Night-restricted feeding of dairy cows modifies daily rhythms of feed intake, milk synthesis and plasma metabolites compared to day-restricted feeding

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Abstract

The timing of feed intake can alter circadian rhythms of peripheral tissues. Milk synthesis displays a daily rhythm across several species, but the effect of feeding time on these rhythms is poorly characterised. The objective of this experiment was to determine if the time of feed intake modifies the daily patterns of milk synthesis, plasma metabolites, and body temperature in dairy cows. Sixteen lactating Holstein dairy 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; feed available from 0700 to 2300 h) and night-restricted feeding (NRF; feed available from 1900 to 1100 h. 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. 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.
Introduction

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 cycles of protein phosphorylation. 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\(^\text{(1)}\). However, food intake can entrain circadian rhythms of peripheral tissues such as liver and adipose tissue, independent of the SCN\(^\text{(2)}\). Desynchronisation of SCN and peripheral rhythms can occur when food is restricted to times outside the animal’s normal active period, and has been heavily implicated in the genesis of metabolic disorders such as obesity and insulin resistance\(^\text{(3)}\).

The circadian clock of the mammary gland is responsible for generating daily rhythms of milk synthesis in lactating mammals. In cattle, humans, and rodents, peak milk yield occurs in the morning, while milk fat and protein concentration peak in the evening\(^\text{(4)}\). 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. Furthermore, molecular evidence suggests that as much as 7% of the of human mammary epithelial cell transcriptome oscillates in a circadian manner\(^\text{(5)}\). The molecular clock also appears to be important for the transition from gestation to lactation in dairy cows, with dramatic changes in core clock gene expression between pre- and post-parturition mammary tissue\(^\text{(2)}\).

While there is evidence supporting a role of the mammary clock in lactation, there is little evidence describing food entrainment of these rhythms. Rottman et al. observed that the amplitudes of milk fat and protein concentration are reduced by feeding cows 4x/d compared to 1x/d\(^\text{(6)}\). However, the response of the mammary gland to altering the timing of nutrient intake has not been examined. Dairy cows (Bos taurus) are a robust model to measure changes in daily rhythms of milk synthesis because of their high milk yield and adaptation to frequent milk collection. Furthermore, dairy cows exhibit daily patterns of intake that are affected by the time of feed delivery, suggesting that they may be responsive to food entrainment\(^\text{(7)}\). The objectives of this study were to determine the effect of the timing of feed intake on daily rhythms of milk synthesis, the daily rhythms of plasma hormones and metabolites and body temperature, and the
daily patterns of eating and lying behavior. 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. We also expect that cows will modify feeding and lying behavior in response to the time of feed availability. Specifically, we expect cows with feed restricted during the night will have increased feeding time and decreased lying time overnight along with an increased bout of activity prior to feed delivery signifying food anticipatory activity. Developing a better understanding the relationship between the timing of feeding and the daily rhythm of the mammary gland may uncover opportunities to improve the efficiency of milk production by matching feed intake to the circadian clock of the nutrient metabolism in the mammary gland.

**Materials and 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. Sample size was based on >80% power of observing a $P < 0.05$ difference in milk yield by time interactions based on the variance observed in previous experiments\(^{(6,8)}\). 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 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, which was confirmed with the presence of a light-sensing data logger (HOBO Pendant Temp/Light; Onset Computer Corp., Bourne, MA). Treatments included day-restricted feeding (DRF) with feed available for 16 h/d from 0700 to 2300 and night-restricted feeding (NRF) with feed available for 16 h/d from 1900 to 1100 (Figure 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
analysed for DM, crude protein, neutral detergent fibre, acid detergent fibre, and ash concentrations according to Rico and Harvatine (Supplemental Table S1\(^9\)). The experiment was conducted from February to March of 2016.

**Eating Behaviour 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.\(^{10}\). 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 characterise feeding behaviour, 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.\(^{11}\). 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 characterise 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\(^{10}\).

**Activity Measurement and Analysis**

The daily pattern of standing and lying was determined on d 13 to 16 of each period using an accelerometer (HOBO Pendant G; Onset Computer Corporation) similar to previously described by Ledgerwood et al.\(^{12}\). Briefly, accelerometers were wrapped in gauze, placed on the outside of the left leg with the x-axis perpendicular to the ground, and secured with a cohesive bandage (Co-Flex; Andover Healthcare, Salisbury, MA). The x, y, and z axes were recorded at 1 min intervals and lying behaviour was determined using an algorithm developed in IGOR Pro 8 (WaveMetrics Inc., Portland, OR).

**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.\textsuperscript{(13)}.

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 analysed for concentrations of individual fatty acids (FA) according to Baldin et al.\textsuperscript{(14)}.

**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.\textsuperscript{(6)}.

Briefly, plasma glucose concentration was analysed using a glucose oxidase/peroxidase enzymatic colourimetric assay (No. P 7119, Sigma-Aldrich, St. Louis, MO), NEFA concentration was measured using an acyl-CoA oxidase/peroxidase enzymatic colourimetric 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 \textsuperscript{125}I-insulin radioimmunoassay kit with correction based on bovine insulin (PI-12K Porcine Insulin RIA, EMD Millipore, Billerica, MA).

**Body Temperature Recording and Analysis**

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 vaginal implant (progesterone-free CIDR; 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 analyses were performed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Daily dry matter intake, milk production, and FA yields were analysed using the fixed effects of treatment, period, and the interaction of treatment by period and the random effect of cow. A separate model including the fixed effects of treatment, time of day, and the interaction of treatment and time of day, as well as the random effects of cow and the repeated effect of time of day was used to test the interaction of treatment and time of day on milk yield and components, rate of feed intake, standing and lying behaviour, plasma metabolite and hormone concentrations and body temperature. Pairwise comparisons of treatment at each time point were performed for rate of feed intake and standing and lying behavior using preplanned contrasts. The model included 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.

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.\(^8\). 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\(^{15}\).
Results

Eating Behaviour

Total daily DM intake was not affected by the time of feed restriction (Table 1). The greatest amount of feed was consumed in the first 2 h following feed delivery in both treatments (Figure 1B). However, night-restricted feeding caused a greater rate of post-feed delivery intake, with NRF 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 S1). 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.

Activity

Treatment did not affect the average percent of each hour spent lying ($P = 0.50$), but an interaction between treatment by lying time occurred ($P < 0.001$);
However, a treatment by time interaction occurred \((P < 0.001)\). Night-restricted feeding caused cows to spend a greater percentage of time lying between 0700 to 0800 and from 1200 to 1600 \((P < 0.01)\), whereas DRF caused cows to have a greater lying time from 0000 to 0100, 0300 to 0400, and 2000 to 2200 \((P < 0.05)\). Treatment did not affect the number of lying bouts per day \((P = 0.75)\), average lying bout length \((P = 0.75)\), or total lying time per day \((P = 0.24)\);
Rhythm of Milk and Milk Components

Milk yield fit a cosine function with a 24 h period in both DRF \((P < 0.0001)\) and NRF \((P < 0.0001)\; \text{Figure 3A})\). The acrophase of milk yield was delayed 8 h by NRF \((P < 0.05)\), and the amplitude of the rhythm was 32\% greater in DRF than NRF (Figure 3A). Total daily milk yield was not affected by treatment \((P = 0.12; \text{Table 1})\).

Night-restricted feeding tended to reduce total daily milk fat yield \((P = 0.08; \text{Table 1})\). Milk fat yield fit a daily rhythm with an amplitude of 13.5 g and an acrophase at 0704 in NRF \((P < 0.0001)\) but did not fit a rhythm in DRF \((P = 0.13; \text{Figure 3B})\). Milk fat concentration fit a 24 h rhythm in NRF \((P < 0.0001)\) and tended to fit a daily rhythm in DRF \((P = 0.06; \text{Figure 3C})\). The
acrophase of milk fat concentration peaked at 1220 in DRF and was phase shifted 10.4 h earlier in NRF ($P < 0.05$). The amplitude of the milk fat concentration rhythm was increased by 64% by NRF ($P < 0.05$). Average daily milk fat concentration was not affected by treatment ($P = 0.86$).

Milk protein yield fit a daily rhythm in both DRF and NRF ($P < 0.001$; Figure 3D). The rhythm of milk protein yield peaked at 0211 h in DRF and was phase shifted 5.6 h later in NRF ($P < 0.05$). The amplitude of the daily rhythm of protein yield was decreased 108% by night-restricted feeding with an amplitude of 19.6 in DRF and an amplitude of 9.4 in NRF ($P < 0.05$).

Both DRF and NRF also exhibited a daily rhythm of milk protein concentration ($P < 0.02$; Figure 3E). Treatment modified the phase of the rhythm with DRF peaking ~9.3 h earlier than NRF (1541 h vs. 0058 h; $P < 0.05$). The amplitude of the rhythm was increased over three-fold by NRF, which had an amplitude of 0.15% compared to 0.04% in DRF ($P < 0.05$). Average daily milk protein yield and milk protein concentration were not affected by treatment.

**Milk FA Yield and Profile**

Compared to DRF, night-restricted feeding decreased FA originating from *de novo* synthesis in the mammary gland by 5% ($\Sigma < 16C; P = 0.03$) and decreased 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 4A). 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 4D-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 NRF delaying the rhythm 6.1 h compared to DRF (0128 vs. 0736; $P < 0.05$), 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 ($P < 0.05$).

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\(^{(16)}\). The concentrations of t10 and t11 were analysed 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 t10 by 32.8\% (\(P < 0.01\)) and t11 by 16.3\% (\(P = 0.001\)) compared to NRF (Figure 4B). Both t10 and t11 fit daily rhythms in both treatments (\(P < 0.0001\); Figure 4G&H). Milk fat t10 was phase delayed 12.1 h by NRF compared to DRF (1038 vs. 2234; \(P < 0.05\)). The amplitude of t10 was increased by NRF compared to DRF (0.08% vs. 0.06%; \(P < 0.05\)). Like t10, night-restricted feeding caused a complete inversion of the daily rhythms of t11 concentration, shifting the peak from 1816 in DRF to 0604 in NRF (\(P < 0.05\)). The amplitude of t11 was not affected by 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\% (\(P < 0.05\); Figure 5A). 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 (\(P = 0.69\); Figure 5B). Night-restricted feeding shifted the acrophase of insulin production 8.0 h and increased the amplitude 0.9 \(\mu\)IU/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 5C). Both treatments exhibited 24 h rhythms of 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 partially attributed to a dramatic rise in NEFA concentration to 214 \(\mu\)Eq/L at 1900 h, 6 hours into the fasting period.

Plasma urea nitrogen fit a 24 h rhythm in DRF (\(P = 0.001\)) and NRF (\(P < 0.001\); Figure 5D). Altering the timing of time-restricted feeding nearly completely inverted the phase of the
daily rhythm with NRF peaking 11.2 h earlier than NRF ($P < 0.05$). Moreover, the amplitude of the PUN rhythm was 58% greater in NRF than DRF ($P < 0.05$).

**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 6). NRF delayed the phase of the rhythm 15.5 h ($P < 0.05$) and increased the amplitude 75% compared to DRF ($P < 0.05$). Average body temperature was not affected by treatment.

**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. Cows naturally exhibit a crepuscular pattern of intake, with greatest intake occurring at dusk and dawn and lowest intake occurring overnight. Cows fed TMR normally have high intake at feed delivery and the late afternoon and low intake in the overnight period. 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. Moreover, rats display circadian rhythms of meal size and meal frequency under constant illumination. 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).

The change in the daily pattern of feed intake was accompanied by a change in standing and lying behaviour. Cows under night-restricted feeding spent less time lying and more time standing overnight (0000 to 0400), which is consistent with their increased rate of feed intake. Meanwhile, cows under day-restricted feeding spent less time lying during the afternoon (1200 to 1600) when their feed intake was greater. Similar to the current study, Niu et al. previously demonstrated that feeding cows at night without time-restricted feeding decreased lying time.
during the overnight period relative to day-feeding\(^8\). In rodent models, time-restricted feeding entrains circadian rhythms of food anticipatory activity, which is an increased bout of activity prior to scheduled feeding time\(^{20}\). Contrary to our hypothesis, cows did not appear to exhibit food anticipatory activity, with no increased activity in the hours leading up to feed delivery.

Dairy cows in commercial settings are usually fed in the morning, and typically have greatest milk yield in the morning and greatest fat and protein concentration in the evening\(^{6,21}\). Our results corroborated these findings in cows under day-restricted feeding but demonstrated that night-restricted feeding shifts this daily pattern 8 hours later in the day. These results are novel and demonstrate that feeding time has a profound effect on the daily rhythms of milk synthesis. The change in the rhythms of milk and milk component synthesis in response to night-restricted feeding is either due to a change in available substrate for milk synthesis or entrainment of circadian clock of the mammary gland. In 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\(^{22}\). 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 tendency for a decrease in total fat yield in NRF appeared to be more influenced by a reduction in \textit{de novo} fatty acid synthesis compared to preformed fatty acid uptake because NRF decreased \textit{de novo} and mixed fatty acid yields but not preformed FA. Notably, the tendency for decrease in milk FA yields appears to be due to the cumulative effects of reductions in milk yield and milk fat concentration because neither milk yield nor milk fat yield were significantly reduced by NRF. The potential decrease in \textit{de novo} fatty acid synthesis in NRF may be related to an increase in \textit{trans}-10 C18:1, a fatty acid isomer that is highly correlated with reduced \textit{de novo} fatty acid synthesis. Notably, \textit{t}11 C18:1 was also increased in NRF indicating overall rumen biohydrogenation was increased, rather than a shift to the alternate biohydrogenation pathway\(^9\). The decrease in apparent ruminal biohydrogenation in NRF may because they consumed a larger percentage of their feed during the first 2 hours after than DRF. Previous research demonstrated that stabilising feed intake through 4x/d feeding increased milk fat yield and decreased \textit{t}10 C18:1\(^6\). The changes in daily patterns of \textit{de novo}, mixed source, and preformed FA yield closely reflected the daily pattern of milk yield. The daily pattern of \textit{t}10 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.

Similar to previous reports in dairy cattle\(^{(28)}\), rodent models\(^{(29)}\), and humans\(^{(30)}\), 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\(^{(31)}\). 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\(^{(32)}\). The results of the current experiment suggest that fasting during the high-intake afternoon period of the day causes greater lipid mobilisation 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\(^{(33)}\). Insulin in a potent inhibitor of hormone sensitive lipase, the enzyme responsible for causing lipid mobilisation from adipose tissue\(^{(34)}\). Expectedly, peak NEFA concentration in both treatment groups coincided with the nadir of insulin release. Shostak et al. reported that lipolysis from white adipose tissue follows a daily pattern, and showed using *Clock*Δ19 and *Bmal*-/- mice that these patterns were controlled by the molecular circadian clock\(^{(35)}\). The current study provides evidence suggesting a similar mechanism may occur in dairy cows.

The shift in the daily rhythm of 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., who reported a 3 hour phase delay after feeding at 2030 compared to 0830, without restricting the time of feed availability\(^{(8)}\). 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 \(^{(36)}\). Interestingly, body temperature began to decline after feed the time of feed delivery and remained low during the period of the day when rumen temperature would be expected to increase due to increased fermentation. Shifts in the rhythm of body temperature are capable of entraining peripheral circadian clocks in mammals\(^{(37)}\). The phase shift in the body temperature due to altering the time of time-restricted feeding may differentially entrain the daily rhythms of organ systems throughout the animal. Future research should examine the role of temperature entrainment in the dairy cow.

In conclusion, timing of feed intake shifted the 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. These results support the hypothesis that timing of nutrient absorption modified the daily rhythms of milk synthesis, perhaps due to entrainment of
the mammary circadian clock. Additionally, 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. Moreover, milk fat yield, particularly the yield of de novo synthesized
fatty acids were reduced by night-restricted feeding, which we speculate was because the greater
rate of feed intake caused an increase in total ruminal biohydrogenation in NRF. These results
reinforce the importance of not limiting the access of dairy cows to feed during the high-intake
afternoon period of the day.

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Conflict of Interest
The authors declare no conflicts of interest.

Author Contribution
K.H. and I.S. formulated the research question. I.S. conducted the study and completed
all sample analyses. K.H. supervised the research project. I.S. wrote the paper. K.H revised the
paper. All authors read and approved the final version of the manuscript.
 References


**Tables**

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

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>DRF</th>
<th>NRF</th>
<th>SEM</th>
<th>P-value</th>
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<td>Milk yield, kg</td>
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</tr>
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</table>

1Treatments 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.
Figure 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 2. Effect of day-versus night-restricted feeding on lying 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]. Panels show (A) the effect of day vs. night-restricted feeding on lying bouts (#/d), lying bout length (min/bout), and daily lying time (min/d); and (B) the daily pattern of lying time (% of h spent lying). Data are presented as LSM. Preplanned contrasts of the effect of treatment at each time point are shown (†0.05 < P < 0.10, *0.01 < P < 0.05, **P < 0.01). Milking times are shown below panel B.
Figure 3. 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 4. 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 5. 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 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 6. 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>
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<th>Trt</th>
<th>Mean</th>
<th>Amp(^1)</th>
<th>Acro(^2)</th>
<th>P-value(^3)</th>
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<td>0.04(^b)</td>
<td>0154(^a)</td>
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<tr>
<td>NRF</td>
<td>38.7</td>
<td>0.07(^a)</td>
<td>1724(^b)</td>
<td>&lt; 0.0001</td>
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</tbody>
</table>

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