.3</td>
</tr>
<tr>
<td>Vitamin/mineral mix(^4)</td>
<td>2.5</td>
</tr>
<tr>
<td>Non-protein nitrogen(^5)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

| Nutrients, % of DM                |             |
| NDF                               | 32.5%       |
| ADF                               | 22.2%       |
| CP                                | 16.8%       |
| Ash                               | 6.9%        |

\(^1\)Contained (% of DM): 6.8% CP, 36.4% NDF, 22.8% ADF.
\(^2\)Contained (% of DM): 20.3% CP, 47.6% NDF, 41.6% ADF.
\(^3\)Contained (% of DM): 9.2% CP, 70.9% NDF, 43.4% ADF.
\(^4\)Composition (DM-basis): 10.6% CP; 43.2% NDF; 7.0% ADF; 14.4% Ca; 0.75% P; 15.1% Cl; 0.28% K; 2.5% Mg; 0.5% S; 9.8% Na; 23.0 mg/kg Co; 651 mg/kg Cu; 796 mg/kg Fe; 54.0 mg/kg I; 1190 mg/kg Mn; 12.8 mg/kg Se; 3434 mg/kg Zn; 195.290 IU/kg vitamin A (retinyl acetate); 62,500 vitamin D (activated 7-dehydrocholesterol); 1864 IU/kg vitamin E (dl-α tocopheryl acetate).
\(^5\)Fed as coated urea (Optigen, Alltech Inc., Lexington, KY; 259% CP, DM basis).
**Supplemental Table S3-2.** Effect of day- versus night-restricted feeding on total daily and daily rhythms of individual milk fatty acids.

<table>
<thead>
<tr>
<th>Name</th>
<th>g/d(^1)</th>
<th>g/6 h(^1)</th>
<th>Amplitude(^2)</th>
<th>Acrophase(^3)</th>
<th>P-Value(^4)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>DRF</td>
<td>NRF</td>
<td>DRF</td>
<td>NRF</td>
<td>DRF</td>
</tr>
<tr>
<td>C4:0</td>
<td>47.1</td>
<td>44.7</td>
<td>11.9</td>
<td>11.7</td>
<td>-</td>
</tr>
<tr>
<td>C6:0</td>
<td>26.1</td>
<td>24.5</td>
<td>6.63</td>
<td>6.44</td>
<td>0.37(^b)</td>
</tr>
<tr>
<td>C8:0</td>
<td>15.1</td>
<td>14.2</td>
<td>3.83</td>
<td>3.73</td>
<td>0.22(^b)</td>
</tr>
<tr>
<td>C10:0</td>
<td>36.4</td>
<td>34.2</td>
<td>9.19</td>
<td>8.97</td>
<td>0.64(^b)</td>
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<tr>
<td>C10:1</td>
<td>3.10</td>
<td>2.97</td>
<td>0.79</td>
<td>0.73</td>
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<tr>
<td>C11:0</td>
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<td>0.92</td>
<td>0.26</td>
<td>0.23</td>
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<tr>
<td>C12:0</td>
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<td>39.7</td>
<td>10.7</td>
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<tr>
<td>C13:0</td>
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<td>1.47</td>
<td>0.40</td>
<td>0.36</td>
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<tr>
<td>C13:0 iso</td>
<td>0.17</td>
<td>0.16</td>
<td>0.042</td>
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<tr>
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<td>0.88</td>
<td>0.83</td>
<td>0.22</td>
<td>0.20</td>
<td>0.021(^a)</td>
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<tr>
<td>C14:0</td>
<td>129.5</td>
<td>120.7</td>
<td>32.7</td>
<td>31.2</td>
<td>2.22(^b)</td>
</tr>
<tr>
<td>C14:0 iso</td>
<td>0.87</td>
<td>0.75</td>
<td>0.23</td>
<td>0.19</td>
<td>0.013</td>
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<tr>
<td>C14:1</td>
<td>10.3(^a)</td>
<td>9.63(^b)</td>
<td>2.61(^a)</td>
<td>2.44(^b)</td>
<td>0.23</td>
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<tr>
<td>C15:0</td>
<td>14.0</td>
<td>13.0</td>
<td>3.52</td>
<td>3.25</td>
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<tr>
<td>C15:0 iso</td>
<td>2.27</td>
<td>2.08</td>
<td>0.57</td>
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<tr>
<td>C15:0 anteiso</td>
<td>4.99</td>
<td>4.80</td>
<td>1.26</td>
<td>1.22</td>
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<tr>
<td>C16:0</td>
<td>320.2(^a)</td>
<td>290.4(^b)</td>
<td>80.5</td>
<td>75.4</td>
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</tr>
<tr>
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<td>2.50(^a)</td>
<td>1.97(^b)</td>
<td>0.61(^a)</td>
<td>0.52(^b)</td>
<td>-</td>
</tr>
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<td>12.7</td>
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<td>6.39</td>
<td>1.60</td>
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<td>-</td>
</tr>
<tr>
<td>C17:0 iso</td>
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<td>3.75</td>
<td>0.89</td>
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<td>0.057</td>
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<tr>
<td>C17:0 anteiso</td>
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<td>C17:1</td>
<td>2.06</td>
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<td>0.044(^a)</td>
</tr>
<tr>
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<td>114.1</td>
<td>29.8</td>
<td>29.9</td>
<td>-</td>
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<td>trans-5 C18:1</td>
<td>trans-6,8 C18:1</td>
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<td></td>
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<td>1044</td>
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<td>0447</td>
<td>0417</td>
</tr>
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</tr>
</tbody>
</table>

1Least squared means of day-restricted feeding (DRF) with feed available from 0700 h to 2300 h and night-restricted feeding (NRF) with feed available from 0700 to 2300 h. Means with different superscripts are different at P < 0.05.

2Difference from mean to peak of 24-h rhythm, g. Amplitudes with different superscripts are different at P < 0.05.

3Time at peak of the 24-h rhythm hh:mm. Acro- phases with different superscripts are different at P < 0.05.

4P-value of the zero-amplitude test corresponding to the fit of a 24-h rhythm. The rhythm fit is significant if P < 0.05 with a tendency for a rhythm at 0.05 < P < 0.10. Amplitude and acrophase are not presented if the P-value of the zero-amplitude test is greater than 0.10.
Chapter 4

The effects of night-restricted feeding on the molecular clock of the mammary gland in lactating dairy cows

Isaac J. Salfer, Paul A. Bartell, and Kevin J. Harvatine
ABSTRACT

Circadian rhythms are generated within tissues through a molecular clock made of a set of transcription factors that oscillate in a 24 h manner. These rhythms can be entrained both by photoperiod and feeding time. Dairy cows display daily rhythms of milk synthesis that are altered by feeding time, but the role of molecular clocks in these rhythms is poorly understood. To determine the impact of feeding on the mammary molecular clock, 11 lactating Holstein cows were used in a crossover design that tested two feeding regimes, either (1) feed available for the entire 24 h day (CON) or (2) night-restricted feeding where feed availability was limited to 16 h/d from 2000 h to 1200 h (NR). All cows were housed in the same 19:5 light:dark cycle. Milk samples were collected at 0700 and 1900 h on d 11 and 17 of each period and analyzed for fat and protein concentration. Mammary tissue representing four times across the day (0400, 1000, 1600, and 2200 h) was collected by needle biopsy. Expression of clock genes including brain and muscle ARNT-Like 1 (BMAL1), circadian locomoter output cycles kaput (CLOCK), cryptochrome 1 (CRY1), period (PER1), period 2 (PER2), and REV-ERBa was determined at each time point using Real-Time RT-PCR. Blood was sampled on d 15 to 17 of each period to represent every 4 h across the day (0200, 0600, 1000, 1400, 1800, and 2200 h), and analyzed for concentrations of glucose, nonesterified fatty acids (NEFA), and plasma urea nitrogen (PUN). Cosinor rhythmometry was performed using the MIXED procedure of SAS 9.4 to determine if expression of clock genes and the concentrations of plasma metabolites fit a 24 h rhythm and if the amplitude and acrophase (time at peak) were different between treatments. Night-restricted feeding tended to reduce dry matter intake ($P = 0.06$) and decreased milk ($P = 0.001$), fat ($P = 0.02$) and protein yield ($P < 0.001$). Milk protein concentration was also reduced by NR ($P = 0.01$), but fat concentration was not changed. The expression of CLOCK fit a 24 h rhythm in CON ($P < 0.001$) and tended to fit a rhythm in NR ($P = 0.06$), with NR increasing the amplitude.
75% and delaying the phase by 6.5 h compared to CON ($P < 0.05$). The expression of CRY1 fit a 24 h rhythm in both treatments and NR increased the amplitude by 53% and delayed the phase by 3 h. A 24 h rhythm of REV-ERBα expression was not present in CON, but a tendency for a rhythm was induced by NR ($P = 0.06$). The expression of BMAL1, PER1, and PER2 did not fit a 24 h rhythm in either treatment. Plasma glucose, NEFA, and PUN concentrations fit a 24 h rhythm ($P < 0.005$). The amplitudes of the daily rhythms of plasma glucose and PUN concentration were increased 124% and 62% by NR, respectively ($P < 0.05$), but the amplitude of NEFA was not altered. Night-restricted feeding phase delayed plasma glucose concentration by 12 h and PUN concentration by 9.9 h, but did not shift the phase of the daily NEFA rhythm. Results indicate that key components of the mammary molecular clock are influenced by the timing of feed intake.

**Keywords**: molecular clock, food entrainment, circadian rhythm.
INTRODUCTION

All organisms have adapted a circadian timing system that allows them to anticipate daily changes in their environment and correspondingly alter their behavior, metabolism, and cell signaling. This system is arranged in a hierarchical manner with a master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, which is normally entrained by photoperiod and sends neural and hormonal signals to synchronize biological clocks located within individual cells of peripheral tissues (Plaut and Casey, 2012). Circadian oscillations in both the master clock and peripheral cells are driven by a core set of clock transcription factors that maintain rhythms of about 24 h by transcriptional-translational feedback loops (Panda, 2016). These transcription factors include positive transcriptional regulators brain and muscle ARNT-like 1 (BMAL1) and circadian locomotor output cycles kaput (CLOCK), which heterodimerize and bind to E-box elements [canonically CANNTG] to activate transcription, and the negative transcriptional regulators period (PER) and cryptochrome (CRY), which feedback to inhibit the expression of BMAL1 and CLOCK. Additionally, an ancillary loop consisting of the orphan nuclear receptors REV-ERBα and RAR-related orphan receptor-α (RORα) stabilize the transcriptional-translational feedback loop by repressing or activating BMAL1 and CLOCK (Takahashi, 2017). Together, these circadian transcription factors and nuclear receptors regulate the expression of a wide host of clock-controlled genes, which are involved in diverse functions including growth, metabolism, and immunity.

In addition to receiving signals from the master clock in the SCN, various other local and systemic signals influence the oscillations of peripheral clocks. One of the most prominent of these signals is the timing of feed intake. In other animals, the daily rhythms of locomotor activity and blood glucocorticoid concentrations are entrained by restricting the time of feed availability to the inactive phase of the day and these rhythms can persist several days after complete food
removal, even with ablation of the SCN (Stephan, 2002). Stokkan et al. (2001) established that
time-restricted feeding shifts the phase of the circadian clock of the liver, without a concomitant
effect in the SCN. This desynchronization of the rhythms of master and peripheral clocks has
been consistently observed during time-restricted feeding experiments in model organisms (Asher
and Sassone-Corsi, 2015).

Circadian rhythms appear to be important for metabolism within the mammary gland. In
several species, including humans, rodents and cattle, milk synthesis follows a daily pattern with
greater milk yield in the morning, and higher milk fat and protein concentration in the evening
(Plaut and Casey, 2012). Maningat et al. (2009) observed that 7% of the mammary epithelial
transcriptome follows circadian rhythms in humans. The circadian rhythm of the mammary gland
appears to be related to stage of lactation. Casey et al. (2014) observed an increase in BMAL1
and CLOCK protein abundance as well as a decrease in PER2 in differentiated cells compared to
undifferentiated cells. This is substantiated by evidence that short photoperiod during the dry
period increases mammary epithelial cell proliferation and increases milk synthesis in the
subsequent lactation (Wall et al., 2005). Furthermore, rate-limiting enzymes for fat, protein, and
lactose synthesis follow circadian rhythms in the mammary gland (Suárez-Trujillo and Casey,
2016).

Recent evidence indicates that the daily pattern of milk synthesis is responsive to the
timing of feed intake. Rottman et al. (2014) determined that feeding four times per day in equal
meals altered the daily pattern of milk synthesis relative to feeding once per day. Furthermore, we
previously established that time-restricted feeding shifts the phase of daily rhythms of milk
synthesis and in dairy cows (Chapter 3). However, it is unknown if these shifts are due to
entrainment of the molecular clock of the mammary gland by feeding or by the timing of nutrient
absorption. In mice, night-restricted feeding altered the expression of circadian transcription
factors. Ma et al. (2013) observed that time-restricted feeding altered clock gene expression and
shifted the rhythms of expression of enzymes and transcription factors related to milk fat synthesis in lactating mice. The objectives of this experiment were to investigate the effect of time-restricted feeding on the molecular circadian clock of the mammary gland. The hypothesis was that restricting the time of feeding to 16 h during the night, which is typically the lowest period of feed intake, will cause a phase shift in the daily patterns of clock genes in mammary tissue, demonstrating a change in the molecular clock of the mammary gland.

MATERIALS AND METHODS

Animals and Treatments

Eleven multiparous mid-lactation Holstein cows (190 ± 31 d postpartum; mean ± SD) from the Pennsylvania State University Dairy Research and Teaching Center were randomly assigned to one of two treatments sequences in a cross-over design. Treatment periods were 22 d long with 10 d of diet adaptation followed by 12 d of sampling. Treatments included (1) control (CON) with feed delivered at 0800 h and available 24 h/d and (2) night-restricted feeding (NR) with feed available for 16 h/d from 2000 h to 1200 h (Figure 4-1). All cows were fed the same total mixed ration (TMR) that was mixed once daily at 0800 h and fed immediately to the CON group (Supplemental Table S4-1). The remaining feed was compressed into plastic barrels, covered with plastic, and stored at ambient temperature until NF feeding at 2000 h. Feed intake was recorded daily and feed was offered at 110% of the previous day’s intake.

Feed samples were collected on d 7, 14, and 21 of each period and composited by period prior to analysis. Dry matter, ash, starch, and CP were analyzed according to the AOAC (2000) Official Methods of Analysis, and NDF and ADF were analyzed according to Van Soest et al. (1991). Dry matter intake was determined on d 15 to 18 of each period. Animals were housed in individual tie-stalls with sawdust bedded rubber mattresses and had ad libitum access to water.
Cows were maintained in a 19 h light: 5 h dark photoperiod with lights on from 0500 to 0000 h.
The experiment was conducted from January to March of 2017. All experimental procedures were approved by the Penn State University Institutional Care and Use Committee.

**Milk Sampling and Analysis**

Cows were milked 2x/d at 0700 and 1900 h and milk yield was measured at each milking using an integrated meter (AfiMilk MPC Milk Meter; Afimilk Agricultural Cooperative Ltd., Kibbutz Afikim, Israel). Milk yields were corrected for stall deviations according to Andreen et al. (2018). Milk was sampled on d 10 and 17 of each period. One subsample was stored at 4°C in preservative (Bronolab-WII; Advanced Instruments, Inc., Noorwood, MA) prior to analysis of fat, protein, lactose, and MUN by Fourier transform infrared spectroscopy (Fossomatic 4000 Milko-Scan and 400 Fossomatic, Foss Electric; Dairy One Laboratory, Ithaca, NY). A second subsample was immediately centrifuged at 2300 x g at 4°C for 15 min and fat cakes were stored at -20°C for analysis of fatty acid (FA) composition. Milk FA were extracted using hexane:isopropanol, transmethylated using sodium methoxide, and FA methyl esters were quantified by GLC with a flame-ionization detector according to Rico and Harvatine (2013) as modified by Baldin et al. (2018).

**Mammary Tissue Collection**

Mammary biopsies were collected to represent every 6 h across the day (0400, 1000, 1600, and 2200 h). On d 11 to 14, one of the rear quarters was randomly chosen for a biopsy collection at 2200 h, followed by a second biopsy on the opposite quarter at 1000 h the following day. Following 7 d of recovery, a third and fourth biopsy was similarly collected from each rear quarter on d 19 to 22 at 1600 and 0400 h. Cows were sedated using intravenous xylazine (0.02 mg/100 kg of BW) and local anesthesia was applied by subdermal block using 10 mL of 2%
lidocaine HCl in a line above the incision site. The biopsy site was disinfected with providone-iodine scrub (Betadine, Avrio Health L.P., Stamford, CT) and ethanol rinse and a surgical blade was used to make a 0.2 to 0.5 cm incision near the midpoint of the rear quarter. Tissue was collected from a depth of 8 to 12 cm using a vacuum-assisted electronic biopsy gun system with a 10 gauge needle (Vacora; Bard Biopsy Systems, Tempe, AZ). Tissue samples were rinsed with 0.9% saline solution, snap frozen in liquid nitrogen, and stored at −80°C until RNA extraction (~80 mg tissue/biopsy). After biopsy collection, manual pressure was placed on the biopsy site for 5 to 10 min until bleeding ceased. Minimal bleeding occurred and milk appeared normal within 3 to 5 milkings after the biopsy. No apparent intramammary infections or production declines occurred as a result of the surgery.

**Plasma Sampling and Analysis**

Blood was collected using potassium EDTA vacuum tubes (Greiner Bio-One North America, Inc., Monroe, NC) by venipuncture of a coccygeal vessel on d 15 to 17 of each period to represent every 4 h across the day (0200, 0600, 1000, 1400, 1800, and 2200 h). Blood was immediately placed on ice and centrifuged at 2300 x g for 15 min at 4°C within 30 min of collection. Plasma was collected and stored at -20°C for analysis of glucose, plasma urea nitrogen (PUN), and non-esterified FA (NEFA) according to Rottman et al. (2014). Briefly, Plasma glucose concentration was analyzed using a glucose oxidase/peroxidase enzymatic colorimetric assay (No. P 7119, Sigma-Aldrich, St. Louis, MO), PUN was assayed using a modified Berthelot methodology (Modified Enzymatic Urea Nitrogen Procedure No. 2050; Stanbio Laboratory, Boerne, TX), and NEFA concentration was measured using a acyl-CoA oxidase/peroxidase enzymatic colorimetric assay [NEFA-HR (2), Wako Diagnostics, Richmond, VA].
Gene Expression Analysis

Approximately 40 mg of mammary tissue was homogenized in 1.5 mL of RNA-Solv (Omega Bio-Tek, Norcross, GA) and total RNA was isolated from approximately 40 mg of mammary tissue using the E.Z.N.A. Total RNA Kit II with on-column DNase treatment (Omega Bio-Tek). RNA concentration and quality were determined using a spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, DE). Total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA) using random primers. Relative gene expression was performed using quantitative PCR (qPCR) using an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Inc.) with 400 nM of gene-specific forward and reverse primers and SYBR Green reporter dye (PerfeCTa SuperMix with ROX, Quanta Biosciences, Gaithersburg, MD). Primers were designed to span exon boundaries and using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD) to amplify a product length between 70 and 150 bp or were from previous publications as indicated (Table 4-1). Reactions were performed in triplicate and transcript abundance was determined relative to a dilution curve of pooled cDNA and normalized using the geometric mean of reference genes [ribosomal protein 9 (RPS9), beta-2 microglobulin (B2M), and beta-actin (ACTB)], which was included as a covariate in the statistical model. The presence of a single product was verified by dissociation curve analysis.

Statistical Analysis

All statistical analysis was performed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Daily DMI, milk, fat, and protein yield, fat and protein concentration, and FA yields were analyzed as a mixed effects model with the fixed effects of treatment and period and the random effects of cow and day nested within period. The autoregressive covariance
structure was used and denominator degrees of freedom were adjusted using the Kenward-Roger method.

Cosinor rhythometry was performed on time course gene expression, plasma metabolites, and body temperature data. Briefly, data were fit to the linear form a cosine function by random regression in SAS 9.4 with a model that tested the fixed effects of treatment, linear form of the cosine function, and the interaction of treatment with cosine function, and the random effects of cow and period. The fit of the 24 h cosine was tested using a zero-amplitude test, and the amplitude and acrophase (time at peak of rhythm) were calculated and significance determined according to Niu et al. (2014). The presence of a 12 h harmonic was tested for plasma hormone and metabolite data and was included for glucose and NEFA because it improved model fit. Secondly, a model testing the fixed effects of treatment, time, and their interaction, and the random effects of cow with preplanned contrasts testing the interaction of treatment and time was performed on time course data. In all analyses, points with Studentized residuals ± 3.5 were removed as outliers. Significance was declared at $P < 0.05$ with trends recognized at $P < 0.10$. High resolution figures were generated using an add-in for Microsoft Excel (Kraus, 2014).

**RESULTS AND DISCUSSION**

**Dry Matter Intake**

Night-restricted feeding tended to reduced DMI with NR cows consuming 1.7 kg (7%) less feed per day than CON ($P = 0.06$; **Figure 4-2A**). The decrease in intake is likely because NR cows were fasted during the high intake period of the day. Dairy cow naturally prefer to eat the greatest amount of feed during the morning and afternoon, with limited feed intake overnight (Albright, 1993). Previous research revealed that night-restricted feeding numerically decreased DMI by 1.6 kg relative to restricting feed availability to 16/d during the day (0900 to 1100), but
this change was not significant (Chapter 3). Niu et al. (2014) also observed a 1.2 kg (4%) decrease in DMI by delivering feed at night (2030) versus the morning (0830) without restricting the time of feed availability. Taken together, fasting during the afternoon when intake is typically high reduces the total amount of feed consumed per day.

*Milk Yield, Milk Composition, and Milk Fatty Acid Yields*

Night-restricted feeding reduced milk yield by 8% \( (P = 0.001; \text{Figure 4-2B}) \). Furthermore, NR decreased milk fat and protein yield by 11% \( (P = 0.02) \) and 10% \( (P < 0.001) \), respectively (Figure 4-2D). Milk fat concentration was not affected by treatment \( (P = 0.91) \), while milk protein concentration was decreased 2% by NR \( (P = 0.01; \text{Figure 4-2C}) \). We previously failed to detect a difference in milk yield and milk fat and protein concentration between cows undergoing time-restricted feeding for 16 h/d either during the day or the night, although night-restricted feeding decreased milk fat yield 64 g (6%) compared to day-restriction (Chapter 3). Rottman et al. (2014) also observed that feeding 4 x/d in equal meals increased the yields and concentrations of both fat and protein compared to feeding 1x/d.

The reduction in milk synthesis in the current experiment may be related to modification of circadian clock of the mammary gland. In rodent models, restricting the time of feeding to the inactive phase without altering feed intake reduces the accretion of adipose tissue (Hatori et al., 2012; Sherman et al., 2012). This change has been associated with increased robustness of daily rhythms of adipocyte clock genes. Moreover, time-restricted feeding reduces adiposity in humans (Manoogian and Panda, 2017). A similar mechanism may occur in cows, whereby alterations in the circadian clock reduce the uptake and/or synthesis of nutrients in the mammary gland. Hu et al. (2017) observed that small interfering RNA (siRNA)-mediated knockdown of *PER2* mRNA expression was associated with a reduction in the synthesis of \( \alpha_{s1} \)- and \( \alpha_{s2} \)-casein in mammary
epithelial cell culture establishing a relationship between the mammary circadian clock and protein synthesis.

The decrease in milk and milk fat and protein yield in the current experiment is possibly confounded by the reduction in feed intake. While milk yield drives intake, unless another limiting factor (rumen fill, energy excess) is present, fasting during the high-intake afternoon of the day may have prevented cows from eating to meet their requirements for milk synthesis (Allen, 2014). Future experiments examining the role of time of feed intake on the mammary circadian clock may consider pair-feeding cows to eliminate this effect.

Night-restricted feeding did not alter the yield of FA originating from de novo synthesis (Σ < 16C; P = 0.50), preformed FA originating from plasma (Σ > 16C; P = 0.41), or mixed-source FA (Σ 16C; P = 0.37; Figure 4-3). These results differ from previous research, which observed a 5% reduction in de novo synthesized fatty acids and an 8% decrease in mixed-source fatty acids in cows restricted to feeding for 16 h/d the night compared to the day (Chapter 3). However, similar to the current paper, altering the timing of feed delivery without restricting the time of feed availability did not affect FA profile (Niu et al., 2014). In the current experiment, individual FA, iso-C14:1, iso-C15:1, iso-C16:1, C22:0 and C20:3-n6 were decreased by NR (P < 0.05), C24:0 tended to be increased by NR feeding (P = 0.09), while NR tended to decrease both trans-10 C18:1 and trans-11 C18:1 (0.05 < P < 0.10; Supplemental Table S4-2).

**Molecular Clock Gene Expression**

The expression of CRY1 fit a daily rhythm in both treatments (P < 0.05), CLOCK fit a rhythm in CON (P < 0.001) and tended to fit a rhythm in NR (P = 0.06), and REV-ERBα failed to fit a rhythm in CON (P = 0.62), but tended to fit a rhythm in NR (P = 0.06; Figure 4-4). However, BMAL1, PER1, and PER2 did not fit daily rhythm in either treatment (P > 0.10). Night-restricted feeding appeared to have a slight effect on the pattern of PER1 expression, with both
treatments having similar baseline expression with a noticeable rise of about 30% at 0400 in CON that was shifted to 1600 in NR. However, this change was not significant. The daily rhythms of CLOCK and CRY1 were affected by treatment. Night-restricted feeding increased the amplitude of CLOCK by 75%, and phase-delayed the rhythm ~4.5 h. Night-restricted feeding did not alter the amplitude of CRY1, but phase-delayed the rhythm by 3 h.

Time-restricted feeding can entrain circadian rhythms of other peripheral tissues in rodent models. Damiola et al. (2000) detected a 12 h phase shift in the liver protein abundance of PER1 and PER2 in mice that had feed availability restricted for 12 h/d during the light phase (0600 to 1800) or the night phase (1800 to 0600), without a concomitant change in SCN clock gene expression. These results were corroborated by Stokkan et al. (2001) in rats feed-restricted to only 4 h during the inactive period. In addition to shifting the phase of the liver molecular clock, they observed that the molecular clock in the lung was shifted by time-restricted feeding, but the clock of the SCN was unaltered. The daily rhythms of mRNA abundance of Bmal1, Per1, Per2, Cry2, and Rev-erba in adipocytes of male C57BL/6 mice were phase shifted by restricting the intake of a high-fat diet to 4 h/d during the inactive period of the day.

There has been limited research describing a role of the circadian clock in the mammary gland. In human mammary epithelial cells, 7% of genes follow a 24 h rhythm (Maningat et al., 2009). Moreover, Casey et al., (2009) suggested a potential role of the mammary clock in modulating the homorhetic response to lactation by discovering an increases in expression and phase shifts in rhythms of positive transcriptional regulators BMAL1, CLOCK, and RORα in lactating compared to nonlactating mammary tissue. In mice, the amplitudes of BMAL1 and CRY1 are increased after the start of lactation (Metz et al., 2006). Rhythms of mammary clock gene expression can be entrained by altering the light-dark cycle (Plaut and Casey, 2012). Furthermore, timing of nursing can entrain the mammary rhythms of PER1 in rabbits. The current...
experiment demonstrates that, in addition to these cues, time-restricted feeding can modestly entrain the molecular clock of mammary gland of dairy cows.

**Plasma Hormones and Metabolites**

There was a treatment by time interaction for plasma glucose ($P = 0.006$) but no main effect of treatment was present ($P = 0.94$; **Figure 4-5A**). The fit of the 24 h rhythm was improved in both treatments by including a 12 h harmonic ($P < 0.001$). A 24 h rhythm with a 12 h harmonic was present in both CON ($P = 0.004$) and NR ($P < 0.0001$). The acrophase of the 24 h rhythm peaked at 0137 in CON, but the rhythm with phase delayed 12 h by NR, which peaked at 1337 ($P < 0.05$). Furthermore, night-restricted feeding increased the amplitude of plasma glucose concentration by 124% from 6.5 mg/dL in CON to 14.6 mg/dL in NR ($P < 0.05$).

Previous research has demonstrated that time of feeding alters the daily rhythms of plasma glucose concentration. We previously detected a phase delay of 10.2 h in plasma glucose concentrations in cows with feed available for 16 h/d from 1900 to 1100 compared to those with feed available for 16 h/d from 0700 to 2300 (Chapter 3). Similar to the current experiment, they observed a 149% increase the amplitude of the plasma glucose rhythms during night-restricted feeding. Moreover, Niu et al. (2014) detected that the daily pattern of plasma glucose concentration was altered by changing the time of feed delivery from 0830 to 2030 h without restricting the time of feed availability.

Unlike previous experiments, we observed a 12 h harmonic in the daily pattern of plasma glucose, suggesting that glucose in our experiment followed a 12 h ultradian rhythm in addition to the daily rhythm. In both treatments, the harmonic approximately corresponded to the acrophase of the opposite treatment. In humans subjected to continuous feeding, glucose concentration has been reported to follow ultradian rhythms of various period lengths, including 12 h (Simon et al., 1987; Shea et al., 2005). To our knowledge, however, there have been no reports of a 12 h
harmonic in cattle. Lefcourt et al. (1993) observed that dairy cows express 3 h ultradian rhythms of plasma cortisol concentration. Cortisol increases plasma glucose concentrations by increasing gluconeogenesis and decreasing tissue glucose uptake, and may be partially responsible for the ultradian rhythm of glucose concentration observed in the current experiment.

Night-restricted feeding did not affect average NEFA concentration \( (P = 0.74; \textbf{Figure 4-5B}) \). A single 24 h rhythm did not fit either treatment, but similar to glucose concentration, both treatments fit a 24 h rhythm that included a 12 h harmonic \((P < 0.01)\). The amplitude of the primary 24 h rhythm was not altered by treatment \((P > 0.05)\). Furthermore, the acrophase of the main rhythm was not affected, with CON peaking at 1125 h and NR peaking at 1237 h \((P > 0.05)\). These results suggest that night-restricted feeding did not alter the daily pattern of lipid mobilization, despite fasting during the typically high-intake afternoon period of the day. Results contrast with previous research from our lab which demonstrated that night-restricted feeding from 1900 to 1100 h shifted the rhythms of NEFA concentration by 10.7 h relative to day-restricted feeding from 0700 to 2300 h (Chapter 3). Furthermore, we detected over a 3-fold increase in the amplitude of the rhythm due to night-restricted feeding. The differences in the current study and previous study may be related to the daily pattern of intake. The previous experiment imposed day-restricted feeding which likely caused more extreme differences in the pattern of feed intake. In the current experiment, maximum NEFA concentration in both treatments corresponded to the period when the NR cows were fasted, with the harmonic occurring at night, during which cows typically have reduced intake. Social interactions have previously shown to have a strong influence on feeding behavior of dairy cattle (Grant and Albright, 2001). Cows on CON may modified their daily pattern of feed intake to correspond to the NR treatment. Moreover, cows on the current experiment may have been in more positive energy balance, resulting in lower lipid mobilization in response to fasting. The results of our experiment correspond more closely with experiments where the time of feed delivery was
altered without restricting the time of feed availability. Nikkhah et al. (2008) observed little
difference in the daily pattern of NEFA concentration when cows were fed at 0900 h versus 2100.
Furthermore, Niu et al. (2014) detected no difference in the daily pattern of NEFA among cows
fed 1x/d at 0830, 1x/d at 2030, or 2x/d at both 0830 and 2030.

Plasma urea nitrogen concentration was not affected by treatment ($P = 0.25$; Figure
4-5C). A 24 h rhythm was present in both treatments ($P < 0.01$), with treatment affecting the
daily rhythm. Night-restricted feeding increased the amplitude of the daily rhythm 62% compared
to CON ($P < 0.05$) and delayed the phase 9.9 h from 1247 to 2241h. A 24 h rhythm of PUN has
been well-established in previous experiments. Our results agreed with a previous experiment in
our lab which demonstrated a 24 h rhythm in cows under night-restricted feeding that was phase
delayed 12.2 h relative to day-restricted feeding for 16 h/d (Chapter 3). Furthermore, Nikkhah et
al. (2008) observed an approximately 12 h delay in the peak of PUN in cows fed at 2100 versus
0900, but only in cows fed low fiber (38:62 forage: concentrate; 28.6 NDF), but not high fiber
diets (51:49 forage: concentrate; 33.8% NDF), suggesting that the effect may be diet dependent.
Results suggest that night-restricted feeding shifts the daily rhythms of amino acid utilization
efficiency.

**CONCLUSIONS**

Restricting feed availability to 16 h during the night increased the amplitude and phase-
shifted the daily rhythms of expression of several molecular clock genes in mammary tissue,
suggesting that nutrients can entrain the mammary molecular clock. These shifts may be partially
mediated by changes in plasma metabolites, because night-restricted feeding shifted the daily
patterns of plasma glucose and plasma urea nitrogen. Results may be partially influenced by a
reduction in total dry matter intake by night-restricted feeding, which was likely responsible for
the decrease in milk, fat and protein yield observed in this treatment. Future studies examining the role of the time of feeding on the molecular clock of the mammary gland may consider pair-feeding cows to ensure intake is consistent among treatments. Additionally, this study was limited to examining clock genes at the mRNA level. Additional post-transcriptional and post-translational modifications of clock-related proteins also affect the manifestation of circadian rhythms in tissues. Further research examining the role of food intake on the protein expression and protein phosphorylation will provide a more complete depiction of the role of nutrients on the mammary clock. However, this study provides initial compelling evidence to suggest that food intake can entrain the molecular clock of the mammary gland.
Figure 4-1. Schedule of feeding, lighting and sampling during the experiment.

Treatments were feed available continuously for 22 h/d starting at 0800 h (CON) or night restricted feeding with feed available for 16 h/d from 2000 to 1200 h (NR). Panels show (A) the times of the day when feed was available, lights were on/off, mammary biopsies were collected, and blood was collected and (B) the days of each period when milk was sampled, biopsies were collected, and blood was collected.
Figure 4-2. Effect of night-restricted feeding on average daily feed intake and milk synthesis.

Treatments were feed available continuously for 22 h/d starting at 0800 h (CON) or night restricted feeding with feed available for 16 h/d from 2000 to 1200 h (NR). Panels show the effect of night-restricted feeding on (A) dry matter intake (kg/d), (B) milk yield (kg/d), (C) milk fat and protein concentration (%), and (D) milk fat and protein yield (g/d).
Figure 4-3. Effect of night-restricted feeding on milk fatty acid yields by origin.

Treatments were feed available continuously for 22 h/d starting at 0800 h (CON) or night restricted feeding with feed available for 16 h/d from 2000 to 1200 h (NR). Fatty acids grouped by biosynthetic origin including FA derived from de novo synthesis in the mammary gland (De novo; Σ < 16C), mixed source from de novo synthesis and preformed uptake (Mixed; Σ 16C), and preformed FA taken up by the mammary gland from plasma (Preformed; Σ >16C).
Figure 4-4. Effect of night-restricted feeding on the daily patterns of expression of molecular clock genes in the mammary gland.

Treatments were feed available continuously for 22 h/d starting at 0800 h (CON) or night restricted feeding with feed available for 16 h/d from 2000 to 1200 h (NR). Panels show mammary mRNA expression of (A) brain and muscle ARNT-like 1 (BMAL1) (B) circadian locomotor output cycles kaput (CLOCK), (C) period 1 (PER1), (D) period 2 (PER2), (E) cryptochrome 1 (CRY1), and (F) REV-ERBα across the day. LSM and SEM bars and cosine fit of the control (solid line) and NR (dotted line) are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test. The black and white bars above the x axis display the light: dark cycle.
Figure 4-5. Effect of night-restricted feeding on the daily patterns of plasma metabolites.

Treatments were feed available continuously for 22 h/d starting at 0800 h (CON) or night restricted feeding with feed available for 16 h/d from 2000 to 1200 h (NR). Panels show the effect of night-restricted feeding on daily patterns of (A) glucose concentration (mg/dL), (B) nonesterified fatty acids (NEFA; μEq/L) and (C) plasma urea nitrogen (mg/dL). Data are presented as relative expression LSM and SEM bars and cosine fit of the control (solid line) and NR (dotted line) are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test. NS: Reduced model fits data significantly better than daily rhythm model. The black and white bars above the x-axis display the light: dark cycle.
Table 4-1. Bovine primers used to quantify mammary gene expression with RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Amplicon Size (nt)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAL1</td>
<td>NC_037342.1</td>
<td>CAGCTCTCCTCCTCCGACAC</td>
<td>TGGCCAGGGTTATATCTG</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>CLOCK</td>
<td>NC_037333.1</td>
<td>TCAGTCTCAGGAGAGGTG</td>
<td>GGAGTGCTAGTATCTGTTGG</td>
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<td></td>
</tr>
<tr>
<td>CRY1</td>
<td>NC_037332.1</td>
<td>TGCAAGTTGCTACTCAAGGGGG</td>
<td>CAATACCTCTGCGATCCTTTG</td>
<td>149</td>
<td></td>
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<tr>
<td>PER1</td>
<td>NC_037346.1</td>
<td>CCAGGAGTTCTACCAGCAATG</td>
<td>GAGACGACCGAGGAGAAG</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>PER2</td>
<td>NC_037330.1</td>
<td>ACGTTTTCCAGTCTCAGT</td>
<td>CGTTTGGACTTCAGTCTTCCG</td>
<td>148</td>
<td></td>
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<tr>
<td>REV-ERBα²</td>
<td>NC_037346.1</td>
<td>TGGTGGGCTCGTGTGAAGGTTG</td>
<td>CCTTTTGACTGGATGTTCTGC</td>
<td>122</td>
<td></td>
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<tr>
<td>ACTB</td>
<td>NC_037352.1</td>
<td>GCGTGCTACAGGCTCCACC</td>
<td>CTTGTGTCACGCCAGATTTTC</td>
<td>55</td>
<td>(Kadegowda et al., 2009)</td>
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<tr>
<td>B2M</td>
<td>NC_037337.1</td>
<td>TTTGGAACACCCCCCGATAG</td>
<td>AAACCTCGATGTTGCCTTC</td>
<td>61</td>
<td>(Urrutia and Harvatine, 2017)</td>
</tr>
<tr>
<td>RPS9</td>
<td>NC_037345.1</td>
<td>CCTCGACCAAGACGCTGAAG</td>
<td>CCTCCGACACCACGGTTC</td>
<td>64</td>
<td>(Urrutia and Harvatine, 2017)</td>
</tr>
</tbody>
</table>

¹Gene names: ACTB = Beta-actin; B2M = Beta-2-microglobulin; BMAL1 = Brain and muscle ARNT-like protein-1; CLOCK = Circadian locomotor output cycles kaput; CRY1 = Cryptochrome 1; PER1 = Period 1; PER2 = Period 2; RPS9 = Ribosomal protein S9.
²Encoded by the nuclear receptor subfamily 1 group d member 1 (NR1D1) gene.
## Supplemental Table S4-1. Diet and nutrient composition of experimental diet.

<table>
<thead>
<tr>
<th>Item</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients, % of DM</td>
<td></td>
</tr>
<tr>
<td>Corn silage(^1)</td>
<td>43.6</td>
</tr>
<tr>
<td>Alfalfa haylage(^2)</td>
<td>20.2</td>
</tr>
<tr>
<td>Canola meal</td>
<td>12.4</td>
</tr>
<tr>
<td>Ground corn</td>
<td>10.9</td>
</tr>
<tr>
<td>Roasted soybeans</td>
<td>5.7</td>
</tr>
<tr>
<td>Grass hay/straw(^3)</td>
<td>2.7</td>
</tr>
<tr>
<td>Vitamin/mineral mix(^4)</td>
<td>2.1</td>
</tr>
<tr>
<td>Molasses</td>
<td>2.0</td>
</tr>
<tr>
<td>Controlled-release N(^5)</td>
<td>0.26</td>
</tr>
<tr>
<td>Nutrients, % of DM</td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>30.6</td>
</tr>
<tr>
<td>ADF</td>
<td>17.9</td>
</tr>
<tr>
<td>Starch</td>
<td>27.8</td>
</tr>
<tr>
<td>Ash</td>
<td>8.6</td>
</tr>
</tbody>
</table>

\(^1\) Contained (% of DM): 33.0% NDF, 16.2% ADF, 42.3% Starch.

\(^2\) Contained (% of DM): 42.5% NDF, 31.3% ADF, 0.7% Starch.

\(^3\) Contained (% of DM): 73.2% NDF, 40.8% ADF, 1.2% Starch.

\(^4\) Contained (%, as-fed basis): 37.0% Calcium carbonate; 29.9% dried corn distillers grains; 24.5% salt; 4.2% magnesium oxide (54% Mg); 2.4% organic phosphorus (15% P); 0.5% zinc sulfate; 0.2% mineral oil. Composition (DM-basis): 7.2% CP; 7.1% NDF; 3.7% ADF; 15.0% Ca; 0.75% P; 0.33% K; 2.6% Mg; 0.5% S; 9.8% Na; 23.0 mg/kg Co; 652 mg/kg Cu; 783 mg/kg Fe; 54.0 mg/kg I; 1190 mg/kg Mn; 12.8 mg/kg Se; 1718 mg/kg Zn; 88,700 IU/kg vitamin A (retinyl acetate); 28,400 vitamin D (activated 7-dehydrocholesterol); 850 IU/kg vitamin E (dl-α tocopheryl acetate).

\(^5\) Fed as coated urea (Optigen, Alltech Inc., Lexington, KY; 259% CP, DM basis).
**Supplemental Table S4-2.** Effect of night-restricted feeding on daily yields of individual milk fatty acids.

<table>
<thead>
<tr>
<th>FA, g/d</th>
<th>Treatment</th>
<th>CON</th>
<th>NR</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4:0</td>
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<td>56.75</td>
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1Least squared means of control (CON) with feed available continuously for 22 h/d fed at 0800, and night-restricted feeding (NR) with feed available 16 h/d from 1900 to 1110 h (n = 11 per treatment.

2Total odd-chain fatty acids (Σ 11:0, 13:0, 13:0 iso, 13:0 anteiso, 15:0, 15:0 iso, 15:0 anteiso, 17:0, 17:0 iso, 17:0 anteiso, cis-9 17:1).

3Total branched-chain fatty acids (Σ 13:0 iso, 13:0 anteiso, 14:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0 iso, 17:0 anteiso).

4Total odd- and branched-chain fatty acids (Σ 11:0, 13:0, 13:0 iso, 13:0 anteiso, 14:0 iso, 15:0, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0, 17:0 iso, 17:0 anteiso, cis-9 17:1).

5Ratio of trans-10 18:1 to trans-11 18:1.
Chapter 5

The effects of time of fatty acid infusion on the daily rhythms of milk synthesis and plasma hormones and metabolites in dairy cows

Isaac J. Salfer, P. A Bartell, and Kevin J. Harvatine
ABSTRACT

Dairy cows display daily rhythms of milk synthesis that appear to be driven by the circadian clock of the mammary gland and are altered by the time of feed availability. Fatty acids have been shown to entrain circadian rhythms in other peripheral tissues such as the liver and adipose tissue in experimental models, but their role in the mammary gland has not been well-investigated. The objective was to determine the effects of the timing of fatty acid absorption on the daily rhythms of milk synthesis. Nine lactating Holstein cows were arranged in a 3 x 3 Latin square design. Treatments were abomasal infusions of 350 g/d of an oil enriched in C18:1 either continuously throughout the day (CON) or over 8 h/d from 0900 to 1700 h (DAY) or from 2100 to 0500 (NGT). Treatment periods were 12 d with a 5 d washout. Cows were milked every 6 h during the final 7 d of each period to determine the daily patterns of milk synthesis. A 24 h rhythm was fit to time-course data using cosine analysis and the amplitude and acrophase (time at peak) were determined. Daily milk, fat, and protein yield and fat and protein concentration were not affected by treatment. Milk yield fit a 24 h rhythm in CON and DAY, and tended to fit a rhythm in NGT. The amplitude of the rhythm of milk yield was increased 29% in DAY compared to CON \((P < 0.05)\), but NGT was not affected. Furthermore, DAY delayed the acrophase of the daily rhythm of milk yield by 2 h compared to CON and NGT \((P < 0.05)\). Fat and protein concentration exhibited a daily rhythms in CON and NGT \((P < 0.05)\), but not DAY. The amplitude of the rhythm of fat concentration was increased 29% by NGT compared to CON. Fat yield tended to fit a 24 h rhythm in DAY \((P = 0.07)\), but not in CON or NGT. Moreover, protein yield fit a 24 h rhythm in all treatments and the amplitude of the rhythm was increased 30% by DAY and decreased 47% by NGT. Both de novo and mixed source FA yield was reduced by DAY and NGT, suggesting that restricting the time of FA infusion may reduce de novo fatty acid synthesis. Glucose failed to fit a daily rhythm in any treatment, while nonesterified fatty acids fit
a rhythm in CON and NGT, but the rhythm was eliminated by DAY. Plasma urea nitrogen fit a
daily rhythm in all treatments, and both the mean and amplitude were increased by DAY. Fatty
acid infusion during the daytime modified the daily rhythms of milk synthesis by increasing the
amplitude of milk yield and decreasing the amplitude of milk fat and protein concentration
whereas infusion at night had little effect. Day-infusion also modified the daily rhythms of
plasma metabolites by reducing the amplitude of nonesterified fatty acids and increasing the
amplitude of plasma urea nitrogen.

**Keywords:** Daily rhythm, milk synthesis, nutrient entrainment, circadian rhythm
INTRODUCTION

Dairy cows exhibit a daily pattern of feed intake that is naturally crepuscular, with high rates of intake during the morning and afternoon and a decline in intake overnight (Albright, 1993). Furthermore, intake is stimulated by delivery of fresh feed and milking (Menzi and Chase, 1994; DeVries et al., 2005). While most cows in the United States are fed a total mixed ration (TMR), which provides a consistent composition of nutrients over the day, the daily pattern of feed intake causes the consumption of nutrients to vary across the day (Ying et al., 2015). The daily pattern of feed intake can be altered by modifying the time of feed delivery, as well as increasing the frequency of feeding (e.g. Niu et al., 2014; Rottman et al., 2014).

In addition to a daily pattern of feed intake, cows display daily rhythms of milk and milk component synthesis. Typically, milk yield is greatest in the morning, while milk fat and protein concentration are greatest in the evening (Rottman et al., 2014; Ying et al., 2015). However, night-restricted feeding shifts these rhythms so that milk yield peaks in the evening and milk fat and protein concentration peak in the morning (Chapter 3). These daily rhythms appear to be driven by the molecular circadian clock of the mammary gland (Chapter 4). The molecular clock is composed of a transcriptional-translational feedback loop that includes a variety of transcription factors that oscillate over 24 h and regulate the expression of gene in a circadian manner (Takahashi, 2017). Moreover, a 24 h pattern post-translational regulation via phosphorylation and dephosphorylation adds another level of regulation to the molecular clock (Robles et al., 2017).

Light is the primary signal that entrains circadian rhythms, but an increasing body of evidence has demonstrating a role for timing of food intake in circadian entrainment of peripheral tissues. Fatty acids, in particular, play an important role in entrainment of circadian rhythms. The lipid-sensing family of nuclear receptors known as peroxisome proliferator-activated receptors
(PPARs) display 24 h rhythms of expression, and possess E-box elements, suggesting direct regulation by BMAL1/CLOCK (Oishi et al., 2005). Moreover, the expression of BMAL1 is activated by PPARα through binding to a PPAR-response element present in the BMAL1 promoter (Ribas-Latre and Eckel-Mahan, 2016). Feeding a high-fat diet has been shown to alter the molecular clock and the daily pattern of adiponectin expression in the liver of male C57BL/6 mice (Barnea et al., 2009; Branecky et al., 2015). Moreover, CLOCK−/− and BMAL−/− double-mutant mice have increased triglyceride accumulation in white adipose tissue, as well as disrupted rhythms of free fatty acids in blood (Shostak et al., 2013).

Evidence suggests that time-restricted feeding alters the molecular circadian clock of the mammary gland. We previously discovered that the daily rhythms of CLOCK and CRY1 mRNA expression were shifted 4 h by restricting the time of feed availability from 7 PM to 11 AM (Chapter 4). Furthermore, the daily rhythms of both clock genes and transcription factors involved in milk fat synthesis were altered by time restricted feeding in mice (Ma et al., 2013). While feed intake entrains the daily rhythms of milk synthesis, the role of individual nutrients, such as FA, is unknown. The objective of this experiment was to determine the effect of time of FA absorption on the daily rhythms of milk synthesis, plasma metabolites, and body temperature. We hypothesize that altering the time of FA absorption through abomasally infusing FA either continuously throughout the day or over 8 h during the day or the night will shift the phase of the daily rhythms of milk fat synthesis. Furthermore, we expect the daily rhythm of plasma nonesterified fatty acid concentration and core body to be shifted by time of fatty acid infusion.

**MATERIALS & METHODS**
**Animals and Treatments**

Nine multiparous mid-lactation (132 ± 90 d postpartum; mean ± SD) Holstein cows from the Penn State University Dairy Research and Teaching Center fitted with rumen cannulas and randomly assigned to treatment sequences in a 3x3 Latin Square design. Treatment periods were 12 d long with 10 d of treatment adaptation and 2 d of sampling. There was a 5 d washout between periods. During the final 7 d of each period, cows were milked every 6 h (4 x/d) to allow a daily rhythm to be fit to milk yield and components data.

Treatments were abomasal infusion of 350 g/d of an oil mixture either continuously throughout the day (CON) or over 8 h/d from 0900 h to 1700 h (DAY) or from 2100 h to 0500 h (NGT; Figure 5-1). The treatment consisted of a high oleate mixture of free fatty acids (KIC Chemicals, Inc, New Paltz, NY; Supplemental Table S5-2) with 10% w/w Tween 80 added to aid in emulsification. Cows in the CON treatment were infused a total of 22 h/d with no infusion occurring for 30 min during each milking. All cows were provided the same basal TMR fed once daily at 0600 h at 110% of the previous day’s intake (Supplemental Table S5-1). Feed samples were collected on day 7 of each period and analyzed for dry matter, ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), starch, and crude protein according to Rico and Harvatine (2013). The experiment was conducted from May 7 to June 22, 2018. All experimental procedures were approved by the Penn State University Institutional Care and Use Committee.

**Milk Sampling and Analysis**

Cows were milked every 6 h on the final 7 d of each period (0100, 0700, 1300, and 1900 h) to observe the daily rhythm of milk synthesis. Milk collected at each time point represented the sum of milk synthesis over the previous 6 h interval and is plotted as the midpoint of the previous milking interval (3 h prior to collection). Milk yield was measured at each milking using an
integrated milk meter (AfiMilk MPC Milk Meter; Afimilk Agricultural Cooperative Ltd., Kibbutz Afikim, Israel). Yields were corrected for the deviation of each individual stall according to Andreen et al. (2018). Milk was sampled at each milking on the final 2 d of each period. One subsample was stored at 4°C with preservative (Bronolab-WII; Advanced Instruments, Inc., Noorwood, MA) prior to analysis of fat and protein concentration by Fourier transform infrared spectroscopy (Fossomatic 4000 Milko-Scan and 400 Fossomatic, Foss Electric; Dairy One DHIA, Ithaca, NY). A second subsample was stored at 4°C and centrifuged at 2300 x g within 24 h. The resulting fat cakes were stored at -20°C and analyzed for concentrations of individual FA according to Baldin et al. (2018).

**Plasma Sampling and Analysis**

Blood was collected by veinipuncture of a coccygeal vessel using potassium EDTA vacuum tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Blood sampling occurred 6 times across d 11 to 12 representing every 4 h across the day (0300, 0700, 1100, 1500, 1900, and 2300 h). Samples were immediately placed on ice and centrifuged within 30 min at 2300 x g for 15 min at 4°C. Plasma was collected and stored at -20°C for analysis of glucose, nonesterified fatty acids (NEFA), and plasma urea nitrogen (PUN) as described by Rottman et al. (2014). Briefly, plasma glucose concentration was analyzed using a glucose oxidase/peroxidase enzymatic colorimetric assay (No. P 7119, Sigma-Aldrich, St. Louis, MO), NEFA concentration was measured with an using acyl-CoA oxidase/peroxidase enzymatic colorimetric assay (NEFA-HR (2), Wako Diagnostics, Richmond, VA), and PUN was assayed using a modified Berthelot methodology (Modified Enzymatic Urea Nitrogen Procedure No. 2050; Stanbio Laboratory, Boerne, TX).
**Body Temperature Analysis**

An intravaginal temperature logger was used to record core body temperature every 10 min on d 10 to 12 of each period as described by Niu et al. (2017). Briefly, a miniature plastic coated thermometer fastened to a vaginal implant was placed centrally in the vagina. Body temperature was averaged over 2 h intervals.

**Statistical Analysis**

All data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Models testing the effect of treatment on daily dry matter intake, milk production, and FA yields included the fixed effect of treatment and the random effects of cow and period. The AR(1) or ARH(1) covariance structure was selected based on convergence criteria, and denominator degrees of freedom were adjusted using the Kenward-Roger method. Individual treatment least squared means were compared using preplanned contrasts of CON versus DAY, CON versus NGT and DAY versus NGT.

Time course data for milk production, plasma metabolites, and body temperature were fit to the linear form of a cosine function with a period of 24 h using random regression in SAS 9.4. The model included the fixed effects of treatment, cosine terms, and the interaction between treatment and cosine terms, as well as the random effects of cow and period. The presence of a 12 h harmonic was tested for body temperature and plasma hormone and metabolite data, and was included if it improved model fit. The fit of the 24 h cosine was determined using a zero-amplitude test, and the amplitude and acrophase (time at peak of rhythm) were determined according to Niu et al. (2014). In all analyses, points with Studentized residuals outside ± 3.5 were removed. A separate model testing the fixed effects of treatment, time, and their interaction, with random effects of cow and period, and preplanned contrasts testing the interaction of
treatment and time was also conducted on all time course data. Statistical significance was declared at $P < 0.05$ and trends acknowledged at $0.05 < P < 0.10$. High resolution figures were generated using an add-in for Microsoft Excel (Daniel's XL Toolbox; Kraus, 2014).

RESULTS AND DISCUSSION

Milk and Milk Components Synthesis

Dry matter intake was not affected by treatment ($P = 0.25$; Figure 5-2). Furthermore, treatment did not affect daily milk or milk fat and protein yield or milk fat and protein concentration ($P > 0.10$; Figure 5-3). High oleic acid oils, like the one used in our experiment, have previously been shown to increase milk yield and milk fat concentration (LaCount et al., 1994). The results of our study suggest that the timing of FA absorption does not affect daily milk or milk component production.

Treatment altered the daily rhythms of milk synthesis (Figure 5-4). Milk yield fit a 24 h rhythm in CON and DAY ($P < 0.002$), and tended to fit a rhythm in NGT ($P = 0.08$). Relative to CON, DAY increased the amplitude of milk yield by 29% ($P < 0.05$), while NGT was unaffected ($P > 0.10$). Milk yield peaked in the morning in all treatments, similar to previous characterizations of the daily rhythm of milk synthesis in dairy cows (Rottman et al., 2014). Day-infusion phase-delayed the rhythm of milk yield by 2 h compared to CON and NGT ($P < 0.05$). Fat yield did not fit a rhythm in CON or NGT, but a rhythm was induced by DAY ($P = 0.02$), with the amplitude being 115% and 200% greater than CON and NGT, respectively ($P < 0.05$; Figure 5-4B). The time of fat infusion did not alter the phase of fat yield. A daily rhythm of protein yield was present in all treatments (Figure 5-4D). The amplitude was increased 30% by DAY, and decreased 32% by NGT. The phase of the rhythm was delayed 2 h by DAY ($P < 0.05$), but was not affected by NGT ($P > 0.10$).
Fat concentration fit a daily rhythm in CON and NGT ($P < 0.05$), but not DAY ($P > 0.10$; Figure 5-4C). Corresponding with the loss of rhythm, the amplitude of fat concentration was dramatically decreased by DAY (67%, $P < 0.05$), but was increased 29% by NGT, relative to CON ($P < 0.05$). Similar to previous reports, fat concentration peaked in the middle of the day in both CON and NGT (Rottman et al., 2014; Salfer et al., 2016). However, the phase of the rhythm was altered by the timing of infusion, with DAY phase-advancing the rhythm 2.75 h and NGT phase-delaying the rhythm 1 h relative to CON ($P < 0.05$). Similarly, protein concentration fit a daily rhythm in CON and NGT ($P < 0.05$), but not DAY ($P > 0.10$; Figure 5-4E). The amplitude was reduced by 50% in DAY ($P < 0.05$), but did not differ between CON and NGT ($P > 0.10$). Furthermore, the acrophase of CON occurred at 1555 h and did not differ from DAY or NGT ($P > 0.10$), but the phase occurred 1 h and 40 min earlier in DAY (1446 h) compared to NGT (1626 h; $P < 0.05$).

Fat metabolism has been previously linked to circadian rhythms of milk synthesis. Ma et al., (2013) observed differences in both the daily rhythms of expression of circadian clock and lipid synthesis genes in wild type mice compared to mice lacking the gene encoding lipid-metabolism regulator Thyroid-Hormone Responsive Spot 14. In the current experiment, phase shifts in the daily rhythms of fat and protein yield and concentration occurred, these effects were small (less than 3 hours) and likely not of biological significance. In contrast, altering the timing of restricted feeding by 8 h caused an approximately 8 h shift in the phases of milk and protein yield and fat and protein concentration (Chapter 3). The larger effects of FA infusion on the daily rhythms of milk synthesis occurred through modification of the amplitude. Fatty acid infusion during the day increased the robustness of the oscillations of milk, fat, and protein yield, while reducing the amplitudes of fat and protein concentration. These changes in amplitude may occur through altering amplitudes of molecular clock genes in the mammary gland. Dietary fat has been shown to reduce the amplitude of daily rhythms of molecular clock expression and expression of
genes related to FA synthesis in the adipose tissue and liver of mice (Kohsaka et al., 2007). However, to our knowledge, the direct effects of FA on mammary circadian clock gene expression have not been investigated. Future research examining the role of the timing of FA infusion on the molecular clock should be conducted.

**Daily Yields and Daily Rhythms of Milk Fatty Acids**

In addition to examining total fat yield, the effect of time of FA infusion on the origin of milk FA was examined. The yield of FA originating from de novo synthesis in the mammary gland (Σ < 16C) was affected by treatment (P = 0.001), with DAY and NGT reducing yields by 12% and 11% compared to CON (Figure 5-5A). Similarly, yields of mixed-source FA originating both from de novo synthesis and preformed FA uptake from plasma (Σ 16C) were decreased 9% by DAY and 6% by NGT compared to CON (P = 0.02). Yield of preformed FA (Σ >16C) was not affected by treatment, however (P = 0.24). These results suggest that limiting the time of FA absorption from the intestine reduced de novo FA synthesis in the mammary gland, but that the time during which it is limited is irrelevant. Altering the timing or frequency of feeding has previously been demonstrated to have no effect on apparent de novo FA synthesis (Niu et al., 2014; Rottman et al., 2014). However, restricting feed intake to 16 h during the night decreased de novo and mixed FA yields relative to 16 h feed restriction during the day, suggesting a role for meal timing in mammary FA synthesis (Salfer et al., 2016).

Of the individual FA, three odd-chain FA (OCFA) emerged as having the greatest decrease from CON in both DAY and NGT treatments (Figure 5-5C). These included C11:0 (28% decrease in DAY; 27% decrease in NGT; P = 0.03), C13:0 (28% decrease in DAY; 23% decrease in NGT; P = 0.002), and C15:0 (23% decrease in DAY; 16% decrease in NGT; P = 0.0004). Along with this, OCFA and total odd- and branched-chain fatty acids (OBCFA) were reduced in DAY and NGT relative to CON (P < 0.02; Figure 5-5B). Odd and branched chain FA
are derived primarily from microbial synthesis in the rumen, and found almost exclusively in adipose tissue and milk of ruminants (Vlaeminck et al., 2006). Moreover, they may have a may have a role in human health because they have been shown to have anti-carcinogenic properties (Yang et al., 2000). In the current experiment, oil was infused post-ruminally and major changes in rumen fermentation would be unexpected. The effect may be indirectly modulated through gut peptides. The gut peptides glucagon-like peptide 1 (GLP-1) and cholecystokinin (CCK) are increased by intestinally-available unsaturated fatty acids and may modify rumen motility (Bradford et al., 2008). Additionally, de novo synthesis of OBCFA can occur in the mammary gland through elongation of the 3-carbon precursor propionyl-CoA, derived from ruminally-produced propionate (Fievez et al., 2012). Similar to reducing total de novo FA synthesis, restricting the time of FA availability appears to inhibit elongation of propionyl-CoA or other OCFA. The effect appears to be fatty-acid specific because previous research demonstrates that altering meal pattern through time-restricted feeding does not affect total or individual OBCFA yields (Salfer et al., 2016; Salfer and Harvatine, 2018).

The daily rhythms of de novo, mixed, and preformed FA were affected by treatment. Similar to previous reports (Rottman et al., 2014), de novo FA yield fit a daily rhythm in CON \( (P = 0.01) \) and DAY \( (P = 0.03) \), but this rhythm was eliminated by NGT \( (P = 0.63; \text{Figure 5-6A}) \). The amplitude of the rhythm was increased 19% by DAY and decreased 46% by NGT. The acrophase of de novo FA yield occurred at 0603 h in CON and was phase-delayed 1.1 h by DAY and phase-advanced 1.3 h by NGT. These small phase shifts are unlikely to be of biological relevance. Similar changes due to time of FA infusion were observed for mixed FA yield, which exhibited a daily rhythm in CON \( (P = 0.03) \) and DAY \( (P = 0.01) \), but not NGT \( (P = 0.68, \text{Figure 5-6B}) \). The amplitude of the daily rhythm of mixed source FA yield was increased 17% by DAY and decreased 42% by NGT. Phase was also modified slightly by treatment with CON peaking at 0632, and being shifted 1 h later by DAY and 30 min earlier by NGT. These results suggest that
in addition to total de novo FA synthesis being affected by time of FA infusion, the daily rhythms of de novo FA synthesis are also affected. Preformed FA failed to fit a rhythm in CON \((P = 0.43)\) or NGT \((P = 0.68)\), but a rhythm was induced by DAY \((P = 0.02; \textbf{Figure 5-6C})\). Corresponding with the induction of a rhythm according to the zero-amplitude test, DAY increased the amplitude of the daily rhythm by 38% compared to CON and 33% compared to NGT. Moreover, the fitted 24 h rhythm was advanced 0.9 h by DAY and delayed 2.8 h by NGT, compared to CON which peaked at 0812 h.

\textit{Daily Rhythms of Plasma Metabolites}

Plasma glucose concentration has been demonstrated to follow a daily rhythm in cows (Giannetto and Piccione, 2009; Niu et al., 2014). However, in our experiment, glucose failed to fit a 24 h rhythm in any treatment according to the zero-amplitude test, nor did it fit a 24 h rhythm with a 12 h harmonic \((P > 0.20; \textbf{Figure 5-7A})\). In model organisms, these rhythms are shown to be influenced by peripheral circadian clocks in the liver and pancreas (Qian and Scheer, 2016). However, nutrient intake has been shown to affect the daily rhythm of cows. Feeding 4x/d has been shown to dampen the daily rhythm (Rottman et al., 2014), and the phase of the rhythm is shifted by altering the time of feed availability (Chapter 3). Our results may suggest that FA infusion reduces the amplitude of the daily pattern of glucose concentration, such that it fails to fit a 24 h rhythm according to the zero-amplitude test, but a negative control was not included in this experiment so this cannot be confirmed.

A daily rhythm of NEFA concentration was present in both CON \((P = 0.003)\) and NGT \((P < 0.0001)\), but this rhythm was eliminated by DAY \((P = 0.35; \textbf{Figure 5-7B})\). In conjunction with an elimination of the rhythm according to the zero-amplitude test, the amplitude of the 24 h cosine function was reduced by DAY \((P < 0.05)\). The phase of the 24 h rhythm was also affected by treatment, with DAY phase advancing the rhythm 4.5 h and NGT phase delay the rhythm \sim 9 h
relative to CON. These results suggest that the time of fat absorption alters the daily patterns of lipid mobilization from adipose tissue. Specifically, infusion during the morning dampens the rhythm, while infusion overnight does not. Moreover, NEFA concentration decreased during infusion in both DAY and NGT treatments, both reaching a nadir near the end of the infusion period. The change in the daily pattern of NEFA concentration may be through entrainment of the adipocyte circadian clock. In laboratory models, plasma free FA concentrations follow daily rhythms that are under the control of the adipocyte molecular clock (Shostak et al., 2013).

Furthermore, lipids can entrain the molecular circadian clock of peripheral tissues through the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors, which can bind to response elements on the promoter regions of circadian clock genes BMAL1 and REV-ERBα and enhance their expression. Interestingly, our study suggests that altering the time of FA absorption may uncouple FA metabolism from glucose metabolism. While the phase of the NEFA rhythm is shifted by the time of infusion during the day, glucose metabolism was not. Direct absorption of FA may desynchronize the circadian clock of adipose tissue from the clocks of the liver and pancreas that drive the daily rhythm of glucose concentration.

Treatment tended to affect concentration of plasma urea nitrogen \( (P = 0.06) \), with DAY increasing PUN 20% compared to CON \( (P = 0.03) \) and 16% compared to NGT \( (P = 0.07; \text{Figure } 5-7C) \). This change in PUN was associated with a 16% increase in milk urea nitrogen in DAY compared to control (Supplemental Figure S5-1), but no difference in milk protein concentration or yield. Plasma and milk urea nitrogen concentrations are measures of nitrogen utilization efficiency (Jonker et al., 1998; Kohn et al., 2005). Results suggest that FA infusion during the day may decrease the efficiency of amino acid utilization by muscle and other non-mammary tissues. A 24 h rhythm fit all treatments \( (P < 0.0005) \), and treatment affected the daily rhythm. These results agree with previous research suggesting that cows exhibit daily rhythms of plasma urea nitrogen concentration (Giannetto and Piccione, 2009; Niu et al., 2017). Day-
infusion increased the amplitude of the daily rhythm 26% compared to CON and 18% compared to NGT \( (P < 0.05) \). This increase in amplitude may be related to the increase in average PUN by NGT. The phase of the rhythm was slightly modified by treatment with the acrophase of NGT occurring 29 min later than CON and 41 min later than DAY \( (P < 0.05) \), but this effect is likely not biologically relevant. Salfer et al. (2016) previously observed that altering the time of time-restricted feeding inverts the daily rhythms of PUN, but our results suggest that this effect was not mediated by FA intake.

**Daily Rhythm of Body Temperature**

The time of FA infusion altered average body temperature \( (P = 0.02) \), with NGT decreasing body temperature compared to CON and DAY (Figure 5-8). Body temperature fit a 24 h rhythm in all treatments \( (P < 0.0001) \) and the rhythm was altered by treatment. The amplitude of the daily rhythm was increased 92% by DAY and 125% by NGT \( (P < 0.05) \). Furthermore, the phase of the daily rhythm of body temperature was delayed 50 min by DAY, but was not affected by NGT.

Previous research has suggested that altering the time of feed restriction dramatically shifts the phase of the daily rhythm of body temperature. We previously observed a \(~9\ h\) phase shift in the body temperature rhythm when feed was restricted to 16 h either during the day or night (Chapter 4). Similar results were observed by Niu et al. (2014) who detected a 3 h shift in the phase of body temperature when cows were fed at 0830 versus 2030 h, without restricting the time of feed availability. The results of the current study contrast this previous work and suggest that the time of fat absorption has very little effect on the phase of the rhythm of body temperature. The discrepancy between the experiments may be related to rumen temperature. Rumen temperature also oscillates in a circadian manner (Piccione et al., 2014). Moreover, rumen temperature is directly correlated to core body temperature, and is related to dry matter intake.
(Beatty et al., 2008). This relationship may explain why only slight differences in the daily rhythm of body temperature occurred after abomasal infusion of fat, whereas changing the time of feed intake has previously been shown to alter the rhythm, perhaps by changing the daily rhythm of body temperature through altered rumen fermentation.

Interestingly, the amplitude of the daily rhythm of body temperature was dramatically altered by time of feed restriction, with night-infusion increasing the rhythm to a greater extent than day infusion. Similarly, we previously reported a 4-fold increase in the amplitude of the daily rhythm when time of feed restricted was limited to the overnight period compared to the day (Chapter 4). Therefore, the timing of fat infusion may influence the daily rhythm of body temperature thorough altering the robustness, but not the phase of the rhythm. The increase in amplitude of the rhythm may be important in modifying the circadian rhythms of other tissues because core body temperature has been shown to be capable of circadian entrainment in mammals (Brown et al., 2002).

**CONCLUSIONS**

The daily rhythms of milk synthesis were altered by modifying the time of FA infusion. Most notably, infusion during the day increased the amplitude of milk yield, but decreased concentration of fat and protein. However, FA do not appear to robustly entrain the mammary clock because the phase of the rhythms of milk, fat, and protein yield and milk fat and protein concentration were not markedly shifted by treatment. De novo FA synthesis was reduced by restricting the time of FA infusion, regardless if time restriction occurred during the day or night. Daily rhythms of glucose and nitrogen metabolism were not greatly affected by time of FA infusion, but total nitrogen efficiency appeared to be decreased by day-infusion. The daily pattern of lipid mobilization was altered by time of fat absorption, however, with day-infusion flattening
the daily rhythm. The amplitude of the daily body temperature was increased both by restricting fat infusion to either the day or night, indicating that time-restricted fat absorption may increase the robustness of the central circadian clock. The effects of time of FA infusion on the daily rhythms of milk synthesis may be related to increasing the robustness of oscillations of the mammary molecular clock. Moreover, the changes in the daily patterns of NEFA concentrations may be related to the adipocyte clock. The role of FA on the molecular circadian clocks of mammary and adipose tissue should be investigated in the future.
Figure 5-1. Schedule of feeding, lighting and sampling during the experiment.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500].
Figure 5-2. Effect of time of fatty acid infusion on dry matter intake.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Data are presented as LSM with SEM bars.
Figure 5-3. Effect of time of fatty acid infusion on daily milk, fat and protein yield and fat and protein concentration.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Panels the effect of time of fatty acid infusion on (A) milk yield (kg), (B) fat and protein yield (g) and (C) fat and protein concentration. Data are presented as LSM with SEM bars.
Figure 5-4. The effects of time of fatty acid infusion on the daily rhythms of milk synthesis.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Panels show the effect of time of fatty acid infusion on the daily rhythms of milk (A) yield (kg), (B) fat yield (g), (C) protein yield (g), (D) fat concentration (%), and (E) protein concentration (%). Data are presented as relative expression LSM and SEM bars and cosine fit are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test. NS: Reduced model fits data significantly better than daily rhythm model. The black and white bars above the x-axis display the light: dark cycle.
Figure 5-5. Effect of time of fatty acid infusion on the daily yields of fatty acids.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day ([DAY]; infused from 0900 to 1700) or for 8 h/d during the night ([NGT]; feed from 2100 to 0500). Panels show the effect of time of fatty acid infusion on the average daily yields of (A) de novo (Σ < 16C), mixed (Σ 16C) and preformed (Σ >16C) sources of fatty acids (g/d), (B) odd and branched chain fatty acids (OBCFA), odd chain fatty acids (OCFA), and branched chain fatty acid (BCFA) (g/d), and (C) C11:0, C13:0 and C15:0 fatty acids (g/d). Data are presented as LSM with SEM bars. Means with differing superscripts are different at $P < 0.05$. 
Figure 5-6. Effect of time of fatty acid infusion on daily rhythms of fatty acids by source.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Panels show the effect of time of fatty acid infusion on the daily rhythms of (A) de novo synthesized (Σ <16C) FA yield (g/d) (B) mixed source (Σ 16C) FA yield (g/d), and (C) preformed (Σ >16C) FA yield (g/d). Data are presented as relative expression LSM and SEM bars and cosine fit are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test. NS: Reduced model fits data significantly better than daily rhythm model. The black and white bars above the x-axis display the light: dark cycle.
Figure 5-7. The effects of time of fatty acid infusion on daily rhythms of plasma metabolites.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Panels include the effects of time of fatty acid infusion on daily rhythms of plasma (A) glucose concentration (mg/dL), (B) nonesterified fatty acid concentration (NEFA; μEq/L), and (C) urea nitrogen concentration (mg/dL). Data are presented as relative expression LSM and SEM bars and cosine fit are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the $P$-value of the zero-amplitude test. NS: Reduced model fits data significantly better than daily rhythm model. The black and white bars above the x-axis display the light: dark cycle.

### A. Plasma Glucose, mg/dL

<table>
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<tr>
<th>Trt</th>
<th>Mean</th>
<th>Amp</th>
<th>Acro</th>
<th>$P$-value</th>
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</thead>
<tbody>
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<td>2.20</td>
<td>0556</td>
<td>0.20</td>
</tr>
<tr>
<td>DAY</td>
<td>54.0</td>
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<td>0502</td>
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<td>NGT</td>
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### B. Plasma NEFA, μEq/L

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<td>32.4</td>
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</tr>
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<td>DAY</td>
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<td>7.53</td>
<td>0329</td>
<td>0.35</td>
</tr>
<tr>
<td>NGT</td>
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### D. Plasma urea N, mg/dL

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<tr>
<td>CON</td>
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<td>0.61</td>
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<td>DAY</td>
<td>4.17</td>
<td>0.77</td>
<td>1026*</td>
<td>0.0002</td>
</tr>
<tr>
<td>NGT</td>
<td>3.59</td>
<td>0.65</td>
<td>1167*</td>
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</tr>
</tbody>
</table>
Figure 5-8. The effect of the time of fatty acid infusion on the daily rhythms of core body temperature.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Data presented as 2 h means and SEM bars of body temperature collected every 10 min by a vaginal temperature data logger, with cosine fit are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test. NS: Reduced model fits data significantly better than daily rhythm model. The black and white bars above the x-axis display the light: dark cycle.
Supplemental Figure S5-1. Effect of time of fatty acid infusion on milk urea nitrogen concentration.

Treatments were 350g/d of fatty acids abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Data are presented as LSM with SEM bars. Means with differing superscripts are different at \( P < 0.05 \).
**SUPPLEMENTAL TABLES**

**Supplemental Table S5-1.** Diet and nutrient composition of the experimental diet.

<table>
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<td>Ingredients, % of DM</td>
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<tr>
<td>Corn silage(^1)</td>
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</tr>
<tr>
<td>Alfalfa haylage(^2)</td>
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</tr>
<tr>
<td>Canola meal</td>
<td>12.5</td>
</tr>
<tr>
<td>Ground corn</td>
<td>11.6</td>
</tr>
<tr>
<td>Roasted soybeans</td>
<td>3.6</td>
</tr>
<tr>
<td>Grass hay/straw(^3)</td>
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</tr>
<tr>
<td>Mechanically-extracted soybean meal(^4)</td>
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<tr>
<td>Vitamin/mineral mix(^5)</td>
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</tr>
<tr>
<td>Controlled-release N(^6)</td>
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</tr>
<tr>
<td><strong>Nutrients, % of DM</strong></td>
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</tr>
<tr>
<td>NDF</td>
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</tr>
<tr>
<td>ADF</td>
<td>18.3</td>
</tr>
<tr>
<td>Ash</td>
<td>5.6</td>
</tr>
</tbody>
</table>

\(^1\)Contained (% of DM): 33.0% NDF, 16.9% ADF  
\(^2\)Contained (% of DM): 45.1% NDF, 34.7% ADF  
\(^3\)Contained (% of DM): 63.2% NDF, 33.5% ADF  
\(^4\)AminoPlus, Ag Processing Inc., Omaha, NE  
\(^5\)Fed as coated urea (Optigen, Alltech Inc., Lexington, KY; 259% CP, DM basis.  
\(^6\)Composition (DM-basis): 7.2% CP; 7.1% NDF; 3.7% ADF; 15.0% Ca; 0.75% P; 0.33% K; 2.6% Mg; 0.5% S; 9.8% Na; 23.0 mg/kg Co; 652 mg/kg Cu; 783 mg/kg Fe; 54.0 mg/kg I; 1190 mg/kg Mn; 12.8 mg/kg Se; 1718 mg/kg Zn; 88,700 IU/kg vitamin A (retinyl acetate); 28,400 vitamin D (activated 7-dehydrocholesterol); 850 IU/kg vitamin E (dl-α tocopheryl acetate).
Supplemental Table S5-2. Fatty acid profile of oil used for abomasal infusion of fatty acids.

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</tr>
<tr>
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<tr>
<td>16:0</td>
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<tr>
<td>cis-9 16:1</td>
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</tr>
<tr>
<td>trans-9 18:1</td>
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</tr>
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Chapter 6

The effect of timing of protein infusion on the daily rhythms of milk production and plasma hormones and metabolites

Isaac J. Salfer, Rebecca A. Bomberger, Cesar I. Matamoros, P.A. Bartell, Kevin J. Harvatine
ABSTRACT

Dairy cows have a daily rhythm of milk synthesis that appears to be driven by the molecular clock of the mammary gland and is modified the time of feed availability. Protein metabolism is intimately linked to circadian rhythms in other species, but the effect of amino acids on the daily rhythm of milk synthesis is not known. The objective of this experiment was to determine the effects of intestinally-absorbed protein on the daily rhythms of milk and milk component synthesis in dairy cows. Nine lactating Holstein cows were randomly assigned to one of three treatment sequences in a 3 x 3 Latin square. Treatments were abomasal infusions of 500 g/d of sodium caseinate either continuous throughout the day (CON), for 8 h/d from 0900 to 1700 h (DAY), or for 8 h/d from 2100 to 0500 (NGT). Treatment periods were 14 d with 11 d of treatment adaptation and 3 d of sampling. Cows were milked every 6 h during the final 8 d of each period. A 24-h rhythm was fit to time-course production data using cosine analysis and the amplitude and acrophase (time at peak) were determined. Daily milk and protein yield were decreased 7.5% and 8% by NGT compared to CON, respectively, while milk fat yield was increased by DAY (P < 0.05). Daily milk fat and protein concentration were not affected by treatment. Milk yield failed to fit a 24 h rhythm in CON or DAY, but a rhythm was induced by NGT (P = 0.03). Neither fat yield nor protein yield fit a rhythm in either treatment. However, fat concentration fit a daily rhythm in all treatments (P < 0.05) and the amplitude of the rhythm was decreased 57% by DAY and 26% by NGT (P < 0.05). The rhythm of milk fat concentration was phase advanced ~2 h by DAY and phase delayed ~1 h by NGT (P < 0.05). Protein concentration fit a daily rhythm in CON and DAY but not NGT. The phase of the rhythm of protein concentration was delayed ~1.25 h by DAY and the amplitude was increased 2-fold relative to CON (P < 0.05). The daily yields of de novo (∑ < C16), mixed (∑ 16C), and preformed (∑ >16C) fatty acids in milk were not altered by treatment (P > 0.10), and none of these sources fit a daily
rhythm in any treatment (P > 0.10). Plasma glucose concentration fit a daily rhythm in CON and NGT, but the rhythm was eliminated by DAY. Furthermore, the phase of glucose concentration was not altered by treatment. Nonesterified fatty acid concentration did not fit a daily rhythm in CON or NGT (P > 0.10), but a rhythm was induced by DAY (P < 0.05). A rhythm of plasma urea nitrogen was present in CON and NGT (P < 0.0001) but not DAY, (P < 0.05), with no difference in phase between CON and NGT. The time of protein infusion influenced the daily rhythms of milk and milk protein synthesis, with night infusion abolishing rhythms of protein concentration and inducing rhythms of milk yield, and day infusion increasing the amplitude of the rhythm of protein concentration. Moreover, infusion of protein during the day damped the rhythms of plasma glucose and PUN concentration, but increased the amplitudes of nonesterified FA, suggesting that the daily rhythms of nutrient metabolism are also altered by the time of protein infusion.

Keywords: Daily rhythm, milk synthesis, nutrient entrainment, circadian rhythm
INTRODUCTION

Dairy cows exhibit rhythms of milk synthesis, with milk yield typically peaking in the morning and milk fat and protein concentration peaking in the evening (Rottman et al., 2014). These daily rhythms appear to be related to changes in the molecular clock of the mammary gland (Plaut and Casey, 2012). The molecular clock is responsible for generating circadian rhythms, which are repeating ~24 h cycles that allow animals to anticipate daily changes in their environment. This molecular clock is comprised of a set of ‘clock’ transcription factors and daily rhythms of phosphorylation and dephosphorylation events that generate 24 h rhythms of gene expression and intracellular signaling (Robles et al., 2017).

A master circadian oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus serves as the central control point for setting the circadian rhythms of peripheral tissues. This master oscillator is entrained by the light-dark cycle and initiates neural and hormonal signaling cascades to entrain the molecular clock of cells throughout the body. In addition to receiving signals from the SCN, the molecular clock in peripheral tissues can be entrained by other signals, including nutrient intake (Stokkan et al., 2001). Entrainment by nutrients can occur independently of the SCN and can cause desynchronization of master and peripheral circadian rhythms (Asher and Sassone-Corsi, 2015). Desynchronization of these rhythms has been implicated in development of metabolic disorders such as obesity and type II diabetes (Vetter and Scheer, 2017).

Nutrient intake appears to influence the circadian rhythms of milk synthesis in dairy cows. Feeding cows four times per day in equal meals dampened the daily rhythms of milk fat and protein concentration, as well as shifted peaks of milk, fat, and protein yield and fat and protein concentration (Rottman et al., 2014). Salfer et al. (2016) discovered that restricting the time of feed availability to 16 h/d at night (1900 to 1100) shifted the daily rhythms of milk yield
and fat and protein concentration approximately 8 h relative to restricting feed availability to the same amount of time during the day (0700 to 2300). The change in these daily patterns of milk synthesis appear to be at least partially modulated through modifying the molecular clock because night-restricted feeding shifts the daily rhythms of some components of the molecular clock (Chapter 4). Similar results were observed in mice under night-restricted feeding, which exhibited shifts in the rhythms of milk fat synthesis, along with changes in the mammary molecular clock (Ma et al., 2013). In addition to modifying the time of feed intake through time-restricted feeding, individual nutrients have been shown to be capable of entraining molecular clocks (Oiike, 2017).

In dairy cows, altering the time of fatty acid (FA) infusion shifts the amplitude and phase of milk, fat, and protein synthesis in the mammary gland (Chapter 5).

While fatty acids appear to entrain daily rhythms of milk synthesis of dairy cows, to our knowledge, other nutrients have not been examined. Amino acids may have a potential role in the circadian rhythm of the mammary gland. Amino acid metabolism is intimately linked with the molecular clock of mammals. In humans, plasma concentrations of total amino acids, and all individual proteogenic amino acids follow circadian rhythms that are entrained by alterations to the sleep-wake cycle (Feigin et al., 1968). Furthermore, Krüppel-like factor 15 (KLF15), a transcription factor responsible for upregulation of pathways related to amino acid mobilization from muscle and their use for gluconeogenesis during fasting, is regulated in a circadian manner by glucocorticoid signaling (Fan et al., 2018). This pathway links amino acid metabolism with circadian rhythms of plasma glucose concentrations.

Amino acids are important for milk protein synthesis in the mammary gland and play a role in the synthesis of lactose because α-lactalbumin is a milk protein and is a regulatory component of the lactose synthase enzyme complex. Altering the time of amino acid absorption may affect the efficiency of protein synthesis in the mammary gland and/or alter the daily rhythms of milk synthesis. The objective of this experiment was to examine the effect of time of
protein absorption on the daily rhythms of milk synthesis and plasma metabolites. We expect that altering the timing of amino acid availability in the small intestine through abomasally infusing sodium caseinate either continuously throughout the day, for 8 h during the day, or for 8 h during the night, will shift the phase of the daily rhythms of milk yield and milk protein concentration and yield and will shift the daily rhythms of plasma glucose concentrations.

MATERIALS & METHODS

Animals and Treatments

Nine cannulated multiparous mid-lactation (128 ± 46 d postpartum; mean ± SD) Holstein cows from the Penn State University Dairy Research and Teaching Center were randomly assigned to treatment sequences in a 3x3 Latin Square design. Treatment periods were 14 d with 11 d of treatment adaptation followed by 3 d of sampling. A 7 d washout was used between treatment periods. During the final 7 d of each period, cows were milked 4 times per day to allow observation of daily rhythm. Treatments included abomasal infusion of 500 g/d day of sodium caseinate dissolved in 6 L of distilled water for either 24 h/d (CON), for 8 h/d from 0900 h to 1700 h (DAY), or for 8 h/d from 2100 h to 0500 h (NGT; Figure 6-1A). Cows on the CON treatment received infusion for approximately of 22 h/d as infusion occurred for 30 min during each milking. Sodium caseinate (Erie Foods International, Erie, IL) was used as a protein source because of its high biological value for milk protein synthesis. Amino acid profile of sodium caseinate was analyzed by The Experiment Station Chemical Laboratories at the University of Missouri (Columbia, MO) according to (AOAC, 2006) (Supplemental Table S6-2). All cows were provided the same basal TMR fed once daily at 0600 h at 110% of the previous day’s intake (Supplemental Table S6-1). Feed samples were collected weekly throughout the course of the experiment, composited by period, and analyzed for dry matter, crude protein, starch, neutral
detergent fiber (NDF), acid detergent fiber (ADF), and ash according to Rico and Harvatine (2013). The experiment was conducted from July 30 to September 23, 2018 and all experimental procedures were approved by the Penn State University Institutional Care and Use Committee.

**Milk Sampling and Analysis**

Cows were milked 4 x/d every 6 h (0500, 1100, 1700, and 2300) on the final 8 d of each period to observe the daily pattern of milk synthesis. Milk collected at each time point represented total milk synthesis over the previous 6 h and is plotted as the midpoint of the previous milking interval (3 h prior to collection). Milk yield was measured at each milking using an integrated milk meter (Afimilk MPC Milk Meter; Afimilk Agricultural Cooperative Ltd., Kibbutz Afikim, Israel), and yields were corrected for the deviation of each individual stall according to Andreen et al. (2018). Milk was sampled at all milkings on d 13 and 14 of each period, with one subsample used for analysis of fat and protein concentration by Fourier transform infrared spectroscopy (Fossomatic 4000 Milko-Scan and 400 Fossomatic, Foss Electric; Dairy One DHIA, Ithaca, NY), and one subsample used for analysis of FA concentrations according to Baldin et al. (2018).

**Plasma Sampling and Analysis**

Blood was collected via venipuncture of a coccygeal blood vessel into potassium-EDTA vacuum tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) on d 13 to 14 of each period. Sampling occurred at 6 time points representing every 4 h across the day (0300, 0700, 1100, 1500, 1900, and 2300 h). Samples were placed on ice immediately after collection and centrifuged at 2300 x g for 15 min at 4°C within 30 min of collection. Plasma was collected and stored at -20°C for analysis of glucose, nonesterified fatty acids (NEFA), and plasma urea nitrogen concentrations (PUN) as described by Rottman et al. (2014). Briefly, glucose was
measured using a glucose oxidase/peroxidase enzymatic colorimetric assay (No. P 7119, Sigma-Aldrich, St. Louis, MO), NEFA were measured using an acyl-CoA oxidase peroxidase enzymatic colorimetric assay (NEFA-HR (2), Wako Diagnostics, Richmond, VA) and urea nitrogen was measured using a modified Berthelot methodology (Modified Enzymatic Urea Nitrogen Procedure No. 2050; Stanbio Laboratory, Boerne, TX).

**Statistical Analysis**

All data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Effects of time of protein absorption on daily dry matter intake, milk production, and milk FA yields were tested using models that included the fixed effect of treatment and the random effects of cow and period. Denominator degrees of freedom were adjusted using the Kenward-Roger method, and the AR(1) or ARH(1) covariance structure was used based on convergence criteria. Preplanned contrasts were used to compare least squared means among individual treatments.

Time course data for milk production and plasma metabolites were fit to the linear form of a cosine function with a 24 h period using random regression in SAS 9.4. Model parameters included the fixed effects of treatment, cosine terms, and the interaction between treatment and cosine terms as well as the random effects of cow and period. The daily patterns of plasma metabolites were tested for the presence of a 12 h harmonic, and the harmonic was included in the 24 h rhythm if it improved model fit. The fit of the 24 h cosine function was determined using a zero-amplitude test and the amplitude and acrophase (time at peak) and their significance were determined according to Niu et al. (2014). Data points with Studentized residuals outside ± 3.5 were removed. Another linear model including the fixed effects of treatment, time, and their interaction, with random effects of cow and period was also tested. Statistical significance was
declared at $P < 0.05$ and trends acknowledged at $0.05 < P < 0.10$. High resolution figures were generated using an add-in for Microsoft Excel (Kraus, 2014).

RESULTS AND DISCUSSION

Daily Milk Yield and Milk Components

Dry matter intake was not altered by treatment ($P = 0.46$; Figure 6-2). Daily milk yield was decreased 7.4% by NGT relative to CON but NGT and DAY were not different ($P = 0.02$; Figure 6-3A). Treatment tended to affect fat yield ($P = 0.07$), with DAY increasing fat yield 4.5% relative to CON and 4.6% relative to NGT (Figure 6-3B). The yield of protein was also affected by treatment ($P = 0.005$; Figure 6-3B), with NGT decreasing protein yield 8.3% relative to CON ($P = 0.01$) and 6.9% relative to DAY ($P = 0.002$). However, daily average concentration of fat ($P = 0.34$) and protein ($P = 0.61$) were not affected by treatment (Figure 6-3C).

The results of the current experiment differ from previous research in our lab examining the timing of FA infusion on milk synthesis, which exhibited no differences in milk or milk components when FA were infused either continuously or over 8 h/d from 0900 to 1700 or 8 h/d from 2100 to 0500 (Chapter 4). Postruminal sodium caseinate infusion consistently increases milk protein yield in dairy cows (Clark, 1975; Mackle et al., 1999). This increase in milk protein is attributed to the high biological value of sodium caseinate, with the amino acid profile of sodium caseinate closely matching that required for milk protein synthesis. Furthermore, specific amino acids, such as leucine and isoleucine can directly increase milk protein synthesis via mechanistic target of rapamycin (mTOR) signaling (Appuhamy et al., 2012). Furthermore, protein supply may increase milk lactose by increasing α-lactalbumin, a regulator of the lactose synthesis pathway, or by providing glucogenic precursors that may increase lactose by increasing plasma glucose availability (Kuhn et al., 1980). Lactose is an osmotic regulator of milk and is
drives milk yield (Schingoethe, 1996). These mechanisms by which protein can increase milk and milk protein synthesis are variable, and depend on the availability of protein and glucogenic precursors. In the current experiment, lactose yield tended to be decreased by NGT, but not DAY (Supplemental Figure S 6-1). This reduction in lactose yield may be responsible for the reduction in milk yield. The effect of time of protein infusion on milk, fat, and protein yield may be related to the mammary clock. Hu et al. (2017) discovered that siRNA-mediated knockdown of the PER2 gene in bovine mammary epithelial cells increased mRNA and protein abundance of αs1- and αs2- casein. Restricting time of protein infusion to the night may similarly affect the mammary clock, which may reduce milk yield.

**Daily Rhythms of Milk Yield and Milk Components**

Previous research has demonstrated that milk yield follows a 24 h rhythm (Gilbert et al., 1972; Rottman et al., 2014). In the current experiment, milk yield fit a daily rhythm in NGT ($P = 0.03$), with an amplitude of 0.72 kg and an acrophase of 0330 (Figure 6-4A). However, milk yield failed to fit a daily rhythm in CON ($P = 0.12$) or DAY ($P = 0.25$). These results imply that infusion of protein throughout the day or during the morning dampened the rhythm of milk synthesis, whereas infusion at night does not. A negative control treatment without protein infusion was not included due to limitations in conducting the intensive experiment and the hypothesis was focused on the timing of absorption.

Fat concentration fit daily rhythms with similar amplitudes in CON ($P = 0.003$) and NGT ($P = 0.01$), but the rhythm was abolished by DAY ($P = 0.26$; Figure 6-4C). The acrophase of the 24 h rhythm was phase-advanced 1.5 h by DAY and phase advanced 1.75 h by NGT, relative to CON ($P < 0.05$). Protein concentration fit a daily rhythm in CON ($P = 0.0004$) and DAY ($P < 0.0001$), but not NGT ($P = 0.49$; Figure 6-4E). Day-infusion increased the amplitude 2-fold compared to CON, while NGT decreased the amplitude by 50% ($P < 0.05$). Similar to previous
reports (Rottman et al., 2014), the acrophase occurred in the afternoon in all treatments, with DAY and NGT phase delaying the rhythm 1.25 and 4 h, respectively ($P < 0.05$).

Fat yield failed to fit a daily rhythm in any treatment ($P > 0.38$ Figure 6-4B). Despite the failure to fit a rhythm via the zero-amplitude test, a post-hoc analysis comparing the amplitude and acrophase of the fitted cosine function were performed. The acrophase of the fitted 24 h cosine appeared to be phase-advanced 5.25 h from 0917 h in CON to 0402 h in DAY ($P < 0.05$). Protein yield similarly failed to fit a daily rhythm in any treatment according to the zero-amplitude test ($P > 0.13$; Figure 6-4D). The amplitude of the 24 h cosine function was increased 106% by NGT, compared to CON ($P < 0.05$). Moreover, the phase of the fitted cosine function was shifted 4.25 earlier by DAY but was not affected by NGT.

The timing of nutrient intake has previously been shown to impact the daily rhythms of milk synthesis in dairy cows. Rottman et al. (2014) observed a reduction in the amplitude of milk fat and protein concentration when cows were fed 4x/d in equal meals compared to 1x/d. Moreover, restricting the time of feed availability to 16 h/d during the night shifted the peak of milk and milk protein yield to the afternoon, and the peak of fat and protein concentration to the morning, relative to restricted feeding for 16 h during the day (Chapter 3). Furthermore, timing of FA infusion has been shown to affect the daily rhythms of milk synthesis, with infusion during the day causing a reduction in the amplitudes of milk and milk fat and protein yield, but increased amplitudes of fat and protein concentration (Chapter 5). Results of the current study demonstrate that the time of protein infusion also modifies the daily rhythms of milk synthesis. Specifically, protein infusion during the day reduces the amplitude of milk fat concentration and increases the amplitude of milk protein concentration, while protein infusion at night reduces the amplitude of milk protein concentration.
Because the timing of protein infusion tended to affect total daily fat yield, we examined the effect of treatment on yields of FA by source. Time of protein infusion did not affect the daily yields of FA originating from de novo synthesis in the mammary gland (Σ < 16C; \( P = 0.12 \)), preformed FA taken up from plasma (Σ >16C; \( P = 0.14 \)), or mixed source FA (Σ 16C; \( P = 0.66 \); Figure 6-5A). Day-infusion numerically increased the yields of de novo, mixed, and preformed FA, suggesting that the increased total FA yield was due to the cumulative effects of slight increases in both de novo FA synthesis and preformed FA uptake from plasma. We have previously observed that the time of feed intake affects de novo FA synthesis, with night-restricted feeding for 16 h/d (1900 to 1100) reducing de novo and mixed FA yields, but not preformed FA, relative to day-restricted feeding for 16 h/d (0700 to 2300; Chapter 3). Furthermore, we observed that restricting the time of FA infusion to the day (0900 to 1700) or night (2100 to 0500) reduced de novo synthesis relative to continuous infusion across the day (Chapter 5). Results of the current study suggest that unlike altering the time of feed restriction or the time of FA infusion, the time of protein infusion does not alter de novo FA synthesis.

In addition to the daily yields of FA, we examined the effect of time of protein infusion on the 24 h rhythms of de novo, mixed, and preformed FA yield. Previous research has demonstrated that these sources of FA oscillate over 24 h (Rottman et al., 2014; Ma et al., 2015). According to the zero-amplitude test, none of the treatments fit daily rhythms of de novo, mixed, or preformed FA (\( P > 0.20 \); Figure 6-5B-D). The present study may indicate that infusion of sodium caseinate eliminates these rhythms, but this cannot be definitively determined without a negative control. Abomasal infusions of FA during the NGT have been shown to eliminate the daily rhythms of de novo, mixed and preformed FA yields, whereas infusion during the day did not (Chapter 4). Despite not fitting a rhythm in any treatment, there were detectable changes in the fitted 24 h cosine functions of de novo, mixed, and preformed FA yield due to treatment. The
amplitude of the rhythm of de novo FA yield was increased over 6-fold by DAY and over 3-fold by NGT, relative to CON ($P < 0.05$), with DAY having a 92% greater amplitude than NGT (Figure 6-5B). Moreover, the phase of the daily rhythm was advanced 11.6 h by DAY and delayed 10.2 h by NGT ($P < 0.05$). However, these results should be interpreted with caution because their low amplitudes hinder the ability to fit a cosine function with an appropriate phase. Relative to CON, the amplitude of mixed FA yield was increased nearly 4-fold by DAY, but was reduced 52% by NGT ($P < 0.05$). Similar to de novo FA, the phase was advanced 10 h by DAY and delayed 10.4 h by NGT, relative to CON ($P < 0.05$). Day-infusion decreased the amplitude of preformed FA yield 48%, but NGT increased the amplitude 7% compared to CON ($P < 0.05$). The phase of the rhythm was shifted 6.8 h earlier by DAY and 3.9 h earlier by NGT ($P < 0.05$).

Taken together, these results imply that although sodium caseinate seemingly causes dramatic reductions in the amplitude of the daily rhythms of the three major sources of FA such that they do not fit a 24 h rhythm, time of protein infusion still causes apparent changes in these low-amplitude rhythms.

**Daily Rhythms of Plasma Metabolites**

Treatment affected the daily rhythms of plasma glucose concentration (Figure 6-6A). A 24 h rhythm was present in CON ($P = 0.03$) and NGT ($P = 0.02$), but was abolished in DAY ($P = 0.85$). Moreover, the amplitude of the rhythm was decreased 66% by DAY, but was increased 47% by NGT, relative to CON. Alternatively, the acrophase of the daily rhythm was not altered by treatment. Furthermore, average plasma glucose concentration was not affected by treatment ($P = 0.14$). Results were similar to previous research demonstrating that altering the time of FA infusion had no effect on average glucose concentrations or the phase of the daily rhythm (Chapter 4). However, altering the time of feed availability has previously been shown to entrain daily rhythms of glucose concentration. Feeding 4x/d reduced the amplitude and phase-delayed
the rhythm 8 h relative to 1 x/d feeding (Rottman et al., 2014). Moreover, we previously demonstrated that restricting the time of feed availability to 16 h/d during the night (1900 to 1100) increased the amplitude 2.5-fold and phase delayed the rhythm by 9.8 h relative to day-restricted feeding (16 h/d from 0700 to 2300 h) (Chapter 3).

In dairy cows, plasma glucose is almost exclusively derived from gluconeogenesis in the liver using propionate as a substrate (Aschenbach et al., 2010). Therefore, the circadian clock of the liver may be linked to daily rhythms of glucose metabolism. In model organisms, the circadian clock of the liver is highly responsive to food entrainment (Stokkan et al., 2001). Maugeri et al. (2018) proposed that the molecular clock of the liver can be entrained by a protein-only diet or treatment with cysteine, and that this effect was mediated through glucagon and IGF-1 signaling. The changes in circadian amplitude due to the time of protein infusion may be through increasing or decreasing the robustness of oscillations of the liver clock. The daily rhythms of glucose metabolism may also be controlled by rhythms of insulin secretion. We have previously demonstrated that insulin concentration follows a 24 h rhythm that is responsive to feeding time (Chapter 3). In model organisms, the daily rhythms of insulin secretion are secreted by a circadian clock in the pancreas, and this clock may be entrained by timing of nutrient absorption (Allaman-Pillet et al., 2004). Further research examining the role of protein and amino acids on the molecular clocks of the liver and pancreas should be conducted. The increase in amplitude of the plasma glucose concentrations due to protein infusion at night may be responsible for the increased amplitude of milk yield. Glucose is required for synthesis of lactose, which is the osmotic driver of milk yield. The increased amplitude of glucose concentration may increase the amplitude of conversion to lactose, and therefore the amplitude of milk yield.

Plasma NEFA concentration has previously been demonstrated to exhibit a 24 h rhythm in dairy cows (Giannetto and Piccione, 2009; Niu et al., 2014). In the current experiment, NEFA concentrated exhibited a daily rhythm in DAY ($P = 0.003$), but this rhythm was abolished by
CON ($P = 0.93$) and NGT ($P = 0.69$; Figure 6-6B). The elimination of the rhythms of CON and NGT corresponded with a 73% and 61% decrease compared to DAY, respectively ($P < 0.05$). The fitted daily rhythm peaked at 0313 in the CON group and was phase-advanced 10.5 h by NGT ($P < 0.05$), but was not affected by DAY ($P > 0.10$). Average plasma NEFA concentration was not affected by treatment ($P = 0.19$).

The time of feed intake has previously been demonstrated to alter the daily rhythm of plasma NEFA concentration in dairy cows. Rottman et al. (2014) observed that the amplitude of NEFA concentration was reduced by feeding 4x/d compared to 1x/d. Moreover, research from our lab demonstrated that night-restricted feeding for 16 h from 1900 to 1100 delayed the phase by 10 h and increased the amplitude over 3-fold compared to day-restricted feeding (Chapter 3). Furthermore, plasma NEFA concentrations are affected by the time of fatty acid absorption. In a previous experiment from our lab, the amplitude of NEFA was reduced 4-fold, and the phase was delayed 9 h when the time of FA infusion was limited to 8 h during the day (0900 to 1700 h) versus continuous infusion (Chapter 5). In contrast, the current experiment demonstrated that protein infusion at during the day increased the amplitude of plasma NEFA nearly 4 fold. Moreover, the previous experiment observed a 5 h phase delay due to day-restricted fat in fusion, whereas the current experiment detected a phase-advance of 9.5 due to protein infusion during the same time period. These disparate results suggest differential regulation of the daily rhythm of lipid mobilization by fat and protein.

The daily rhythms of NEFA mobilization may be regulated by the molecular clock of adipocytes. In model organisms, lipolysis follows a daily rhythm, and this rhythm is modulated by the adipocyte clock (Shostak et al., 2013). However, to our knowledge, there has been no research examining the role of amino acids on the circadian clock of adipose tissue. Future research using explant or cell culture to examine the direct effects of protein on the adipocyte clock should be conducted. Similar to previous research from our lab testing the time of fatty acid
infusion (Chapter 3), the time of protein infusion uncouples the daily rhythm of glucose concentration from the daily rhythm of NEFA concentration. This suggests that protein infusion during the night may desynchronize the peripheral circadian clocks governing glucose and fatty acid metabolism.

Similar to previous reports (Giannetto and Piccione, 2009; Niu et al., 2014), a daily rhythm of plasma urea nitrogen concentration was present in CON ($P < 0.0001$) and NGT ($P < 0.0001$), but the rhythm was ablated by DAY ($P = 0.22$; Figure 6-6C). The amplitude of the daily rhythm was decreased 30% by DAY and increased 66% by NGT. Furthermore, relative to CON the daily rhythm was phase-delayed 6.1 h and phase-advanced 1.4 h by DAY and NGT, respectively. Treatment did not affect the concentration of PUN ($P = 0.81$). Unlike protein infusion, previous unpublished research from our lab demonstrated that the time of FA infusion alters PUN concentration, with 8 h of night-infusion causing a 20% increase compared to continuous infusion (Chapter 5). In the same experiment, however, the phase of the rhythm of PUN was only slightly (less than 1 h) shifted by treatment. Plasma urea nitrogen concentration is an indicator of nitrogen utilization efficiency, with higher PUN values indicating poorer efficiency (Kohn et al., 2005). Results of the current experiment suggest that the daily rhythm of efficiency of nitrogen use is dependent on the time of protein absorption. Nitrogen homeostasis has previously been shown to display endogenous circadian rhythmicity in humans (Minami et al., 2009). Research conducted in model organisms revealed an integral role for Krüppel-like factor 15 (KLF15), a zinc-finger DNA-binding protein, in circadian regulation of nitrogen metabolism (Jeyaraj et al., 2012). Branched-chain amino acids suppress KLF15 expression, and may be a potential avenue for entrainment of circadian rhythms of nitrogen utilization (Liu et al., 2017).
CONCLUSIONS

The time of sodium caseinate absorption affected the daily rhythms of milk synthesis. This effect appears to be primarily through affecting the robustness of the rhythm without entraining the phase. In particular, restricting infusion to the night increased the amplitude of the milk yield and decreased the amplitude of protein concentration, while day-infusion damped the rhythms of milk yield and fat concentration. The changes in amplitude of milk yield may be driven by changes in the daily rhythms of glucose concentration, because, similar to milk yield, the amplitude of plasma glucose was decreased by day-infusion and increased by night-infusion. The time of protein infusion also appears to affect the daily pattern of lipogenesis, with the peak of the NEFA rhythm corresponding to the time of protein infusion, and day-infusion dramatically increasing the amplitude. The nadir of PUN was shifted to the time when protein was infused, suggesting that protein may entrain the daily rhythms of efficiency of nitrogen utilization. The changes in the daily rhythms of milk synthesis and plasma metabolites may be mediated through alterations in the circadian clock of peripheral tissues. Future research examining the role of amino acids on the molecular clocks of the mammary gland, liver, and adipose should be conducted.
Figure 6-1. Schedule of feeding, lighting and sampling during the experiment.

Treatments were 500g/d of sodium caseinate abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500].
Figure 6-2. Effect of time of protein infusion on dry matter intake.

Treatments were 500g/d of sodium caseinate abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Data are presented as LSM with SEM bars.
Figure 6-3. Effect of time of protein infusion on daily milk, fat and protein yield and fat and protein concentration.

Treatments were 500g/d of sodium caseinate abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Data are presented as LSM with SEM bars. Means with differing superscripts are different at P < 0.05.
Figure 6-4. The effects of time of protein infusion on daily rhythms of milk synthesis.

Treatments were 500g/d of sodium caseinate abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Panels show the effect of time of fatty acid infusion on milk (A) yield (kg), (B) fat yield (g), (C) protein yield (g), (D) fat concentration (%), and (E) protein concentration (%). Data are presented as relative expression LSM and SEM bars and cosine fit are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test. The black and white bars above the x-axis display the light: dark cycle.
Figure 6.5. Effect of time of protein infusion on daily yields and daily rhythms of milk FA by source.

Treatments were 500g/d of sodium caseinate abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Data are presented as LSM with SEM bars Panels show the effect of protein infusion on (A) daily yields of de novo (Σ < 16C), mixed (Σ 16C) and preformed (Σ >16C) sources of fatty acids (g/d). (B) daily rhythms of de novo synthesized (Σ < 16C) FA yield (g/d) (c) daily rhythms of mixed source (Σ 16C) FA yield (g/d), and (C) daily rhythms of preformed (Σ >16C) FA yield (g/d). Data are presented as relative expression LSM and SEM bars and cosine fit are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test. The black and white bars above the x-axis display the light: dark cycle.
Treatments were 500g/d of sodium caseinate abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Panels show the effects of time of fatty acid infusion on daily rhythms of plasma (A) glucose concentration (mg/dL), (B) nonesterified fatty acid concentration (NEFA; μEq/L), and (C) urea nitrogen concentration (mg/dL). Data are presented as relative expression LSM and SEM bars and cosine fit are shown. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the \( P \)-value of the zero-amplitude test. The black and white bars above the x-axis display the light: dark cycle.

**Figure 6-6.** The effects of time of protein infusion on daily rhythms of plasma metabolites.
Supplemental Figure S 6-1. Effect of time of protein infusion on lactose yield.

Treatments were 500g/d of sodium caseinate abomasally infused continuously for 22 h/d (CON); for 8 h/d during the day [(DAY); infused from 0900 to 1700] or for 8 h/d during the night [(NGT); feed from 2100 to 0500]. Data are presented as LSM with SEM bars. Treatments with different superscripts tend to be different at $P < 0.10$. 

![Supplemental Figure S 6-1](image-url)
### SUPPLEMENTAL TABLES

**Supplemental Table S6-1.** Diet and nutrient composition of the experimental diet.

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<th>Item</th>
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<td>Alfalfa haylage(^2)</td>
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<td>Canola meal</td>
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\(^1\)Contained (% of DM): 42.3% NDF, 21.7% ADF  
\(^2\)Contained (% of DM): 47.2% NDF, 33.5% ADF  
\(^3\)Contained (% of DM): 69.4% NDF, 35.8% ADF  
\(^4\)AminoPlus, Ag Processing Inc., Omaha, NE.  
\(^5\)Fed as coated urea (Optigen, Alltech Inc., Lexington, KY; 259% CP, DM basis).
**Supplemental Table S6-2.** Amino acid profile of sodium caseinate.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>g/100 g AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic Acid</td>
<td>21.1</td>
</tr>
<tr>
<td>Proline</td>
<td>10.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.04</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.59</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>6.80</td>
</tr>
<tr>
<td>Valine</td>
<td>6.52</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.32</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.22</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.99</td>
</tr>
<tr>
<td>Serine</td>
<td>4.37</td>
</tr>
<tr>
<td>Threonine</td>
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<tr>
<td>Arginine</td>
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<td>Alanine</td>
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<td>Histidine</td>
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<tr>
<td>Methionine</td>
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<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>Tryptophan</td>
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</tr>
<tr>
<td>Cysteine</td>
<td>0.36</td>
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</table>
Chapter 7

Annual rhythms of milk and milk fat and protein production in dairy cattle in the United States

Isaac J. Salfer, Chad D. Dechow, and Kevin J. Harvatine

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ABSTRACT

An annual pattern of milk composition has been well appreciated in dairy cattle, with highest milk fat and protein concentration observed during the winter and lowest occurring in the summer. However, rhythms of milk yield and composition have not been well quantified. Cosinor rhythmometry is commonly used to model repeating daily and annual rhythms and allows determination of the amplitude (peak to mean), acrophase (time at peak), and period (time between peaks) of the rhythm. The objective of this study was to use cosinor rhythmometry to characterize the annual rhythms of milk yield and milk fat and protein concentration and yield using both national milk market and cow-level data. First, ten years of monthly average milk butterfat and protein concentration for each Federal Milk Marketing Order (FMMO) were obtained from the USDA Agricultural Marketing Service database. Fat and protein concentration fit a cosine function with a 12-month period in all milk markets. There was an interaction between milk marketing order and milk fat and protein concentration ($P < 0.01$). The acrophase (time at peak) of the fat concentration rhythm ranged from December 4th to January 19th in all regions, while the rhythm of protein concentration peaked between December 27th and January 6th. The amplitude (peak to mean) of the annual rhythm ranged from 0.07 to 0.14 percentage points for milk fat and from 0.08 to 0.12 percentage points for milk protein. The amplitude of the milk fat rhythm generally was lower in southern markets and higher in northern markets. Secondly, the annual rhythm of milk yield and milk fat and protein yield and concentration were analyzed in monthly test day data from 1684 cows from eleven tie-stall herds in Pennsylvania. Fat and protein concentration fit an annual rhythm in all herds, while milk and milk fat and protein yield only fit rhythms in 8 of the eleven herds. On average, milk yield peaked in April, fat and protein yield peaked in February, fat concentration peaked in January, and protein concentration peaked in December. Amplitudes of milk, fat, and protein yield averaged 0.82 kg, 55.3 g, and
30.4 g, respectively. Milk fat and protein concentration had average amplitudes of 0.12 and 0.07, respectively, similar to the results of the milk market data. Generally, milk yield and milk components fit annual rhythm regardless of parity or DGAT1 K232A polymorphism, with only cows of the low frequency AA genotype (5.2% of total cows) failing to fit rhythm of milk yield. In conclusion, the yearly rhythms of milk yield and fat and protein concentration and yield consistently occur regardless of region, herd, parity, or DGAT1 genotype, and supports generation by a conserved endogenous annual rhythm.

**Keywords:** Annual rhythm, seasonal rhythm, milk synthesis, milk fat
INTRODUCTION

Milk production and milk component concentrations follow a seasonal pattern across the year that is well-recognized by dairy producers and nutritionists. Milk yield and milk fat and protein concentrations are typically greatest in the winter and reach a nadir in the summer. Seasonal variation has long been quantified and accounted for by animal breeders, but has not been well integrated into dairy management (Wood, 1970, 1976). The causes of these yearly changes are not fully understood and are often attributed to environmental factors such as heat stress or changes in forage quality. However, these yearly patterns may be the result of an endogenous annual rhythm controlling milk synthesis.

In nature, annual rhythms occur as a mechanism for organisms to predict seasonal environmental changes before they occur to allow proactive adaptations. Many animal species exhibit yearly cycles of reproductive activity, hibernation, migration, hair growth, and feeding behavior, allowing them to better prepare for changes in weather conditions and food supply (Lincoln et al., 2006; Schwartz and Andrews, 2013). A classic example of annual rhythmicity in production animals is seasonal breeding observed in ewes, which exhibit estrus only from early autumn to late January, restricting lambing to the spring (Robinson, 1951; Shelton and Morrow, 1965). These annual rhythms of estrus are the result of interactions between photoperiod and an endogenous physiological timekeeping mechanism (Malpaux et al., 1989). Feed intake of sheep may also be regulated via annual rhythms, as the secretion of leptin, ghrelin, and orexin is impacted by photoperiod length (Kirsz et al., 2012). In dairy cattle, the secretion of prolactin (Chew et al., 1979) and serotonin (Philo and Reiter, 1980) follow melatonin-controlled annual rhythms, with prolactin levels peaking in summer and serotonin peaking in winter. Furthermore, Piccione et al. (2012) determined that circulating concentrations of β-hydroxybutyrate, bilirubin, creatinine, and triglycerides followed annual rhythms in Italian Brown dairy cattle.
Biological rhythms, including annual rhythms, can be analyzed using cosinor rhythmometry. This technique fits a linear form of the cosine function with a 12-month period to multiple years of data to determine if it fits an annual rhythm. The degree to which the data follow an annual rhythm is assessed using a zero-amplitude test, which determines if the linear form of the cosine function models that data significantly better than the linear effect of time (Went, 2006). Cosinor rhythmometry also determines the amplitude, difference from mean to peak, and acrophase, or time at peak, of a biological rhythm (Bourdon et al., 1995). Quantifying the annual rhythms of milk and milk component production can improve management decisions and may provide insight into physiological mechanism governing annual rhythms. The objective of this study was to quantify the annual rhythms of milk and milk component production in dairy cattle in the USA and examine cow specific factors affecting these rhythms.

**MATERIALS & METHODS**

**USDA Milk Market Data**

Milk composition data from January 2000 through October 2015 were obtained from the Agricultural Marketing Service (AMS) agency of the United States Department of Agriculture (USDA). Monthly milk butterfat and protein concentration from 2000 through 2015 were downloaded for each US federal milk marketing order (FMMO). Orders included: Northeast (Order 1), Appalachian (5), Florida (6), Southeast (7), Upper Midwest (30), Central (32), Mideast (33), Pacific Northwest (124), Southwest (126), Arizona-Las Vegas (131), and Western (135; **Figure 7-1**). Butterfat data were available for all 11 orders; however, milk protein percentages were not available for the Appalachian, Florida, Southeast, or Arizona-Las Vegas regions. Only four years of data were available from the Western region because the order was terminated in 2004 (Tosi, 2004).
All statistical analysis was performed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Monthly butterfat and protein concentration were fit to the linear form of a cosine function with a 12-month period according to Bourdon et al. (1995) using random regression (Seltman, 1997) as described by Niu et al. (2014). The model tested the random effect of year and the fixed effects of marketing order, the linear form of a cosine function with a period of 12-months, and the interaction of order and the linear form of cosine functions. Denominator degrees of freedom were estimated using the Kenward-Roger method and the AR(1) covariance structure was used. The fit of the 12-month rhythms was determined using a zero amplitude test, which employs an F-Test to compare the full model containing the linear form of the cosine function to a reduced linear model testing the linear effect of month (Went, 2006). The acrophase and amplitude of each marketing order were calculated, with subsequent significance tests, using a spreadsheet program developed in Microsoft Excel® (Bourdon et al., 1995).

**Cow-Level Data**

Data from a previously published experiment by Vallimont et al. (2010) were used to examine the effect of seasonal rhythms on milk production at the individual cow level. Briefly, test day milk yield and butterfat and protein concentration were collected from 1684 individual cows in eleven Pennsylvania tie-stall dairy herds during the years 2002 to 2011. Of these cows, 731 had been tested for the diacylglycerol O-acyltransferase 1 (DGAT1) K232A polymorphism using a panel of 121 candidate gene markers (Dekleva et al., 2012; Igenity, Neogen Corporation, Lincoln, NE). Butterfat and protein yield were calculated as the product of milk yield and butterfat and protein concentration. Test days when cows were less than 40 days in milk (DIM) were removed from the analysis as high milk fat is expected during early lactation.

Milk, fat, and protein yield and milk fat and protein concentration were fit to the linear form of a cosine function with a 12-month period using the MIXED procedure of SAS 9.4. Fixed
effects included herd, DGAT1 genotype, lactation group (1, 2, 3+ lactations), and days in milk, along with the random effects of cow and test year. The levels of each main effect (herd, DGAT1 genotype, and lactation group), along with the average of all herds, were fit to a 12-month cosine function and rhythm fit, amplitude and acrophase were determined using the methodology described above for the milk market data.

**RESULTS**

**USDA Milk Market Data**

A cosine function with a 12-month rhythm was used to test for an annual rhythm in milk composition using milk fat and protein concentration data from USDA federal milk marketing orders. There was an interaction of milk marketing order and the cosine function on fat concentration so the fit, amplitude, and acrophase of the 12-month cosine function were determined for each milk market order (Table 7-1). Fat concentration in all marketing orders fit the 12-month rhythm ($P < 0.0001$). Mean fat percent was lowest in the Arizona-Las Vegas region (3.56%) and greatest in the Pacific Northwest (3.73%). The amplitude of the rhythm of fat concentration ranged from 0.07 to 0.14 percentage units between orders. The Southwest, Central (Figure 7-2C), Southeast, Mideast, Appalachian, and Western regions had 12-month rhythms with the greatest amplitudes (0.13 to 0.14 percentage units) and did not differ between each other ($P > 0.10$). The Northeast, Upper Midwest (Figure 7-2B), and Pacific Northwest marketing orders all exhibited an amplitude of 0.11 percentage units, which was lower than the Southwest, Central, Southeast, Mideast, Appalachian, and Western regions ($P < 0.05$). The amplitudes of fat concentration rhythms were lowest in Arizona-Las Vegas (0.09 percentage units) and Florida (0.07 percentage units), with Florida having a lower amplitude than Arizona-Las Vegas ($P < 0.05$; Figure 7-2A).
The acrophase, or time at peak, of butterfat concentration ranged from December 4\textsuperscript{th} to January 18\textsuperscript{th} across milk marketing orders. In six of eleven marketing orders, including Arizona-Las Vegas, Northeast, Upper Midwest, Mideast, Southwest, and Southeast, the 12-month rhythm of fat concentration peaked within 4 days of January 1\textsuperscript{st} ($P > 0.10$). No difference was observed between the acrophases of the Western, Central, and Appalachian regions, and all peaked between January 17\textsuperscript{th} and January 19\textsuperscript{th} ($P > 0.10$). Florida’s annual rhythm peaked on December 4\textsuperscript{th}, markedly earlier than the other regions ($P < 0.05$).

An interaction between milk marketing order and the cosine function was observed for protein concentration, so the fit, amplitude, and acrophase of the 12-month rhythm were compared between marketing orders. Protein concentration fit the 12-month function in all seven regions with protein data available ($P < 0.001$; Table 7-1). Mean protein concentration was greatest in the Pacific Northwest, Southwest, and Western Regions, followed by Central and Mideast ($P < 0.05$). The Northeast and Upper Midwest had the lowest mean protein concentrations. The Northeast and Upper Midwest averaged 3.04% protein, the lowest of all regions ($P < 0.05$). The amplitudes of annual rhythms in all regions were between 0.08 and 0.10 percentage units. The greatest amplitudes (0.10 percentage units) were observed in the Central and Southwest (Figure 7-3B) regions. The Upper Midwest and Mideast had intermediate amplitudes (0.09 percentage units), while the Northeast (Figure 7-3A), Pacific Northwest and Western regions displayed amplitudes of 0.08 percentage units ($P < 0.05$). The acrophases of annual protein concentration rhythms occurred between December 27\textsuperscript{th} and January 6\textsuperscript{th} in all regions ($P > 0.10$). These results are consistent with those observed by Bailey et al. (2005), who reported that three-year average milk production peaks were greatest in December and January in the Mideast milk market.
**Cow-Level Data**

Data from 11 tie-stall herds in Pennsylvania were analyzed to characterize seasonal patterns of yield of milk and milk fat and protein, determine how cow-level factors including parity or DGAT1 genotype influence annual production rhythms, and examine variation in annual rhythms between individual herds. Milk yield of the 11 herds fit an annual rhythm with an amplitude of 1.26 kg and an acrophase of March 13th. A yearly rhythm of milk yield was observed in ten of the eleven herds ($P < 0.05$; **Supplemental Figure S7-3A&B**). Eight of the ten herds that exhibited a yearly rhythm of milk yield peaked between March 13th and May 5th, while the remaining two herds peaked in mid-summer. These results agree with previous data suggesting that the average amount of milk shipped from farms is greatest in March, April, and May (Bailey et al., 2005). The amplitudes of the milk yield rhythms ranged from 0.84 to 1.64 kg/d, suggesting that annual rhythms may be responsible for as much as 3.3 kg/d difference in milk production across the year.

Overall, the average fat yield of all herds fit a 12-month rhythm with a mean of 1313 g/d, an amplitude of 55.3 g/d, and an acrophase occurring on February 23rd ($P < 0.0001$; **Supplemental Figure S7-3C&D**). All but one of the eleven herds exhibited a yearly rhythm, while one exhibited a rhythm with a drastically lower amplitude than the other nine herds (16.5 g/d; $P < 0.0001$). The nine remaining herds had amplitudes ranging from 97.2 to 47.3 g/d. The non-rhythmic and low amplitude herds peaked on July 5th and 6th, respectively, in contrast to the other 9 herds, which peaked between January 22nd and April 3rd. Average protein yield also fit a yearly rhythm, with a mean of 1081 g, an amplitude of 30.4 g, and an acrophase occurring on February 19th (**Supplemental Figure S7-3E&F**). A yearly rhythm was observed in 10 of the 11 herds. The amplitudes of annual rhythms of protein yield were highly variable between herds and ranged from 14.3 to 47.0 g/d. There was, however, low variability in the acrophases, with 8 herds peaking between January 31st and March 1st and one herd peaking on April 24th.
Fat and protein concentration fit 12-month rhythms in all herds as expected and exhibited much more consistent yearly patterns than milk, fat, and protein yield ($P < 0.0001$; Supplemental Figure S7-4A&B). The average rhythm of fat concentration for the 11 herds peaked on January 25th with an amplitude of 0.12 percentage units. Of the 11 herds, 9 peaked within 30 days of January 25th, with the remaining two peaking on March 27th and April 7th. The amplitude of the yearly rhythm averaged 0.12 percentage units, equivalent to a 0.24 unit change in fat percent throughout the year and was highly variable between herds (0.07 to 0.19 units). Milk protein concentration similarly fit an annual rhythm in all herds ($P < 0.001$), with an average amplitude of 0.07 units and the average acrophase occurring on December 30th (Supplemental Figure S7-4C&D). All herds peaked between December 8th and January 23rd with amplitudes ranging from 0.04 to 0.11 percentage units, suggesting that annual rhythms of protein percent are highly consistent between herds.

The effect of parity on fit of the cosine function and the mean, amplitude and acrophase of annual production rhythms was evaluated (Figure 7-4). Groups included cows in first, second, or third and greater lactations. Milk yield increased with increasing parity ($P < 0.05$) and fit an annual rhythm in all three parity groups ($P < 0.0001$). The amplitude of the 12-month milk yield rhythm was greater for cows in their third and greater lactations than 1st and 2nd lactation ($P < 0.05$), but was not different between 1st and 2nd lactation cows ($P > 0.10$). Furthermore, acrophase did not differ between 1st and 2nd lactation, but occurred over a month earlier in third and greater lactation cows ($P < 0.05$).

A yearly rhythm of fat yield was also observed in all lactation groups ($P < 0.0001$). Mean fat yield increased with increasing parity, while cows in third and greater lactation had 80% and 55% greater amplitudes than 1st and 2nd lactation cows, respectively ($P < 0.05$). The acrophase of fat yield did not differ between cows in second or third and greater lactations ($P > 0.10$), but occurred 23 and 35 days later in 1st lactation cows compared with 2nd and 3rd lactation,
respectively ($P < 0.05$). Protein yield fit a 12-month rhythm in all lactation groups ($P < 0.0001$) and mean protein yield increased from 936 g in 1\textsuperscript{st} lactation to 1130 g in 2\textsuperscript{nd} lactation and 1260 g in 3\textsuperscript{rd} and greater lactations ($P < 0.05$). Amplitude was greatest in third and greater lactation (73.1 g; $P < 0.05$), but did not differ between cows in first and second lactation ($P > 0.10$). The acrophase of the yearly rhythm was similar between second and third and greater lactations, and occurred on March 11\textsuperscript{th} and March 1\textsuperscript{st}, respectively ($P > 0.10$). Alternatively, the acrophase of the annual rhythm of protein yield occurred later in first lactation cows (April 10\textsuperscript{th}; $P < 0.05$).

Milk fat and protein concentration fit a 12-month rhythm in all three parity groups ($P < 0.0001$). Mean milk fat concentration decreased with increasing parity ($P < 0.05$), but no difference in amplitude was observed between lactation groups ($P > 0.10$). Similarly, parity exerted no effect on the acrophase of fat concentration rhythms, with all peaking between January 14\textsuperscript{th} and January 25\textsuperscript{th} ($P > 0.10$). Milk protein concentration fit a yearly rhythm in all three lactation groups ($P < 0.05$). Mean protein concentration did not differ between 1\textsuperscript{st} and 2\textsuperscript{nd} lactation ($P > 0.10$), but was 8\% and 6\% lower in 3\textsuperscript{rd} and greater lactation cows than 1\textsuperscript{st} and 2\textsuperscript{nd} lactation, respectively ($P < 0.05$). No difference occurred in the acrophase or amplitude of the rhythms of protein concentration between lactation groups, with amplitudes ranging from 0.08 to 0.09 percentage units and all acrophases occurring between December 12\textsuperscript{th} and December 26\textsuperscript{th} ($P > 0.10$).

DGAT1 genotype influenced average milk fat concentration, with the cows of the AA genotype averaging 4.19\% milk fat, KA genotype averaging 3.94\% fat, and KK genotype averaging 3.58\% fat ($P < 0.05$; \textbf{Figure 7-5}). Fat yield did not differ between the AA (1296 g) and KA genotype (1256 g; $P > 0.10$), but was lower the KK genotype (1178 g; $P < 0.05$). These results are consistent with previous reports showing that the K232A polymorphism is associated with milk fat percent and yield (Schennink et al., 2007). An annual rhythm of fat concentration was observed regardless of DGAT1 genotype ($P < 0.0001$) and DGAT1 genotype did not affect
the amplitude or acrophase of the rhythm ($P > 0.10$). Similarly, fat yield fit a rhythm in all three genotypes ($P < 0.0001$) with no difference in mean fat yield. Amplitude was similar between all genotypes and ranged from 54.6 to 70.0 g ($P > 0.10$). Likewise, acrophase was unaffected by DGAT1 genotype and all rhythms peaked between February 15th and March 1st. Average milk yield did not differ between the AA and KA genotypes ($P > 0.10$), but was 2.4 and 1.2 kg greater in cows with the KK genotype than AA and KA ($P < 0.05$). Milk yield fit a 12-month rhythm in cows with the KA and KK genotype, but not AA. The failure of AA to fit a rhythm may have been because of its low frequency within the population limited observations within the dataset (only 5% of cows are AA). Between the KA and KK groups, no difference was observed for either amplitude or acrophase.

**DISCUSSION**

Regional milk market and cow-level data both confirmed the presence of 12-month rhythms of fat and protein concentration and cow level data demonstrated a rhythm in milk and milk fat and protein yield, which is biologically and economically important. The consistency of these rhythms between years, regions and herds suggests that the annual patterns of production seen on dairy farms may be the result of a biological rhythm, rather than the outcome of environmental factors. Heat stress is the most notable environmental factor correlated to seasonal production declines, and begins to occur when the temperature-humidity index rises above 70 (Bohmanova et al., 2007). Cows in the United States are most likely to experience during July and August. If the summer decrease in production were strictly due to the acute effects of heat stress, yearly production would be expected to remain relatively stable, with a sharp decline in the summer, followed by recovery in the fall when the stress is alleviated. However, the results of this study suggest a consistent and predictable cosine decrease from the peak of production to the
nadir. For example, fat and protein concentration, which peak in January in the majority of regions, begins to decrease in February and March, much earlier than would be expected if the effect is simply a consequence of heat stress. Furthermore, milk yield was shown to reach a minimum between September and November, rather than mid-summer when heat stress is expected to be the greatest. Lastly, the rhythm is very consistent between years and the degree heat stress is expected to differ between years, especially in more northern regions (Supplemental Figure S7-5).

The annual rhythms of fat and protein concentration consistently peaked in mid-winter in both the milk market and cow level datasets. These results are consistent with previous reports showing that maximal fat and protein concentration occur during December and January (Bailey et al., 2005). Research in grazing cattle has alternately suggested that the occurrence of milk fat depression is greatest in May, however this may be influenced by changes in the fermentability of grass throughout the year (Carty et al., 2017). Dunshea et al. (2008) observed that milk concentrations of trans-10 C18:1 isomers, associated with biohydrogenation-induced milk fat depression, were greatest in August and September which is consistent with the trough of fat yield production observed in the current experiment.

The yearly change in photoperiod with lengthening and shortening days is a strong cue for entraining annual rhythms and greater amplitude rhythms are often associated with greater variation in the light-dark cycle across the year (Hut et al., 2013). In the Federal Milk Market Order data the lowest amplitude annual rhythm occurred in Florida, followed by Arizona –Las Vegas. Florida has the lowest latitude (Tallahassee, FL: 30.4° N) of all the regions investigated, and consequently has the smallest change in photoperiod across the year. Similarly, Arizona-Las Vegas has a lower latitude (Las Vegas, NV: 36.2° N) than the majority of the other marketing orders. However, the amplitude of the other nine orders did not follow a pattern based on photoperiod length. For example, the Southeast and Southwest regions had the greatest amplitude
rhythms, along with the Appalachian, Mideast and Western regions. The northernmost regions including the Northeast, Upper Midwest, and Pacific Northeast had intermediate amplitudes. One potential cause for the inconsistency between regions is the differences in housing systems among various U.S. regions. In Florida and Arizona a large proportion of cows are housed in shaded dry lots with access to natural lighting, while in northern states cows are more likely to be housed in enclosed tie-stall or free-stall barns under the influence of artificial lighting. However, this does not account for the high amplitudes observed in the Southeast and Southwest, where cows are also commonly housed in open dry lots. Data from dairy herds in the southern hemisphere would provide additional insight into the role of change in yearly photoperiod on annual rhythms of production. If the effects is a direct effect of photoperiod, rhythms in the southern hemisphere would be expected to be inverted compared to those in the northern hemisphere. Auldist et al. (1998) demonstrated that in New Zealand, milk fat and protein concentration are greatest during their winter (June-August), while milk yield peaks in their spring and early summer (September-December). Results from data in the southern hemisphere are difficult to interpret, however, because grazing systems are common in those countries, and effects of the annual rhythm may be confounded by differences in the nutrient composition of pasture.

Herd level data suggested that, unlike fat and protein concentration, which peak between December and February, fat and protein yield reach an acrophase between February and April and milk yield reaches an acrophase between March and May in most herds. Between herds, variability was present in the annual rhythms of milk, fat, and protein yield. Two herds in particular stood out for having no annual rhythms, a low amplitude of fat and protein yield, and acrophases of fat, protein, and milk yield that occurred during the summer, about four to six months later than the other herds. One herd also notably failed to demonstrate a yearly rhythm of milk yield. These herds possessed no identifiable feeding or management characteristics to differentiate them from the other eight (Vallimont et al., 2010). Results suggest that management
factors such as changes in diet or feed intake across the year may mask an endogenous annual rhythm driving yearly changes in milk production.

We examined the genotype at the diacylglycerol O-acyltransferase 1 (DGAT1) locus to determine its influence on the yearly rhythms of fat concentration, fat yield, and milk yield. The K2332A polymorphism in the DGAT1 gene is responsible for up to 50% of the genetic variation in milk fat production (Schennink et al., 2007). The AA genotype is associated with high milk fat concentration, the KK genotype is associated with low milk fat, and the KA heterozygote results in an intermediate phenotype (Winter et al., 2002). The current experiment confirmed these previous results, with AA cows producing milk with a 0.25% greater milk fat concentration than KA cows, which was 0.36% greater than KK. Annual rhythms of nearly all production variables persisted regardless of DGAT1 genotype, with a lone exception being milk yield in cows with the AA genotype. The reason for the lack of an annual rhythm in this group is unclear, but may be a consequence of a lower prevalence of this genotype. Only 1196 test day observations from 40 unique cows carried the AA genotype, which was equivalent to 5% of the total cows in the study. This low number of cows, combined with management variability between herds may have contributed to the inability to detect an annual rhythm. However, despite the low number of observations, annual rhythms were detected for fat concentration and yield within the AA group. The DGAT1 genotype of the animals did not affect the shape of the annual rhythms of fat percent or fat yield, with similar amplitudes and phases in all groups. These results are similar to those observed by Duchemin et al. (2013), who observed that milk fat concentration was not affected by the interaction of DGAT1 genotype and season, although they did observe an interaction between DGAT1 and summer vs winter milk for unsaturated fatty acid concentration.

Lactation number had little effect on the shape of annual production rhythms. Increased amplitudes of milk, fat, and protein yield occurred in cows in their third or greater lactation. These results suggest that as cows age, greater oscillation in the yearly rhythms of production
occurs. Furthermore, greater lactation number was associated with slightly earlier peaks in yearly rhythms of milk protein and fat yield. The cause of changes is unclear, but may be related to overall increase in production observed as lactation number increases.

Previous experiments have supported that dairy cattle are influenced by annual rhythms. Although cows are not generally considered to be seasonal breeders, Hansen (1985) observed modest effects of season and photoperiod length on reproduction in cattle. Furthermore, Kendall and Webster (2009) observed greater daily fluctuations in body temperature in the summer compared to winter. Several circulating hormones and metabolites also follow annual rhythms across the year. Petitclerc et al. (1983) observed that circulating prolactin concentrations are over six times greater in summer than winter, and that this effect occurs even when melatonin signaling is blocked through blinding or pinealectomy. Plasma concentrations of bilirubin, creatinine, triglycerides, and β-hydroxybutyrate (BHBA) follow 12-month rhythms, with BHBA peaking on April 1st, bilirubin peaking on July 14th, creatinine peaking on June 12th, and triglycerides peaking June 16th (Piccione et al., 2012). The annual rhythms observed in these metabolites may be regulated by the same mechanism as the annual rhythms of production. The annual patterns of production may also be a consequence of seasonal changes in feed intake. Cattle typically increase feed intake in the winter and Ueda et al. (2016) reported dry matter and fiber in the rumen of dairy cows is greater in autumn than spring, with a reciprocal relationship for ruminal VFA concentration. Endocrine factors regulating intake have been reported to follow an annual rhythm in other animals. Circulating leptin concentrations vary across the year in sheep and Siberian hamsters (Rousseau et al., 2003; Henry et al., 2010) and leptin, orexin and ghrelin appear to be modulated by photoperiod in sheep (Kirsch et al., 2012).

A seasonal rhythm in milk yield and composition may be adaptive to improve nutrition of the calf. From a biological perspective, there is a clear advantage to providing nursing offspring with high-energy milk during the winter months. In other organisms, seasonal rhythms function
to maximize reproductive success by scheduling parturition to allow neonates to be born during periods of high food availability and favorable climatic conditions (Lincoln et al., 2003). As an important component of mammalian reproduction, it is reasonable to expect lactation to similarly follow an annual rhythm and provide more milk and higher fat and protein to neonates in the winter when energetic demands are greater. Seasonal reproduction in sheep and hamsters appears to be coordinated by interactions photoperiod and circadian clock genes located within the hypothalamus. Melatonin is released from the pineal gland during the dark phase of the photoperiod and binds with high affinity to the pars tuberalis (PT) of the hypothalamus (Johnston et al., 2006). The duration of melatonin release affects the phase of the core clock genes Per and Cry within the PT, and which acts downstream to influence the pulsatile frequency of GnRH, thus affecting reproductive cyclicity (Misztal et al., 2002). Although this mechanism is well-characterized for controlling seasonal reproduction, there is a dearth of research examining the presence of mechanisms governing annual rhythms of lactation.

The amplitude, acrophase, and period length of annual rhythms can be manipulated through photoperiod alterations. Gwinner (1981) demonstrated that yearly cycles of testes growth and molting in migrating common starlings (Sturnus vulgaris) can be shortened by accelerating changes in photoperiod. Furthermore, accelerated photoperiods can speed up the reproductive cycles of rainbow trout (Oncorhynchus mykiss; Bon et al., 1997). Photoperiod manipulation has been extensively studied for its effects on milk production (Peters et al., 1978; Dahl et al., 1997; Miller et al., 1999). Cows consistently increase milk yield when placed in a photoperiod with over 12 h of light, with maximal response occurring in a 16L:8D photoperiod (Reksen et al., 1999). This effect of may be related to hormonal changes caused by long-day lighting. Dahl et al. (1997) established that increased milk synthesis due to an artificial 18L: 6D photoperiod is associated with increased concentrations of circulating IGF-1. While the direct effect of IGF-1 on milk synthesis is unclear, its concentration is increased after exogenous treatment with
recombinant bovine somatotropin, suggesting it may increase milk yield (Bauman and Vernon, 1993). Plasma prolactin concentrations are also consistently elevated under long-day lighting (Tucker et al., 1984; Stanisiewski et al., 1988; Lacasse et al., 2014). Prolactin is an important driver of seasonal physiological changes in other mammalian species, drives changes in reproduction and molting (Martinet et al., 1984; Gómez-Brunet et al., 2008). While an association between prolactin and milk synthesis has been suggested, the effect does not appear to be direct because no response to exogenous prolactin on milk yield has been observed in cattle (Plaut et al., 1987; Lacasse et al., 2012).

There appears to be a paradox between the effects of long-day lighting and annual rhythms of production. In natural conditions milk increases across the winter when the duration of the light cycle is less than 12 h, whereas photoperiod research suggests that milk yield should be greatest when more than 12 h of light is present. The effect may be explained by induction of photorefractoriness to the annual rhythm by constant-long day photoperiods. In other mammalian species, long-term exposure to a constant photoperiod causes spontaneous reversion, or photorefractoriness, of a seasonal physiological response to the state expected in the opposite photoperiod (Lincoln et al., 2005). Suffolk sheep exposed to a fixed short 8L: 16D photoperiod exhibit summer-type physiology, characterized by a decrease in serum luteinizing hormone concentrations and anestrous (Robinson and Karsch, 1984). In long-day breeding Syrian hamsters (Mesocricetus auratus) gonadal degeneration spontaneously occurs after long-term administration of a fixed 14L: 10D photoperiod. The mechanism for this phenomenon is thought to be through dissociation of the endogenous annual timer in the PT with melatonin-based signals from the light-dark cycle (Lincoln et al., 2005). The long-term treatment needed to elicit the effects of long day photoperiod on milk synthesis provides additional support for this mechanism. In other species, a fixed photoperiod must be applied for 4 to 12 weeks prior to induction of photorefractoriness, which is consistent with data suggesting that the effect of long day lighting
on milk yield does not manifest until 4 weeks after administration (Dahl et al., 2000). While this mechanism seems like a promising explanation for the incongruence between the annual rhythm and photoperiod research, it has not been well-examined in cows and further research should be performed to investigate this effect.

CONCLUSIONS

Milk yield and milk fat and protein concentration and yield fit annual rhythm at both the regional and cow level. These rhythms appear to exist independent of environmental effects such as heat stress and may be the result of endogenous circannual rhythms. Rhythms of fat and protein concentration peak in the middle of winter, and reach a nadir in the summer, whereas yields of milk, fat and protein peak in the spring. Region of the U.S. appears to have mild effects on annual rhythms, with lower latitude regions having lower-amplitude rhythms. Moderate variability in annual rhythms exists between herds, but DGAT1 genotype and lactation group exert little effects. Dairy producers and consultants should consider the annual rhythms of production when making management decisions. An understanding of these rhythms will allow better benchmarking across the year.
Figure 7-1. Map of United States Federal Milk Marketing Orders.

Image provided courtesy of the USDA Agricultural Marketing Service. The Western (135) marketing order was terminated in 2004.
Figure 7-2. Annual rhythms of fat concentration in selected Federal Milk Market orders.

Orders include (A) Florida (Order 6), (B) Upper Midwest (Order 30) and (C) Central (Order 32). Data presented as least-squares means with error bars representing SEM of each month and the line representing a fitted cosine function with a 12-month period. Characteristics of fitted rhythm are displayed including: Mean, \(^1\)Amplitude (peak minus mean), \(^2\)Acrophase (time at peak), and \(^3\)P-value for the zero-amplitude test corresponding to the fit of the 12-month cosine rhythm. Annual rhythms of fat concentration for the other milk market orders are displayed in Supplemental Figure 1.
Figure 7-3. Annual rhythms of protein concentration in selected Federal Milk Market orders.

Orders include (A) Northeast (Order 1) and (B) Southwest (Order 126). Data presented as least-squares means with error bars representing SEM of each month and the solid line representing a fitted cosine function with a 12-month period. Characteristics of fitted rhythm are displayed including: Mean, $^1$Amplitude (peak minus mean), $^2$Acrophase (time at peak), and $^3$P-value for the zero-amplitude test corresponding to the fit of the 12-month cosine rhythm. Annual rhythms of fat concentration for the other milk market orders are displayed in Supplemental Figure 2.
Figure 7-4. The effect of parity on annual rhythms of milk yield and composition.

Annual rhythm by parity shown for (A) milk yield, (B) fat yield, (C) fat concentration, (D) protein yield, and (E) protein concentration. Data presented as least-squares means with error bars representing SEM of each month and the line representing a fitted cosine function with a period of 12-months. Mean, Amplitude (peak minus mean), Acrophase (time at peak), and P-value for the zero-amplitude test corresponding to the fit of a 12-month cosine rhythm are displayed in tables below each corresponding graph.
Figure 7-5. The effect of DGAT1 K232A polymorphism on annual rhythms of milk and milk fat concentration and yield.

Effect of DGAT1 polymorphism on annual rhythms of (A) milk yield, (B) fat concentration, and (C) fat yield are shown. Data presented as least-squares means with error bars representing SEM of each month and the line representing a fitted cosine function with a 12-month period. Mean, ¹Amplitude (peak minus mean), ²Acrophase (time at peak), and ³P-value for the zero-amplitude test corresponding to the fit of a 12-month cosine rhythm are displayed in tables below each corresponding graph.
Table 7-1. The acrophase and amplitude of a cosine function with a 12-month period fit to the regional monthly averages of milk fat and protein concentration.

<table>
<thead>
<tr>
<th>Item</th>
<th>FMMO&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Region</th>
<th>N</th>
<th>Mean&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Amplitude&lt;sup&gt;3&lt;/sup&gt; (%)</th>
<th>Acrophase&lt;sup&gt;4&lt;/sup&gt; (date)</th>
<th>P-value&lt;sup&gt;5&lt;/sup&gt;</th>
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<tr>
<td>Fat, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td></td>
<td>Northeast</td>
<td>189</td>
<td>3.71&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Dec 31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Appalachian</td>
<td>190</td>
<td>3.66&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Jan 17&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>Florida</td>
<td>189</td>
<td>3.61&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Dec 4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>7</td>
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<td>Southeast</td>
<td>189</td>
<td>3.66&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Jan 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>30</td>
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<td>Upper MW</td>
<td>189</td>
<td>3.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Dec 31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>Central</td>
<td>189</td>
<td>3.67&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Jan 19&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>33</td>
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<td>Mideast</td>
<td>189</td>
<td>3.69&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Dec 31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>124</td>
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<td>Pacific NW</td>
<td>189</td>
<td>3.73&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Jan 12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>126</td>
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<td>Southwest</td>
<td>188</td>
<td>3.64&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>AZ-LV</td>
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<td>3.56&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Dec 29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
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<td>Western</td>
<td>51</td>
<td>3.62&lt;sup&gt;de&lt;/sup&gt;</td>
<td>0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Jan 18&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

| Protein, % |                 |              |    |                 |                           |                             |                   |
| 1    |                 | Northeast    | 190| 3.04<sup>c</sup>  | 0.08<sup>c</sup>         | Dec 31                    | < 0.001           |
| 30   |                 | Upper MW     | 190| 3.04<sup>c</sup>  | 0.09<sup>bc</sup>        | Dec 30                    | < 0.001           |
| 32   |                 | Central      | 189| 3.07<sup>b</sup>  | 0.10<sup>ab</sup>        | Jan 6                     | < 0.001           |
| 33   |                 | Mideast      | 190| 3.06<sup>bc</sup>| 0.09<sup>ab</sup>        | Dec 30                    | < 0.001           |
| 124  |                 | Pacific NW   | 189| 3.10<sup>a</sup>  | 0.08<sup>c</sup>         | Dec 27                    | < 0.001           |
| 126  |                 | Southwest    | 189| 3.09<sup>ab</sup>| 0.10<sup>a</sup>         | Dec 30                    | < 0.001           |
| 135  |                 | Western      |  51| 3.09<sup>ab</sup>| 0.08<sup>abc</sup>       | Jan 2                     | < 0.001           |

<sup>1</sup>Federal Milk Marketing Order.
<sup>2</sup>Means that do not share a superscript differ $P < 0.05$.
<sup>3</sup>Distance from mean to peak of the 12-month rhythm. Values that do not share a superscript differ $P < 0.05$.
<sup>4</sup>Date at peak of the 12-month fitted rhythm as month and day of month. Values that do not share a superscript differ $P < 0.05$.
<sup>5</sup>P-value for the zero-amplitude test corresponding to the fit of a 12-month cosine rhythm.
Supplemental Figure S7-1. Annual rhythms of fat concentration in Federal Milk Market orders.

Orders include (A) Northeast (Order 1) (B) Appalachian (Order 5), (C) Southeast (Order 7), (D) Mideast (Order 33), (E) Pacific Northwest (Order 124), (F) Southwest (Order 126), (G) Arizona-Las Vegas (Order 131), and (H) Western (Order 135). Data presented as least-squares means with error bars representing SEM of each month and the solid line representing a fitted cosine function with a period of 12-months. Characteristics of fitted rhythm are displayed including: Mean, $^1$Amplitude, $^2$Acrophase, and $^3$P-value for the zero-amplitude test corresponding to the fit of a 12-month cosine rhythm.
Supplemental Figure S7-2. Annual rhythms of protein concentration in Federal Milk Market orders.

Orders include (A) Upper Midwest (Order 30), (B) Central (Order 32), (C) Mideast (Order 33), (D) Pacific Northwest (Order 124), and (E) Western (Order 135). Data presented as least-squares means with error bars representing SEM of each month and the solid line representing a fitted cosine function with a period of 12-months. Characteristics of fitted rhythm are displayed including: Mean, $^1$Amplitude, $^2$Acrophase, and $^3$P-value corresponding to the zero-amplitude test of the 12-month rhythm.
Supplemental Figure S7-3. Variability in acrophase and amplitude of daily rhythms of milk, fat and protein yield among 11 herds in Pennsylvania.

Panels show: (A&C) Acrophases (date at peak) of the 12-month (A) fat concentration and (C) protein concentration rhythms among herds. The average acrophase of the 11 herds are shown at the top of the figures in grey. Fit of a 12-month rhythm as determined by zero-amplitude test is shown on the right side of the figures. (B&D). Amplitudes of the 12-month (B) fat concentration and (D) protein concentration rhythms among herds. The average amplitude of the 11 herds is shown at the right side of the figures in grey. Data are presented as means with error bars representing the 95% confidence interval.
Supplemental Figure S7-4. Variability in acrophase and amplitude of daily rhythms of milk fat and protein concentration among 11 herds in Pennsylvania.

Panels show: (A & C) Acrophases (date at peak) of the 12-month (A) fat and (C) protein concentration rhythms among herds. The average acrophase of the 11 herds are shown at the top of the figures in grey. Fit of a 12-month rhythm as determined by zero-amplitude test is shown on the right side of the figures. (B & D). Amplitudes of the 12-month (B) fat concentration and (D) protein concentration rhythms among herds. The average amplitude of the 11 herds is shown at the right side of the figures in grey. Data are presented as means with error bars representing the 95% confidence interval.
Supplemental Figure S7-5. Repeatability of annual rhythms across years.

Panels show monthly average fat and protein concentration in (A) the Northeast region (Order 1) and the (B) Central region (Order 32) from 2000 to 2015.
Chapter 8

Annual rhythms of milk synthesis in dairy herds in four regions of the United States and their relationships to environmental predictors.

Isaac J. Salfer, Paul A. Bartell, C.D. Dechow, Kevin J. Harvatine
ABSTRACT

Annual rhythms of milk fat and protein concentration are well described, but the rhythm of milk and milk component yield is not well described and is important to dairy management. Recent analysis of Federal Milk Marketing orders in the USA suggested that the amplitude and time at peak (acrophase) of the rhythm differ between regions. Our objective was to determine the annual rhythm of milk production and the effect of U.S. region at the herd level, and examine potential environmental factors entraining this rhythm. Monthly DHIA records of all available herds in Pennsylvania, Minnesota, Texas and Florida from the years 2003 to 2016 were obtained from Dairy Records Managements Systems. Milk yield, fat and protein yield, and fat and protein concentration were fit to the linear form of the cosine function with a 12-month period using a linear mixed effects model. Model parameters included the fixed effects of state, cosine parameters, the interaction of state and cosine parameters, and breed and the random effects of herd and year. A zero-amplitude test was performed to determine cosine function. The fit of models containing either the cosine function or environmental temperature were compared using an F-test. Additionally, raw milk production responses and the predicted response from the cosine function were correlated with daylength, change in daylength, and maximum temperature. Milk yield and fat and protein yield and concentration fit a cosine function in all four states, indicating an annual rhythm ($P < 0.001$). The amplitude of the rhythm of milk yield varied by state and was lower in PA (2.8 kg) and MN (2.4 kg) compared to TX (6.9 kg) and FL (8.1 kg; $P < 0.05$). Fat and protein yield similarly showed a greater amplitude in the southern versus northern states ($P < 0.05$). The fat and protein concentrations was opposite, with greater amplitudes occurring in MN and PA than in TX and FL ($P < 0.05$). The acrophase of milk yield, fat and protein yield, and concentration also varied by state, but all peaked between October and March ($P < 0.05$). The annual rhythm fit the data better than environmental temperature for all responses in all states ($P$
< 0.001), except for fat and protein concentration in Florida. Both raw milk yield (r = 0.19) and the annual rhythm (r = 0.85) were highly correlated with the change in daylength (P < 0.0001), while raw milk fat and protein concentration and their annual rhythms were highly correlated with absolute daylength. Results suggest that region of the U.S. impacts annual production rhythms, with a greater yearly variation in milk, fat and protein yield occurring in the south. Moreover, the annual rhythms of milk yield and milk components appear to be controlled by two separate oscillatory mechanisms with change in daylength controlling milk yield and absolute daylength controlling milk fat and protein concentration.

**Keywords:** Annual rhythms, milk synthesis, yearly pattern
INTRODUCTION

Annual rhythms commonly occur in nature as a mechanism for animals to anticipate seasonal changes in their environment before they occur and improve adaptation to environmental changes. These rhythms drive remarkable biological changes including migration, hibernation, and seasonal reproduction. The change in photoperiod across the year is a primary driver of annual rhythms for organisms living in temperate climates. For example, in short day breeders like sheep, estrus is triggered as photoperiod shortens resulting in mid-spring lambing (Woodfill et al., 1991). True annual rhythms are endogenously generated and continue even if animals are placed under constant photoperiods. The most extreme documented example of this occurred the African stonechat (Saxicola torquatus axillaris), a species of thrush, which maintained annual rhythms for 15 years when held in a constant 12.25 light: 11.75 dark photoperiod (Gwinner, 1996). Recent evidence suggests that specialized cells within the hypothalamus integrate changes in photoperiod with a molecular circadian clock, causing time-of-year specific signals that can be maintained even after the photoperiod becomes fixed (Hazlerigg et al., 2018).

Dairy cattle display seasonal changes in milk production that are well-appreciated by dairy producers and nutritionists. We have previously characterized annual rhythms of milk and milk components using USDA milk market data and test day data from 1684 cows in 11 Pennsylvania dairy herds (Salfer et al., 2019). Fat and protein concentration peaked near early January in all regions within the USDA milk market dataset, although the amplitudes varied with southern regions having a lower amplitude rhythm than northern regions. Moreover, cow-level data revealed that milk yield peaked in late March and fat and protein yield peaked in late February and that neither parity nor diacylglycerol O-acyltransferase 1 (DGAT1) K232A polymorphism, associated a majority of the genetic variation in milk fat concentration, exert substantial influence over annual rhythms of production (Salfer et al., 2019).
While U.S. milk market and cow-level data have been insightful for characterization of annual rhythms, a more comprehensive dataset was desired to better quantify these rhythms. The objective of this study was to model the annual rhythms of milk and milk fat and protein yield and milk fat and protein concentration using a robust dataset containing a large number of herds in northern and southern states. The hypothesis was that the annual rhythms of milk production would vary by latitude, with Florida and Texas having lower amplitude rhythms than Pennsylvania and Minnesota. Furthermore, the fit of a cosine function was compared to the effects of environmental temperature, period, and change in photoperiod to determine which factors correspond to the annual rhythms of milk production.

**MATERIALS & METHODS**

*Data Collection*

Monthly DHIA test day records from January 2004 to October 2016 were obtained from Dairy Records Management Systems (DRMS) for all available herds in Florida (FL), Minnesota (MN), Pennsylvania (PA), and Texas (TX). These states were selected because they represented four distinct geographic regions of the U.S. and had a large amount of data available within the DRMS system. Herd codes were blinded and records included test date, herd size, average DIM, percent of cows in 1st, 2nd, and 3rd or greater lactation, milk yield, fat concentration, protein concentration and somatic cell count. For comparisons between states, data were filtered to include only herds designated as having Holsteins to eliminate the potentially confounding effect of breed. A total of 764,196 records from 9757 Holstein herds were included in the dataset (5,021 records from 98 herds in FL, 184,521 records from 2708 herds in MN, 552,542 records from 6647 herds in PA, and 15,825 records from 303 herds in TX).
**Cosinor rhythmometry**

The fit, amplitude, and time at peak of a 12 month rhythm was determined among selected states and breeds using cosinor rhythmometry as described by (Salfer et al., 2019). Briefly, response variables were fit to the linear form of cosine functions with a 12 month period in ASREML version 14 with the following model:

\[ y_{ijklm} = \mu + S_i + A + B_k + S_i \times A_j + S_i \times B_k + M_l + Y_m + \epsilon_{ijklm} \]

Where \( y_{ijklm} \) is the response variable of interest, \( \mu \) is the overall mean, \( S_i \) is the fixed effect of state, \( A_j \) is the linear form of the sin function, \( B_k \) is the linear form of the cosine function, \( M_l \) is the fixed effect of average days in milk, \( Y_m \) is the random effect of year (I = 2004 to 2016) and \( \epsilon_{ijklm} \) is the residual error. The fit of the linear form of cosine functions was compared to a reduced model testing the linear effect of day of year:

\[ y_{ijkl} = \mu + S_i + D_j + S_i \times D_j + M_k + Y_l + \epsilon_{ijkl} \]

Where \( y_{ijkl} \) is the response variable of interest, \( \mu \) is the overall mean, \( S_i \) is the fixed effect of state, \( D_j \) is the fixed effect of day of year (i= 1 to 365), \( M_k \) is the fixed effect of average days in milk, \( Y_l \) is the random effect of year (i= 2004 to 2016), and \( \epsilon_{ijkl} \) is the residual error. The fit of the annual rhythms was determined by a zero amplitude test comparing the residual sums of squares between the full model containing linearized cosine functions and the reduced model (Cornelissen, 2014). The amplitude (peak minus mean) and acrophase (time at peak) and their significance tests were determined using a script written in R ver. 3.4.3 according to equations of Bourdon et al. (1995).

**Effect of breed on annual rhythms of production**

The effect of breed was tested based on breed designation codes in the database. Breeds within fewer than 50 herds were removed, leaving Holsteins (n = 8,790 herds, 675,068 records), Jerseys (n = 398 herds, 21,510 records), Brown Swiss (n = 67 herds, 2,913 records), and crossbreds (n = 1463 herds; 53,444 records). A full model containing the fixed effects of breed,
cosine terms, the interaction of breed with cosine terms, and average days in milk and the random
effect of year was compared to a reduced model containing the fixed effects of breed, day of year,
and average days in milk, and the random effect of year.

Comparison of annual rhythm with temperature

To compare the fit of the annual rhythm with the fit of a model testing the effect of
environmental temperature, daily climate data were collected from the U.S. National Weather
Service from representative weather stations centrally located near heavy populations of dairy
herds in each state (Tallahassee, FL; Litchfield, MN; Harrisburg, PA, Abilene, TX). The effect of
daily maximum temperature on milk and milk fat and protein yield and milk fat and protein
concentration was tested using the following model:

\[ y_{ijkl} = \mu + S_i + T_j + S_i \times T_j + M_k + Y_l + \epsilon_{ijkl} \]

Where \( y_{ijkl} \) is the response variable of interest, \( \mu \) is the overall mean, \( S_i \) is the fixed effect of state \( T_j \)
is the maximum daily temperature, \( M_k \) is the fixed effect of average days in milk, \( Y_l \) is the random
effect of year (i= 2004 to 2016), and \( \epsilon_{ijkl} \) is the residual error. The coefficient of determination
\((R^2)\) was calculated for both the model containing cosine functions with a 12-month period
(Equation 1) and the model with maximum temperature (Equation 3). An F-test was performed to
compare the fit of the two models (Glatting et al., 2007).

Relationships among environmental variables and milk production responses

The relationship between daylength, change in daylength, and maximum temperature and
the annual rhythms of milk synthesis were tested to better understand which environmental
predictors best explain these annual rhythms. Daylength (min of daylight) for each day of the year
was determined using the latitude of the weather stations used for collection of temperature data
(Tallahassee, FL: 30.4°N; Litchfield, MN: 45.1°N; Harrisburg, PA: 40.3°N, Abilene, TX: 32.4°N)
The change in day length across the year was computed for each region as described by Forsythe et al. (1995). These environmental predictors were correlated with both the raw milk and milk component responses and with the fitted 12 month cosine function as described by Roenneberg and Aschoff (1990). Pearson correlations were performed using the cor.test() function of R ver. 3.4.3 (R Development Core Team, 2013).

RESULTS

Annual Rhythms within Selected States

An interaction between state (PA, MN, TX, and FL) and the cosine function with a 12 month period occurred for milk and milk fat and protein yield and milk fat and protein concentration so the fit, amplitude, and acrophase of the rhythm were determined for each state (\(P < 0.001\)). Average milk yield was greatest in Pennsylvania (26.8 kg), followed by Minnesota (26.7), Texas (25.8), and Florida (23.9; \(P < 0.001\)). In all four states, milk yield fit the 12 month cosine function, indicating the presence of an annual rhythm (\(P < 0.001\); Figure 8-1A). The amplitude of the 12-month rhythm of milk yield differed by state, with Florida (3.31 kg) and Texas (3.06) having amplitudes over twice that of Pennsylvania (1.35) and Minnesota (1.29; \(P < 0.001\)). The acrophase, or day at peak, occurred on April 11 for both Minnesota and Pennsylvania, while Florida peaked 4 days earlier on April 7, and Texas peaked 8 days earlier on April 3.

Milk fat and protein concentration also fit an annual rhythm in all states (\(P < 0.002\); Figure 8-1C&E). The amplitude of the 12-month rhythm of milk fat concentration was greatest in PA (0.16%), followed by MN (0.14), TX (0.09), and FL (0.06). The acrophase of the annual
rhythm occurred near the first of the year and was similar between PA (Jan 7) and TX (Jan 8), but
occurred over one week earlier in MN (Dec 29), and nearly a month earlier in FL (Dec 3). The
annual rhythm of milk protein concentration differed between regions similar to milk fat
concentration, with FL having a lower amplitude (0.05%) than MN (0.10), TX (0.10), and PA
(0.09; \(P < 0.001\)). The acrophase of the protein rhythm was similar between MN, PA, and TX,
with all occurring within one day of Dec 21, while FL had an acrophase occurring 19 d earlier on
Dec 2.

The annual rhythms in milk yield and milk fat and protein concentration caused a rhythm
in milk fat and protein yield, which fit an annual rhythm regardless of state, with differences in
amplitude and acrophase among states (\(P < 0.001\); Figure 8-1B&D). The amplitude of the fat
yield rhythm was greatest in TX (117 g), followed by FL (110), with much lower amplitudes
observed in PA (61.6) and MN (52.6). The acrophase of the 12-month rhythm of fat yield
occurred on April 2 in FL and was 10 days earlier in TX (March 23). The annual rhythm of fat
yield peaked about a month earlier in PA and MN, occurring on March 1 and March 2,
respectively (\(P < 0.001\)). The annual rhythm of protein yield did not differ in amplitude between
FL (94.3 g) and TX (93.1), which both oscillated more than MN (39.4) and PA (39.3; \(P < 0.001\)).
The acrophase of protein yield was different between all states, peaking on March 31 in FL,
March 17 in TX, March 7 in FL, and March 5 in MN (\(P < 0.001\)).

**Annual Rhythms among Breeds**

Annual rhythms were detected for milk and milk fat and protein yield and milk fat and
protein concentration regardless of breed (\(P < 0.001\)). As expected, average milk yield was
greatest for Holsteins (26.6 kg), followed by crossbreds (21.6), Brown Swiss (21.1), and Jerseys
(17.7; Figure 8-2A). The amplitude of milk yield varied by breed with Holsteins having a 106,
54, and 40%, greater amplitude than crossbreds, Jerseys, and Brown Swiss, respectively (\(P <
However, no differences were detected among the other three breeds \( (P > 0.05) \). The greater amplitude in Holsteins is likely related to greater milk yield, and when amplitude was examined as a percent of average milk yield, there was little difference among breeds (3\% for Brown Swiss, 5\% for Holsteins, 5\% for Jerseys, and 4\% for crossbreds). Breeds also varied in acrophase of the annual rhythm of milk yield with Holsteins (Apr. 1) and crossbreds (Apr. 22) peaking earlier than Jerseys (May 11) and Brown Swiss (May 16). The annual rhythms of fat and protein concentration also varied by breed (Figure 8.2D&E). Fat concentration was greatest in Jerseys (4.71\%), followed by Brown Swiss (4.07\%), crossbreds (3.95\%), and Holsteins (3.72\%). The amplitude of the annual rhythm of fat concentration was greatest in Jerseys (0.27\%) and lowest in Holsteins (0.14\%; \( P < 0.05 \)), with Brown Swiss (0.23\%) and Crossbreds (0.20\%) having a similar rhythm amplitude \( (P > 0.05) \). All breeds had an acrophase of milk fat concentration that occurred between Jan 5th and 7th \( (P > 0.05) \). Similar to fat concentration, the amplitude of protein concentration was greatest for Jerseys (0.15\%), followed by Brown Swiss (0.14\%), crossbreds (0.12\%), and Holsteins (0.08\%). The acrophase of the protein concentration rhythm occurred on Dec. 21 for both Holsteins and Jerseys, two days earlier than crossbreds and 8 d earlier than Brown Swiss.

Milk fat yield was greatest in Holsteins (990 g; \( P < 0.05 \)), followed by Brown Swiss (899 g) and crossbreds (850g), and was lowest in Jerseys (836 g; \( P < 0.05 \)). Similar to fat yield, protein yield was greatest in Holsteins (811 g), did not differ between Brown Swiss (745 g) and crossbreds (689 g), and was lowest in Jerseys (635 g). The annual rhythm of milk fat yield had an amplitude of 64.2 g in Holsteins 69, 55, and 42\% greater than Brown Swiss, Jerseys and crossbreds, respectively \( (P < 0.05) \). The acrophase of fat yield did not differ among Brown Swiss (Feb. 6), crossbreds (Feb. 23), or Holsteins (Feb. 24 \( P > 0.05 \)), but the acrophase of Jerseys (Mar 3) acrophase was delayed 25 d compared to Brown Swiss \( (P < 0.05) \). Holsteins also had a larger amplitude of milk protein (42.8 g) than the other three breeds. Protein yield peaked
on Feb 12 in Brown Swiss, which was 16 d earlier than Holsteins, 18 d earlier than crossbreds and 31 d earlier than Jerseys ($P < 0.05$; Figure 8-2C).

**Comparison of Annual Rhythm and Temperature Models**

To determine the influence of environmental temperature versus the annual rhythm, a model containing the daily maximum temperature of a representative weather station in each state was compared to the cosine function with a period of twelve months. Milk yield fit a 12 month cosine function better than the model including the effect of maximum temperature ($P < 0.0001$; Table 8-1). Furthermore, among the four states (Florida, Minnesota, Pennsylvania and Texas), all fit a cosine function better than maximum temperature ($P < 0.0001$). Model $R^2$ was increased from 0.16 in the temperature model to 0.19 in the 12 month cosine model for the combined dataset, and was also greater for the cosine function in all individual states. Florida (0.14 vs 0.32) and TX (0.23 vs. 0.35) had the greatest improvements in model $R^2$ from the temperature model to the cosine function model.

Fat concentration fit a cosine function better than the temperature model in the combined data set ($P < 0.0001$) and in MN, PA, and TX ($P < 0.0001$; Table 8-1). However, for FL, the temperature model fit fat concentration better than the cosine function ($P < 0.0001$). Model $R^2$ of the combined dataset was improved from 0.30 in the temperature model to 0.31 in the cosine model. Furthermore, the $R^2$ of the cosine model was 0.01 units greater in MN, PA, and TX, but were the same for both models in FL. Similarly, milk protein concentration fit a 12 month cosine function better than maximum temperature in the combined dataset and in MN, PA, and TX ($P < 0.0001$). Like fat concentration, milk protein concentration fit temperature better than the cosine function in FL ($P < 0.0001$). Model $R^2$ of protein concentration was improved from 0.36 in the temperature model to 0.39 in the cosine model in the combined dataset. Among the individual
states the $R^2$ of the cosine model was greater than the temperature model for protein concentration in MN, PA, and TX, but was not different between models in FL.

Fat and protein yield also both fit a cosine function better than temperature model for the combined model ($P < 0.0001$) and each individual state ($P < 0.0001$). Model $R^2$ of the combined dataset was improved from 0.12 in the maximum temperature model to 0.15 in the cosine function model for fat yield and improved from 0.12 to 0.14 for protein yield.

**Relationships among Environmental Variables and Milk Production**

Daylight and change in daylight change across the year, and appear to follow similar daily patterns to milk component concentrations and milk yield, respectively (Figure 8-3B&C). There was a significant relationship among daylength, change in daylength, and environmental temperature with all production responses (milk yield, milk fat concentration, milk protein concentration, milk fat yield, milk protein yield; $P < 0.0001$), with the exception of daylength and milk protein concentration ($P = 0.14$; Figure 8-3A). Change in daylength was the environmental predictor with the strongest relationship to both milk yield ($r = 0.19$) and the fitted cosine function ($r = 0.85$). Maximum temperature had a negative correlation with both milk yield ($r = -0.06$) and cosine-adjusted milk yield ($r = -0.14$), while daylength had a weak positive relationship (raw: $r = 0.04$; fitted: $r = 0.33$).

For both raw data and the fitted cosine function, absolute day length had a strong negative relationship with milk fat (raw: $r = -0.31$; fitted: -0.88) and protein (raw: $r = -0.40$; fitted: -0.92) concentrations (Figure 8-3A). Maximum temperature also exhibited a strong negative correlation with both milk fat (raw: $r = -0.30$; fitted: -0.85) and protein concentration (raw: $r = -0.36$; fitted: -0.77). Alternatively, the correlation between change in daylength and raw and fitted milk fat concentration was relatively low (raw: $r = 0.07$; fitted: 0.23). Milk protein concentration
had a weak negative relationship with change in daylength ($r = -0.003$) and the fitted cosine function was not correlated ($r = 0.002; P = 0.14$).

Fat yield, like milk yield, was the most strongly correlated with change in daylength ($r = 0.22$). Moreover, milk fat yield was also negatively correlated with maximum temperature ($r = -0.19$) and daylength ($r = 0.10$). The fitted cosine function of fat yield also had a strong positive relationship with change in daylength ($r = 0.83$) and had a reasonably strong negative relationships with maximum temperature ($r = -0.62$) and daylength ($r = -0.28$). Similarly, both raw protein yield and the fitted cosine function had the strongest relationship with change in daylength (raw: $r = 0.19$; fitted: $0.77$), followed by a strong negative relationship with maximum temperature (raw: $r = -0.14$; fitted: $-0.58$), and a weaker negative relationship with absolute daylength ($r = -0.07$; $-0.27$).

*Regression Equations to Adjust for Seasonal Changes in Production*

The consistency of annual rhythms among years allowed for the derivation of regression equations to adjust expected production for each month. Equations derived from the linear form of the cosine function significantly predicted actual milk and milk fat and protein yield and milk fat and protein concentration for each of the 4 regions ($P < 0.001$; Table 8-2). Furthermore, the expected deviations from mean responses were computed from these equations for rapid adjustment of milk and milk fat and protein yield and milk fat and protein concentration to account for the seasonal rhythm (Supplemental Table S8-1 - Supplemental Table S8-4).

**DISCUSSION**

Milk synthesis followed an annual rhythm in all four states that were examined that represent different regions of the U.S. In all states, milk yield peaked in early April, whereas fat
and protein concentration peaked at the start of the year. These results were similar to previous accounts of seasonal variation in milk yield. Wood (1970) detected a ‘spring hump’ in milk yield across multiple years. Moreover, Bailey et al. (2005) determined that the maximal quantity of milk shipped per farm in the Mideast Federal Milk Market Order occurred in March, April, and May. Salfer et al. (2019) observed that in 11 Pennsylvania dairy herds, the yearly rhythm of milk yield peaked on March 13, while fat concentration peaked on January 25, and protein concentration peaked on December 20.

The region of the U.S. had a clear effect on the robustness of the annual rhythms of milk synthesis. States in the southern U.S. (FL and TX) had greater amplitudes of milk and milk fat and protein yield, whereas states in the northern U.S. (MN and PA) had greater amplitudes of milk fat and protein concentration. These results concurred with those observed by Salfer et al. (2019), which demonstrated that the Milk Marketing Order located in Florida had a lower amplitude of fat and protein concentration than other orders. Together, these results suggest that the amplitude of annual rhythms is directly influenced by the latitude, perhaps related to differences in photoperiod. While data from the southern hemisphere would be insightful for better understanding the relationship between photoperiod and annual rhythms of milk synthesis, these data are limited and confounded by management system. In New Zealand, milk yield peaks in September through January, and fat and protein concentration peak from June through August (Auldist et al., 1998). This would suggest that annual rhythms of milk synthesis are opposite in the southern compared to the northern hemisphere. However, the cows in this experiment were housed on pasture and results were likely affected by changes in pasture composition across the year and perhaps by seasonal breeding.

Of the four breeds examined, all displayed annual rhythms of production with little differences in the rhythms among breeds. Milk yield peaked approximately one month later in Brown Swiss and Jerseys than Holsteins and crossbreds, but this difference is unlikely to be
biologically significant. All of major breeds studied are of the *Bos taurus* species and were
developed in northern Europe and are relatively genetically similar. An interesting comparison
would be between *Bos taurus* breeds and animals of the *Bos indicus* species, which were
developed in tropical climates where the change in photoperiod throughout the year is smaller,
but data is not available to our knowledge. Chital (*Axis axis*), a species of deer native to the
Indian subcontinent near the equator, show asynchronous annual rhythms of gonadal activity both
in the wild and captivity, whereas these rhythms are well-synchronized in related species of deer
native to Europe (Loudon and Curlewis, 1988).

The annual changes in milk production are often attributed to heat stress. Heat stress
occurs during mid-to late summer because of high ambient temperature and humidity (West,
2003). While the effects of heat stress on milk production are well-established, several factors
suggest that the annual rhythm of milk synthesis is influenced by factors beyond heat stress alone.
First, if the summer decrease in production was caused only by heat stress, yearly production
would be expected to remain stable during the fall through spring, with an acute decline in
production during the summer. Furthermore, the nadir of milk yield occurs during early October,
well past peak temperature, but could be a carry-over effect. Interestingly, average milk, fat and
protein yield fall below the fitted cosine function in June, July, and August, particularly in Texas
and Florida. This suggests that there is an additional depression in milk yield below the annual
rhythm during the summer, likely due to the additive effect of heat stress.

In the current study, the effect of heat stress was directly compared to the annual rhythm
using an *F*-test that tested the fit of a model testing the main effect of maximum daily
temperature, to a model testing the linear form of the cosine function. Milk, fat and protein yield
were better predicted by the annual rhythm than by maximum temperature in all regions
examined. Furthermore, the annual rhythm was a better predictor of fat and protein concentration
in Minnesota, Pennsylvania and Texas. In Florida, however, fat and protein concentration were
better predicted by maximum temperature. This result may be because Florida has a lower amplitude annual rhythm than other states, so variability in environmental temperature may have a greater influence on production. Maximum temperature was highly correlated with fat and protein concentration and their respective fitted cosine functions. However, this effect appears to be confounded with daylength. Daylength follows a similar yearly pattern to maximum temperature (Figure 8-3B&D). Furthermore, daylength has a stronger negative relationship with fat and protein concentration and their fitted cosine functions than maximum temperature. Importantly, a summary of controlled experiments testing the high ambient temperature using environmental chambers demonstrate that, while milk yield is consistently decreased by heat stress, fat concentration is consistently increased, and the effects on protein yield are small and inconsistent (Table 8-3). While milk yield was decreased by heat stress in all 6 experiments, with an average decrease of 6.7 kg, fat concentration was increased by heat stress in 5 out of the 6 experiments and was increased 0.22 percentage units on average. Protein concentration was decreased in 4 and increased in 2 experiments and was decreased 0.12 percentage units on average. The decrease in milk yield is consistent with the annual rhythm of milk yield, but the increase in milk fat during heat stress is opposite of the rhythm. These results suggest that the yearly pattern of milk fat is regulated by a different mechanism.

The pattern of milk synthesis appears to represent an endogenous annual rhythm rather than a consequence of heat stress. Annual rhythms occur in a wide variety of organisms as a mechanism to predict seasonal changes in the environment before they occur (Lincoln, 2019). Commonly, these rhythms are involved in optimizing reproductive processes to improve the likelihood of offspring survival. For example, ewes express estrous only during late fall and early winter, restricting the time of lambing to the spring (Shelton and Morrow, 1965). While dairy cows are not generally considered to be seasonal breeders, Hansen (1985) detected modest effects of season and photoperiod length on postpartum calving interval. As a component of
reproduction, annual rhythms of milk fat and protein concentration may exist to provide neonates with nutrient-dense milk during the winter when energetic demands are higher. Evidence suggests that other physiological responses follow annual rhythms in dairy cows. Piccione et al. (2012) discovered that the concentrations of creatinine, triacylglycerides, and β-hydroxybutyrate follow 12-month rhythms. Moreover, Petitclerc et al. (1983) observed that circulating prolactin follows an annual rhythm that persists even after blinding or pinealectomy. The core body temperature is also influenced by season, with greater daily fluctuation in temperature during the summer compared to winter (Kendall and Webster, 2009).

Annual rhythms are governed by endogenous circannual clocks that convert environmental signals to time-of-year cues that influence physiology in a seasonal manner. In mammals and birds, a central circannual pacemaker located in the pars tuberalis (PT) of the pituitary gland has been described (Wood and Loudon, 2018). Notably, annual rhythms can persist for long periods of time after organisms are placed in constant conditions (Pengelley et al., 1976; Gwinner, 2003). When annual rhythms are allowed to freerun in the absence of environmental stimuli, they typically have a period of only 10 to 11 months and require entrainment of circannual clocks to maintain a 12 month period (Lincoln, 2019). Several entrainment cues such as photoperiod, social stimuli, and nutritional cycles can synchronize endogenous annual rhythms to the external environment (Lincoln et al., 2006).

To study the potential role of photoperiod on entrainment of annual rhythms of milk synthesis, the relationships among milk production responses and both absolute photoperiod (daylength) and change in daylength were tested. In addition to correlating these predictors with raw production responses, they were correlated with the fitted response from the linear form of the cosine function. Fat and protein concentration were most highly correlated with daylength, and in particular the 12 month fitted cosine function was very highly correlated. These results suggest that fat and protein concentration may be controlled by a circannual oscillator that is
entrained by photoperiod. A wealth of research has described the relationship between photoperiod length and milk synthesis in dairy cattle. Placing cows in an long days (> 12 h daylight) consistently stimulates milk yield, with the optimal response occurring with a 16 h light:8 h dark (16L: 8D) photoperiod (Dahl et al., 2000). However, there are a few interesting contrasts between the results of studies using artificial photoperiod manipulation and the results of the current study examining the naturally-occurring annual rhythm. First, while long-day lighting stimulates milk yield, it does not alter fat and protein concentration. In the current study, not only are fat and protein concentration strongly correlated to daylength, the long days are associated with decreases in fat and protein concentration. Moreover, milk yield is poorly correlated with daylength, but maximal milk yield occurs during the spring, when daylength is shorter than 12 h.

The paradox between controlled photoperiod manipulation trials and the annual rhythm of milk yield may be caused by animals becoming photorefractory to artificial long-day lighting. In seasonally-breeding mammals such as Suffolk sheep (Ovis aries) and Syrian hamsters (Mesocricetus auratus), exposure to a fixed photoperiod for long periods of time causes a spontaneous reversion to a physiological state expected during the opposite photoperiod (Robinson and Karsch, 1984; Lincoln et al., 2005). In short-day breeding sheep, estrus is inhibited by fixed 8L:16D photoperiods, while the estrus of long-day breeding hamsters is conversely inhibited by fixed 14 light:10 dark photoperiods. Induction of photorefractoriness requires 4 to 12 weeks of a fixed photoperiod prior to a physiological change, which is consistent with results of long-day lighting experiments, which require 4 weeks of adaptation before increased milk yield is observed (Dahl et al., 2000).

As mentioned above, milk, fat and protein yield were poorly correlated with absolute daylength, but had a strong positive relationship with change in daylength. This suggests that, rather than being entrained by the absolute daylength, a separate circadian oscillator controlled
change in daylength may be responsible for entraining the annual rhythms of milk yield. Furthermore, the annual rhythm of milk yield may be related to the phase-relationship between an oscillator entrained by photoperiod, and a daily circadian oscillator. Bartell and Gwinner (2005) proposed that the degree of synchronization between a circannual and a circadian oscillator can entrain seasonal responses.

The consistency of annual rhythms among years and herds allowed for the derivation of regression equations to normalize milk and milk fat and protein yield and milk fat and protein concentration. Furthermore, these regression equations were used to calculate adjustment factors to standardize these responses across the year. Using these adjustment factors may allow dairy producers to remove the annual variation in milk production to make better-controlled nutritional and management decisions. A spreadsheet developed in Microsoft Excel that uses these regression equations for rapid calculation of yearly rhythm-adjusted milk and milk fat and protein yield and milk fat and protein concentration are included in the supplement.

CONCLUSIONS

The milk synthesis of dairy cows follows an annual rhythm that is affected by region of the United States. The annual rhythm of milk yield appears to be related to, and is perhaps entrained by the change in daylength. Fat and protein concentration have a strong relationship to absolute daylength, suggesting that they may be entrained by photoperiod. While maximum environmental temperature is also highly correlated to fat and protein concentration, the cosine function fits the data better, suggesting it better explains their yearly pattern. Fat and protein concentration closely align with the lengthening and shortening of days, while milk yield aligns with the change in daylength. These annual rhythms may to be driven by endogenous circannual oscillators, and may not be able to be influenced through management interventions. Further
research must be conducted to understand the physiological mechanisms governing the annual rhythms of milk synthesis. Dairy producers can account for the annual rhythms of production by standardizing milk production for this rhythm across the year.
Figure 8-1. Annual rhythms of milk, fat and protein yield and fat and protein concentration in Holstein dairy herds in Florida (FL), Minnesota (MN), Pennsylvania (PA), and Texas (TX).

Data includes Dairy Herd Information Association (DHIA) test day results from individual herds in FL (98 herds, 5,021 records), MN (2708 herds, 184,521 records), PA (6647 herds, 552,542 records), and TX (303 herds, 15,825 records) acquired from Dairy Records Management Systems (DRMS). Panels show: (A) Effect of state on annual rhythms of milk yield, (B) Effect of state on annual rhythms of fat yield, (C) Effect of state on annual rhythms of fat concentration, (D) Effect of state on annual rhythms of protein yield, (E) Effect of state on annual rhythms of protein concentration. Data are presented as the least squares mean of each month with error bars representing the SEM and the line is the fitted cosine function with a period of 12 months. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the $P$-value of the zero-amplitude test.
Figure 8-2. Annual rhythms of milk and milk fat and protein yield and milk fat and protein concentration by breed.

Data includes Dairy Herd Information Association (DHIA) test day results from individual herds designated to contain Brown Swiss (BS; 67 herds, 2,913 records), Holsteins (HO, 8790 herds, 675,071 records), Jerseys (JE; 398 herds, 21,510 records), and crossbreds (1463 herds, 53,444 records) acquired from Dairy Records Management Systems (DRMS). Panels show: (A) Effect of breed on annual rhythms of milk yield, (B) Effect of breed on annual rhythms of fat yield, (C) Effect of breed on annual rhythms of fat concentration, (D) Effect of breed on annual rhythms of protein yield, (E) Effect of breed on annual rhythms of protein concentration. Data are presented as the least squares mean of each month with error bars representing the SEM and the line is the fitted cosine function with a period of 12 months. Cosinor analysis is shown below each panel and include the amplitude (Amp; difference between peak and mean), acrophase (Acro; time at peak of the rhythm), and the P-value of the zero-amplitude test.
Figure 8-3. Relationship among environmental predictors and annual rhythms of milk and milk fat and protein yield and milk fat and protein concentration by breed.

Panels show: (A) Correlogram showing relationship among environmental variables [daylength, change in daylength (ΔDaylength), and maximum temperature (Max. Temp)] and milk production responses (Milk yield, fat concentration, protein concentration, fat yield and protein yield). For each relationship, the correlation is listed first, followed by the $P$-value, (B) daylength, (C) change in daylength, and (D) average maximum temperature across the year for Florida (FL), Minnesota (MN), Pennsylvania (PA), and Texas (TX). Fitted correlations show relationship of milk and milk fat and protein yield and fat and protein concentration with the response predicted by a the model: $\gamma_{ijklm} = \mu + S_i + A_j + B_k + S_i \times B_k + M_i + Y_m + \varepsilon_{ijklm}$ where $\gamma_{ijklm}$ is the response variable of interest, $\mu$ is the overall mean, $S_i$ is the fixed effect of state, $A_j$ is the linear form of the sin function, $B_k$ is the linear form of the cosine function, $M_i$ is the fixed effect of average days in milk, $Y_m$ is the random effect of year (i.e. 2004 to 2016) and $\varepsilon_{ijklm}$ is the residual error.
Table 8-1. Comparison between models testing the fit of a cosine function versus maximum temperature.

<table>
<thead>
<tr>
<th></th>
<th>R² Cosine Model¹</th>
<th>R² Temperature Model²</th>
<th>F-Value</th>
<th>P-value</th>
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<td>Milk yield, kg/d</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
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<td>0.12</td>
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</tr>
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¹Cosine model: \( y_{ijklm} = \mu + S_i + A + B_k + S_i \times A_j + S_i \times B_k + M_l + Y_m + \epsilon_{ijklm} \) where \( y_{ijklm} \) is the response variable of interest, \( \mu \) is the overall mean, \( S_i \) is the fixed effect of state, \( A_j \) is the linear form of the sin function, \( B_k \) is the linear form of the cosine function, \( M_l \) is the fixed effect of average days in milk, \( Y_m \) is the random effect of year (i= 2004 to 2016) and \( \epsilon_{ijklm} \) is the residual error.

²Temperature model: \( y_{ijkl} = \mu + S_i + T_j + S_i \times T_j + M_k + Y_l + \epsilon_{ijkl} \) where \( y_{ijkl} \) is the response variable of interest, \( \mu \) is the overall mean, \( S_i \) is the fixed effect of state \( T_j \) is the maximum daily temperature, \( M_k \) is the fixed effect of average days in milk, \( Y_l \) is the random effect of year (i= 2004 to 2016) and \( \epsilon_{ijkl} \) is the residual error.

³F-test of temperature model fits data better than the cosine model (P < 0.0001).
Table 8-2. Equations for cosine regression equations for milk and milk fat and protein yield and milk fat and protein concentration for Florida (FL), Minnesota (MN), Pennsylvania (PA), and Texas (TX).

<table>
<thead>
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<th>Response</th>
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1Parameters derived from fitting Dairy Herd Information Association (DHIA) test day data acquired from Dairy Records Management Systems (DRMS) to a 12 month cosine function with the following equation: $y = x - A \cdot \cos\left(\frac{2\pi m}{12}\right) + B \cdot \sin\left(\frac{2\pi m}{12}\right)$. A cosine by state interaction was included so parameters could be derived within each state.
Table 8-3. A summary of milk yield and milk fat and protein concentration responses in experiments testing the effects of heat stress in environmental chambers.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Milk, kg</th>
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<th>Protein, %</th>
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<td>Rhoads et al. (2009)</td>
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<td>Schwartz et al. (2009)</td>
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<td>0.06</td>
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<td>Wheelock et al. (2010)</td>
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<td>Zimbelman et al. (2010)</td>
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<td>Baumgard et al. (2011)</td>
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### SUPPLEMENTAL TABLES

**Supplemental Table S8-1.** Adjustment factors to correct for annual rhythms of milk production in Florida.

<table>
<thead>
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<th></th>
<th>Milk, kg/d</th>
<th>Milk lb/d</th>
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<th>Protein, %</th>
<th>Fat, g/d</th>
<th>Fat, lb/d</th>
<th>Protein, g/d</th>
<th>Protein, lb/d</th>
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<tr>
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<td>-0.20</td>
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<tr>
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<td>0.05</td>
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<td>-0.14</td>
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<tr>
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<td>0.02</td>
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<th>Protein, %</th>
<th>Fat, g/d</th>
<th>Fat, lb/d</th>
<th>Protein, g/d</th>
<th>Protein, lb/d</th>
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**Supplemental Table S8-3.** Adjustment factors to correct for annual rhythms of milk production in Pennsylvania.

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<th>Fat, lb/d</th>
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**Supplemental Table S8-4.** Adjustment factors to correct for annual rhythms of milk production in Texas.

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<th>Fat, g/d</th>
<th>Fat, lb/d</th>
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Chapter 9

Integrative Discussion

Dairy producers are well aware of daily and seasonal changes in milk production. Moreover, differences in milk and milk components between morning and evening milking (Gilbert et al., 1972), and among different seasons of the year (Wood, 1976) have been characterized in the literature. However, the regulation of these changes have been largely unexplored and are not considered in modern dairy management. Developing a better understanding of the biological rhythms governing milk synthesis may uncover opportunities to improve the efficiency of dairy production. Therefore, the research conducted for this dissertation sought to examine the nutritional and environmental factors affecting daily and annual rhythms of milk synthesis.

The first four experiments focused on the relationship between feed intake and daily rhythms of milk synthesis using an in vivo approach with lactating cows. Previous research has provided groundwork to suggest that a relationship between feed intake and daily rhythms of milk production exists. Rottman et al. (2014) demonstrated that increasing the frequency of feeding from 1x to 4x/d damped the rhythms of milk yield, while Niu et al. (2014) observed differences in the ratio of morning to evening milk production and daily patterns of plasma metabolites when cows were fed at 8:30 AM versus 8:30 PM. Based on these initial reports, we wanted to investigate the impact of the time of feed intake of the rhythms of milk synthesis more completely. To do this, we employed time-restricted feeding to impose a more dramatic shift in the feeding pattern of cows, while milking cows 4x/d to determine the daily rhythm of milk synthesis (Chapter 3). Time-restricted feeding caused a substantial shift in the daily rhythms of
milk yield and milk components corresponding with the 12 h difference in feed delivery. These results confirmed in a very clear fashion that feeding time does indeed affect the daily rhythms of milk synthesis. Plasma hormone and metabolite concentrations were similarly shifted by the time of feed intake, corresponding with the fasting cycle. Most interestingly, the response to fasting was much greater in the night-restricted feeding group, who had much greater fasting non-esterified fatty acid concentrations, indicating greater lipid mobilization. Cows typically increase feed intake during the afternoon (Albright, 1993). Our results demonstrate that this daily pattern of intake appears to be an inherent component of the cow’s physiology, because the cow appears to continue to want to eat in the afternoon, even after being adapted to night-feeding. Although not quantified, the cows on the night-restricted treatment also exhibited more food seeking behavior and greater irritability than day-restricted cows.

In laboratory models, time-restricted feeding can shift physiological rhythms by modifying the daily pattern of molecular clock gene expression (Stokkan et al., 2001). We wanted to determine if this mechanism was responsible for the changes in the daily rhythms of milk synthesis in Chapter 3. We used mammary biopsies to collect tissue from the mammary gland at four times across the day in cows that were either fed 24 h/d or 16 h/d at night. Of the 6 clock genes measured, 3 followed a daily rhythm. Time-restricted feeding shifted the daily rhythms of expression of two clock genes CLOCK and CRY1, and induced a rhythm in REV-ERBa.

To our knowledge, this is the first demonstration that feeding can entrain the mammary molecular circadian clock in cattle, and one of the first to demonstrate this in any species. On many large-scale modern dairy farms, milking parlors are operating 24 h/d, leading to cows being milked at all hours of the day and night. However, feeding of all cows is usually performed within the same timeframe. Differences in the relationship between feeding and milking time among individual cows may cause differences in the entrainment of their mammary gland. Future research should be done to better elucidate this relationship. The results of this experiment not
only has exciting implications for dairy production, but human lactation as well. In humans 7% of the transcriptome follows a daily rhythm (Maningat et al., 2009). Because humans have a less consistent daily eating pattern than cows, they may be even more responsive to food entrainment. Therefore, nutritional composition of breastmilk may be affected by meal pattern of the mother. Research into this area may develop potential avenues to improve post-natal nutrition.

Unfortunately, while we observed compelling evidence to suggest the circadian clock of the mammary gland is entrained by food intake, the results were not as clear cut as we hoped. Unexpectedly, 3 of the measured clock genes failed to follow a daily rhythm according to the zero-amplitude test. One potential cause of this is that we had an unexpectedly high variability in the samples. Visually, all of the genes analyzed appear to be somewhat rhythmic. Moreover, PER1 seems to be influenced by treatment with a large spike in expression at 0400 in control that occurs 12 h later under night-restricted feeding, but again this change is not statistically significant. The high sample variability may have been due to several potential factors. First, the mammary tissue collected may have been more heterogeneous than anticipated. The mammary gland is a complex organ containing multiple cell types including epithelial cells, smooth muscle cells, and adipocytes (Inman et al., 2015). While biopsy collection was performed as consistently as possible, variation in the proportions of individual cell types and their potential entrainment may have contributed to the variation in the gene expression results. More frequent sampling could have been employed to develop a better characterization of the daily pattern of gene expression and perhaps helped us detect a rhythm, but mammary biopsy is an invasive species and we wanted to avoid excess stress on the cows for both animal welfare and research purposes. An increased number of experimental subjects could have also been used to increase the power of the experiment, and this will likely be done in the future.

Recent research has illustrated the importance of post-transcriptional and post-translational regulation of circadian rhythms (Robles et al., 2014, 2017). In Chapter 4, the
molecular circadian clock was only examined at the mRNA level, limiting its scope. In the future, research should consider using proteomic and phosphoproteomic analyses, or antibody-based techniques to understand these additional levels of regulation.

In Chapters 5 and 6, the effects of the time of fat and protein availability on the daily rhythms of milk synthesis were studied by infusing cannulated cows with these nutrients post-ruminally. The time of protein infusion had considerable effects on daily milk yield. If maintained in commercial herds, the 7 lb reduction in milk yield by restricting protein infusion to the night would have momentous implications for dairy farmers. This experiment provides compelling evidence that if protein availability is limited during the day, milk synthesis will also be limited. Alternatively, the time of fat infusion did not affect milk or milk components. Day infusion of fat increased the robustness of milk yield and decreased the robustness of fat and protein concentration (Chapter 5). Alternately, night infusion of protein increased the robustness of milk yield and decreased the robustness of protein concentration (Chapter 6). These experiments were the first to study individual nutrients on daily rhythms in the mammary gland. Interestingly, the response of the daily rhythms of milk synthesis differed between infusions of fat and protein, suggesting that these nutrients differentially affect the clock. Future research looking at the direct effect of these nutrients on the mammary circadian clock should be performed.

In Chapters 5 and 6, mixtures of fatty acids or amino acids were used. Presumably, individual fatty acids and/or amino acids may regulate the mammary clock differently. The choice to use nutrient mixtures was made partially because no previous characterizations of the role of nutrients on the mammary clock had been made. Providing a mixture of nutrients allowed for detection of a general effect of each nutrient on the daily rhythms. Furthermore, performing in vivo experiments with cows is expensive and time-consuming, and obtaining quantities of pure fatty acids or amino acids large enough to perform these experiments would be very difficult and costly. Therefore, experiments using readily available sources of fat and protein were used.
Because we have demonstrated that these nutrients do affect daily rhythms, future research examining specific fatty acids and amino acids would be compelling. The most cost-effective approach would be to test individual nutrients in a cell culture system first, before developing an in vivo experiment.

Altering the time of fatty acid and protein infusion also influenced the daily rhythms of plasma glucose, nonesterified fatty acid and urea nitrogen concentrations. These changes may have been due to entrainment of peripheral tissues besides the mammary gland. Future research should consider rhythms in other tissues. The liver of dairy cows is an incredible metabolic organ that is responsible for producing nearly all of the glucose used by the animal via gluconeogenesis. Additionally, adipose tissue and muscle are important metabolic organs for dairy cows. Currently the molecular clocks in these tissues are poorly characterized but developing a better understanding of them and their relationship to metabolism could reveal opportunities to improve dairy cow efficiency. Another unexplored aspect of circadian biology in dairy cows is circadian rhythms of the rumen microbiome. As ruminants, dairy cows are dependent on microbial fermentation to meet their nutritional requirements. Recent research has shown that microbes in the hindgut of laboratory models display daily rhythms that are related to the circadian rhythms of the host (Heath-Heckman, 2016). However, this has been unexplored in ruminants. Understanding the daily rhythms of microbial metabolism and their relationship to the host could uncover promising nutritional strategies to improve dairy cow efficiency.

In Chapter 6, milk synthesis failed to exhibit a daily rhythm in the control treatment with continuous infusion of sodium caseinate. This may suggest that sodium caseinate for 24 h/d abolishes the rhythm of milk synthesis, but this cannot be confirmed without a negative control where no sodium caseinate was infusion. Unfortunately in both Chapters 5 and 6, the availability of cannulated cows was limited so no negative controls were included.
Importantly, the daily rhythms of milk synthesis observed in these experiments do not represent true circadian rhythms. Detection of a true circadian rhythm requires that subjects be placed in constant lighting conditions. Maintaining lactating cows in a constant lighting environment is technically challenging because they would need to be housed in an environment with no access to outside light with the necessary equipment to milk cows in place. At the Pennsylvania State University, all cows must be moved through an alleyway exposed to natural light on their way to the milking parlor. Development of a facility where cows could be placed under constant lighting conditions would open up interesting avenues of research. Not only could the presence or absences of a true circadian rhythm of milk synthesis be determined, research into the relationships between light entrainment and food entrainment could be performed.

The characterizations of the rhythms of milk synthesis in Chapters 3, 5, and 6 were limited by the frequency of milking. Practically, at the Penn State facility it was only possible to milk cows 4x/d without drastically altering behavior. A facility where cows are milked in-place would allow more frequent milking and better characterization of the rhythm.

Chapters 7 and 8 of this dissertation characterize annual rhythms of milk and milk component production. In the Chapter 7, results demonstrated that annual rhythms are highly conserved among individual cows, but vary by geographic region. To our knowledge, this experiment was the first to describe yearly changes of milk production as an annual rhythm. Using a much more robust dataset, we discovered that the annual rhythms differed between the northern and southern U.S, with the amplitude of milk yield being greater in the south and the amplitude of milk fat and protein concentration being greater in the north. Moreover, milk yield was found to be highly related to change in daylength, while milk component concentrations were highly correlated with absolute daylength, suggesting that two separate circannual oscillators may regulate these responses. The observations made in these experiments represent a dogmatic shift in the way that the dairy industry views seasonal changes in milk production. Previously, the
changes were thought to be strictly a consequence of environmental conditions, such as heat stress. Our results clearly suggest that there is an additional effect of an annual rhythm. So far in the dairy industry, this work has received significant attention. We hope that it will allow dairy farmers and consultants to better manage cows across the year.

While characterizing the annual rhythms of milk synthesis is a strong initial step, mechanistic work really must be performed in order to both better understand these rhythms and develop approaches to modify them. Across all species, there is limited research examining the mechanisms governing annual rhythms due to the long time-course required to conduct experiments. In dairy cows, this is complicated even further because of the 9 month lactation period, which would conflict with the annual rhythm. In other model organisms, ‘accelerated year’ experiments with controlled lighting have been conducted that allow an entire annual cycle to be compressed into about 3 months. This approach could be used to better understand the annual rhythms governing milk production in dairy cows. Additionally, if the mechanisms causing these rhythms become more well-understood, lighting strategies could be developed to modify them and potentially improve milk production.
REFERENCES


Auldist, M.J., B.J. Walsh, and N. a Thomson. 1998. Seasonal and lactational influences on bovine


Castro, C.U. 2016. Hypothalamic concentration of kisspeptin and GnRH during breeding season (BS) and non breeding season (NBS) in sheep. Colorado State University.


623.


Ivleva, N.B., T. Gao, A.C. LiWang, and S.S. Golden. 2006. Quinone sensing by the circadian


Ma, P. 2011. Searching for a circadian clock in Rhodopseudomonas palustris Strain TIE-1 by oxygen entrainment. Vanderbilt University.,


Salfer, I.J., C.D. Dechow, and K.J. Harvatine. 2019. Annual rhythms of milk and milk fat and


Stevenson, T.J., and G.A. Lincoln. 2017. Epigenetic mechanisms regulating circannual rhythms. V. Kumar, ed. Springer India, Delhi, India, India.


VITA

Isaac James Salfer was born on February 6, 1991 in Maplewood, Minnesota. He began showing dairy heifers at the age of 11 and working on commercial dairy farms during high school, which ignited his passion for the dairy industry. Isaac enrolled in the University of Minnesota in the fall of 2009. As an undergraduate he was highly active within the campus community, including being a member of the university dairy judging team, the dairy challenge team, serving as the treasurer of Gopher Dairy Club, and being the president of FarmHouse Fraternity. He completed his bachelor’s degree in Animal Science in spring of 2013 and stayed at the University of Minnesota to start master’s degree program in the laboratory of Dr. Marshall Stern. His master’s research used dual-flow continuous culture fermenters to study potential modifiers of rumen fermentation and the rumen microbiome. Isaac finished his master’s degree in Animal Science in the summer of 2015, and immediately began a Ph.D. at The Pennsylvania State University. At Penn State, his Ph.D. dissertation focused on the nutritional and environmental factors influencing daily and annual rhythms of milk synthesis in dairy cows. After graduation, Isaac will remain in academia as an assistant professor of dairy science at South Dakota State University.