Advances in Dairy Research
ISSN: 2329-888X

Casey, et al., J Adv Dairy Res 2014, 2:2
DOI: 10.4172/2329-888X.1000119

Research Article Open Access

Continuously Changing Light-Dark Phase Decreases Milk Yield, Fat, Protein and Lactose in Dairy Cows

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Rec date: Apr 28, 2014, **Acc date:** July 18, 2014, **Pub date:** July 20, 2014

Abstract

Photoperiod impacts feeding behavior, health, growth and milk production of dairy cattle. In humans behaviors that are asynchronous with the natural light-dark cycle, such as shift work or jet lag, are strongly associated with metabolic disorders as well as impaired reproductive performance. The objective of this study was to determine the effects of exposing mid-lactation dairy cows to chronically shifting 8h light (L)-8h dark (D) cycles on feed intake, milk yield, milk composition and mammary gene expression. Six first lactation Holsteins ~90d in milk were maintained on a 16 h L: 8 h D cycle and milked at 5AM and 4PM for 7d (control, Period 1). During Period 2, 7 d immediately following Period 1, cows were exposed to continuous cycles of 8 h L: 8 h D, but maintained on the same milking and feeding schedule. Exposure to chronic 8 h LD cycles significantly depressed milk yield ($P<0.05$), but did not affect daily feed intake. Percent milk fat, protein and lactose were not different, but milk urea nitrogen (MUN) significantly increased. On the last day of each period mammary gene expression was measured by Q-PCR of total RNA isolated from the cytosolic components of milk fat globules. Expression of the core clock gene *BMAL1* in addition to *Beta-casein*, *Alpha-lactalbumin*, *Fatty acid synthase* and *Acetyl CoA-carboxylase* were all decreased ($P < 0.05$) after 7 d of chronic 8 h LD cycles. The results from this study show for the first time that exposing lactating cows to chronic light shifts decreases milk production and may alter metabolism. Further, experimental design may provide a paradigm to study the effects of changing lighting schedules on milk production and a potential model to study effects of disrupting circadian system on production efficiency.

Keywords: Lactation; Mammary; Dairy management; Lighting

Introduction

Environmental factors, including thermal stress and photoperiod, impact the feeding behavior [1-3], health [4], growth [5] and milk production of dairy cattle [6, 7]. To more effectively control environmental factors and improve production efficiency, housing domestic food-producing animals in close confinement has become the norm. Extension bulletins promote housing lactating cattle under long day photoperiod (16h light 8h of dark) conditions, as multiple studies have shown it enhances milk production [8]. However, many intensive dairy operations do not follow lighting recommendations, often providing varying light levels with exposure to light throughout the night [9].

In humans, daily behaviors that are asynchronous with the natural light-dark cycle, such as shift work or jet lag, have been strongly associated with acute and chronic metabolic disorders as well as impaired reproductive performance [10-12]. Exposure of rodents to models of shift work or jet lag also results in the development of metabolic disorders and negatively impacts reproduction. Further studies with rodents have demonstrated that exposure to acute or chronic disruptions in light-dark cycles result in desynchronization of the circadian system [13-17]. Circadian rhythms are roughly 24h cycles of physiology (e.g. plasma hormone levels and core body temperature) or behavior (e.g. sleep-wake cycle) that appear to have evolved as a common strategy among animals to coordinate internal systems and synchronize these systems to the environment [18,19]. In mammals, the circadian system is comprised of the master circadian clock in the suprachiasmatic nuclei (SCN) of the hypothalamus and peripheral clocks that are distributed in every organ of the body. The intrinsic rhythmicity of the SCN is entrained by synchronization to the 24h day by regularly occurring environmental cues [20]. The light-dark (LD) cycle is the most important environmental cue for entraining the SCN [21]. Other cues include exercise, food availability, temperature and stress.

Virtually all aspects of mammalian physiology are controlled by the circadian system including homeostasis, thus disruption of clocks by abruptly altering light-dark cycles and feeding schedules or exposure to stress has the potential to negatively impact every system of the body. Although early studies showed that exposing lactating cows to continuous light had no effect on milk production or eating behavior [22], the effect of frequent changes in the light-dark cycle during lactation has not been investigated until now. The objective of this study was to determine the effects of exposing mid-lactation dairy cows to chronically shifting 8h light-8h dark cycles on feed intake, milk yield, milk composition and mammary gene expression.

Materials and Methods

Animal management and experimental design

All animal care, use, and handling protocols were approved by the Purdue Animal Care and Use Committee (PACUC) prior to the start of the study. The study began in Spring 2012 when the natural light cycle was 13h light (L) and 11h dark (D). Six first- lactation Holsteins approximately 90 ± 7.3 days in milk were moved to a tie- stall research barn that was devoid of natural light. The temperature range in the

facility was 12-15°C with mean 14.6°C ± 0.9, and no greater than a 1°C difference between sampling points (5AM, 1PM, 9PM) within a day.

The experiment was comprised of two contiguous 7-d periods; period 1 comprised a LD cycle of 16h:8h, which was similar to farm's artificial light schedule. Period 2 cows were exposed to a continuously alternating 8h LD cycle (Figure 1). During period 1, cows were milked at 0500 and 1600. Lights were turned on just before the 0500 milking and were turned off at 2100. During period 2, the second 7d of the experiment, which immediately followed period 1, cows were exposed to alternating cycles of 8h L and 8h D, and maintained on the same milking and feeding schedule used in period 1. For milking scheduled during a dark cycle in period 2, the lights were turned on to move the cows to the parlor where they were milked and then returned to the tie- stalls at which point the lights were turned off. The use of alternating 8h LD cycles was intended to disrupt the "normal" cycle of light and dark within a 24h period, similar to models of jet lag and rotating shift work developed for rodents, consequently the introduction of light to facilitate milking during a dark phase can be considered part of this disruption.

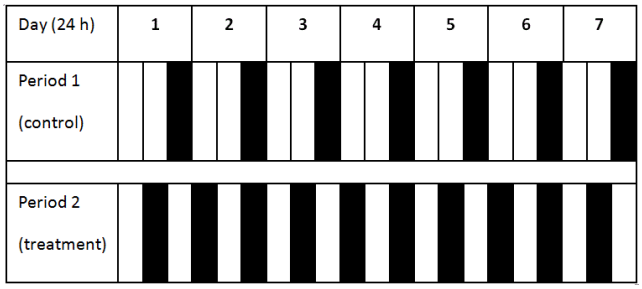

Figure 1: Experimental light (L) and dark (D) schedules for Period 1 (16 h L: 8 h D) and Period 2 (8h L: 8 h D) of the study. During Period 1, lights were turned on at 0500 and off at 2100 each day. On the first day of Period 2, the lights were turned on at 0500 and then turned off at 1300. This 8h L: 8h D cycle was maintained for the duration of the treatment period. Each of the three boxes within a day represents 8h; open boxes represent light and black boxes represent dark phase.

Cows were fed a total mixed ration to meet NRC recommendations [23], based on milk production and stage of lactation; water was always available when cows were in their tie stalls. A sample of the diet was analyzed for nutrient composition by Dairy One (Ithaca, NY; Table 1). Cows were fed for ad libitum intake after both the 0500 and 1600 milking. Daily feed intake was determined by difference between feed offered and feed refusals.

Milk collection and analysis

Individual milk yields were recorded electronically at each milking. Milk samples were analyzed for percent fat, lactose, and protein by near infrared reflectance and for milk urea nitrogen (MUN) by the Bentley Chemspec method at the DHIA Laboratory (Ithaca, NY).

Mammary gene expression analysis of total RNA isolated from milk fat globule

A homogenous milk sample was collected during the 1600 milking on the last day of each treatment period. Total RNA from the cytosolic component of milk fat globule was isolated as described, [24], with the following modifications: A 20 ml aliquot of milk was divided equally among four 15 ml snap top polypropylene tubes wrapped with aluminum foil and swirled in a 70°C water bath for 15 s. Heat-treated milk was combined into a single 50 ml conical tube and centrifuged at 3,000 rpm for 10 min at 4°C. The fat layer was transferred to a 15 ml conical tube using a sterile disposable spatula, and 2 ml of Qiazol (Qiagen Inc, Valencia, CA) was added to the fat and thoroughly mixed by pipetting. Samples were stored at -80°C until completion of RNA isolation, following manufacturer's protocol. QIAGEN's RNeasy Mini kit (Qiagen Inc, Valencia, CA) and Rnase-free DNase kits were used to purify RNA and degrade DNA in samples, respectively. Quantity of total RNA was assessed with the Nano drop® ND-1000 UV-Vis Spectrophotometer (Nano drop Technologies, Wilmington, DE) and quality on the Nano chip using the Bio analyzer 2100 (Agilent Inc., Palo Alto, CA).

Diet composition and chemical analysis	% Dry matter
Corn silage	34.40%
Haylage	16.10%
Orchard grass hay	3.60%
Wheat straw	0.90%
Chemical composition	
Crude protein (CP)	16.20%
Acid detergent fiber (ADF)	23%
Neutral detergent fiber (NDF)	33.40%
Net energy of lactation (NE_L)	1.48 Mcal/kg
Calcium	0.90%
Phosphorous	0.41%
Magnesium	0.32%
Potassium	1.68%
Sodium	0.43%

Table 1: Diet composition and chemical analysis

Quantitative polymerase chain reaction (Q-PCR) gene expression analysis

Total RNA of the same cow from both period 1 and 2 were available for four cows and used for Q-PCR analysis. RNA was reverse transcribed into cDNA using the QuantiTect Whole Transcriptome kit (Qiagen Inc., Valencia,CA). Q-PCR analysis was performed using the StepOnePlus™ Real-Time PCR System (Life Technologies Corporation Carlsbad, CA) and a unique TaqMan® Gene Expression Assay (Life Technologies Corporation) specific for bovine: aryl hydrocarbon receptor nuclear translocator-like (*ARNTL* aka *BMAL1*, cat. no. Bt04302500_m1), period homolog 2 (*PER2*, cat. no. Bt04311406_m1), acetyl Co-A Carboxylase (*ACACA*, cat. no. Bt03213366_m1), fatty acid synthase (*FASN*, cat. no. Bt 03210490_g1), sterol regulatory element binding transcription factor 1 (*SREBF1*, cat. no. Bt03276371_m1), sterol regulatory element binding transcription factor 2 (*SREBF2*, cat. no. Bt 04283469_m1) beta-casein (*CSN2*, cat.

no. Bt03217428_m1), alpha lactalbumin (*LALBA*, cat. no. Bt 03213963_m1); Ribosomal protein S18 (*RPS18*, cat. no. Bt03225196_g1) was used as a reference. Relative gene expression (RQ) was calculated according to the following equations: ΔCT (individual animal) = CT (target gene) – CT (reference gene); ΔΔCT (individual animal) = ΔCT (individual animal period 2) – ΔCT (individual animal period 1); relative expression (RQ) = $2^{-\Delta\Delta CT}$.

Statistical analysis

Based on previous human and rodent jet-lag studies, the statistical design a *priori*, was 5 days of housing acclimation and 2 days of sampling. Mean and standard error of the mean (SEM) of both AM and PM milk yield plus total daily feed intake were calculated for each period. To evaluate the effect of treatment on feed intake, milk yield, and milk composition, the mean of these variables during the last 48h in each period were calculated for each cow and the differences between periods were analyzed with a paired t-test (n=6 cows) using statistical analysis software (Minitab 16, State College, PA). Similarly, differences in relative mammary gene expression between period 1 and period 2 were analyzed using a paired t-test (n=4 cows). Means were different if $P < 0.05$, and tended to differ if $0.05 \leq P \leq 0.15$. Values reported are means and associated standard error of mean.

Bioinformatics analysis of E-box in promoter sequence

Promoter regions of *FASN*, *ACACA*, *CSN2* and *LALBA* were analyzed for presence of canonical, CACGTG, and non-canonical: CANNTG (i.e. CAAGTG, CAAATG, CAATTG, CAACTG, CACATG, CACTTG, CACCTG, CAGGTG, CAGATG, CAGCTG, CAGTTG, CATTTG, CATATG, CATCTG, and CATGTG) E-Box sequences. The 2,000 base nucleotide sequences upstream from transcription start sites were obtained from the 2011 assembly of the cow genome (Baylor Btau_4.6.1/bostau7) available through the genome browser tool on the UCSC Genome Bioinformatics page (http://genome.ucsc.edu/).

Results and Discussion

Exposure to continuous changes in light-dark cycle caused a significant decrease in morning (0500) and total daily milk yield relative to control period (Figure 2). Percent milk fat, protein and lactose were not significantly ($P > 0.05$) impacted by exposure to chronic light-dark shifts (Figure 3A). Analysis of milk components by weight produced at each milking revealed that exposure to chronic light shifts decreased yield of total protein, fat and lactose in morning (0500) milking, but not afternoon (1600) milking (Figure 3B). Decreased yield of milk components resulted in a significant reduction in total daily lactose produced (period 1=1.54 ± .07 kg, period 2=1.44 ± .06 kg; $P < 0.05$), and tended to reduce amount of total milk protein produced (period 1=0.92 ± .05 kg, period 2=0.87 ± .04 kg; $P = 0.15$). However, daily yield of milk fat was not different between treatment periods.

Comparison of change in milk yield during the experiment to control cows that remained in herd (matched by lactation and days in milk with experimental animals) showed no change in milk yield between the study time course that occurred between control and light-shift periods (data not shown). Further, there was no difference in average daily milk yields in the week prior to beginning study (31.5 ± 1.6 kg/day) and control period (31.6 ± 1.5 kg/day) milk production levels within experimental animals.

Milk urea nitrogen (MUN) was significantly increased ($P < 0.001$; Figure 4) in cows exposed to chronic light-dark shifts. MUN closely correlates to the concentration of urea found in the blood (BUN) [25] and is an indicator of protein status. Because MUN mirrors BUN concentrations [26], changes in MUN concentrations reflect differences in efficiency of ammonia capture by rumen microbes as well as tissue metabolism of absorbed amino acids. The increase in MUN for cows exposed to chronic 8h LD shifts therefore may reflect a decrease in rumen N capture, a decrease in efficiency of absorbed amino acids, or a combination of both processes. The lack of change in feed intake between control (period 1) and the chronically shifted LD (period 2) periods does not support a change in rumen N capture, and therefore the observed changes in MUN are likely due to a decrease in use of absorbed amino acids. These data are consistent with an increase in protein accretion observed in heifers exposed to long-day compared with short-day length [27]. Disruptions in management practices that impinge on light-dark cycles therefore appear to impact N metabolism in dairy cows. It is interesting to speculate that management inconsistencies in lighting may be partly responsible for the elevated MUN observed in some dairy herds and the associated reductions in profitability [28].

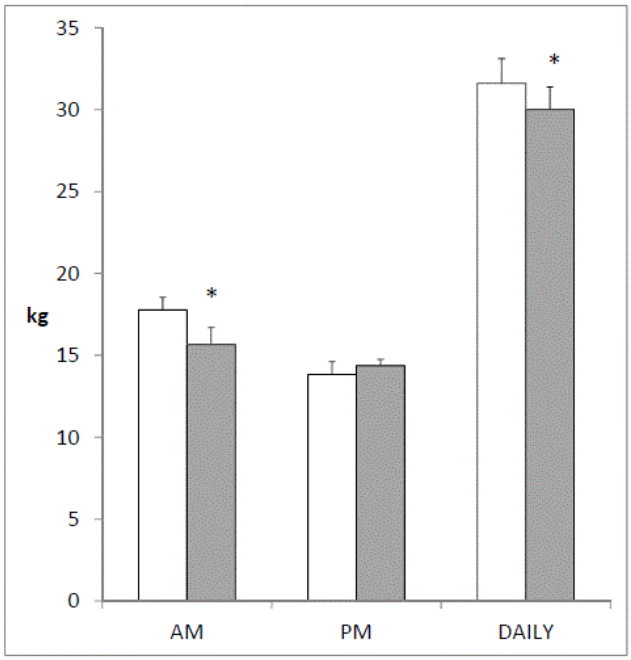

Figure 2: Mean AM (0500), PM (1600) and daily milk yield during last 48 hr of period 1 (white) and period 2 (gray). Values are mean ± SEM; differences were analyzed used paired t-test,*indicates difference of $P < 0.05$

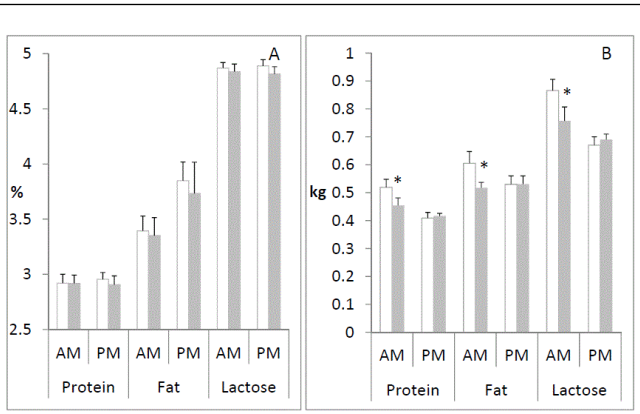

Figure 3: Composition of milk collected at AM (0500) and PM (1600) milking during the last 48h in period 1 (white) and period 2 (gray) expressed as A) mean percent and B) mean kg produced of total protein, fat and lactose. Values are mean ± SEM; differences were analyzed used paired t-test,*indicates difference of P<0.05

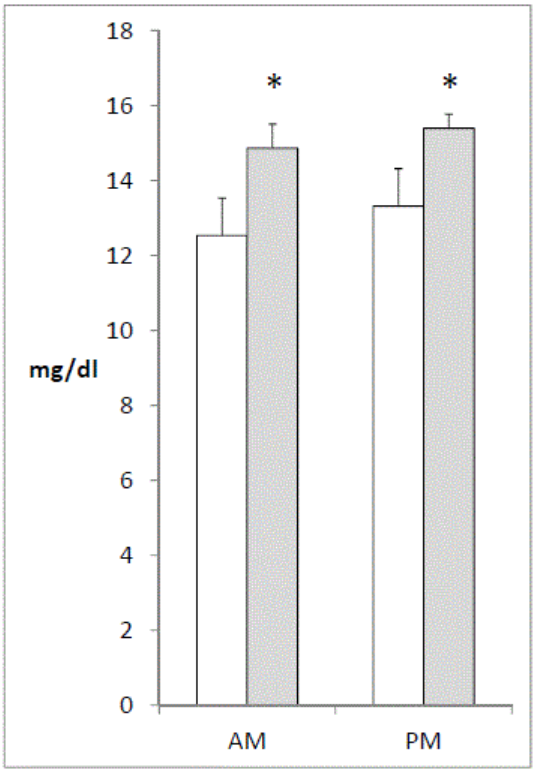

Figure 4: Mean concentration of milk urea nitrogen of AM (0500) and PM (1600) milk samples were calculated for last 48h of Period 1 (white) and Period 2 (gray) and analyzed for differences using paired t-test. Values are mean ± SEM; *different between periods at P=0.001

Chronic light-dark shifts did not significantly affect daily feed intake (Period 1=37.8 ± 1.5 kg/day; Period 2 38.4 ± 1.5 kg/day). These data suggest treatment impacted feed efficiency (i.e. milk produced per pound of dry matter intake).

Studies in humans and rodents have demonstrated that disruption in natural light-dark cycles impact the circadian system at the molecular level. To determine if exposure to chronic light-dark shifts impacted the mammary circadian clock, a homogenous milk sample was collected at the afternoon milking on the last day of each treatment period and total RNA was isolated from cytosolic components of milk fat globules which was then used to measure expression of the core clock genes *BMAL1* and *PER2*. *BMAL1* expression following 7 days of chronic 8h LD shifts was 38% of the control period (Figure 5). This finding, although above the significance level (P=0.06), was based only on 4 cows, which is justified as number was limited by matched good quality RNA. Thus data suggest that the treatment impacted circadian clock in the mammary gland. Since samples were taken at one time point during each treatment period, it is not known whether phase, period or amplitude of circadian rhythms was impacted. No difference was detected in expression of *PER2* between the treatment periods.

Following exposure to 7 d of chronic 8h LD shifts, expression of mRNA from genes whose products regulate fatty acid synthesis in the mammary gland, *FASN* and *ACACA*, were reduced relative to the control period (Figure 5). Sterol regulatory element binding protein 1 and 2 (*SREBF1* and *SREBF2*) are transcription factors that regulate mammary fatty acid synthesis in part through their regulation of *ACACA* and *FASN* gene expression [29], and function as key integrators of circadian and nutritional cues in the liver [30]. Chronic light shifts had no effect on *SREBF1* expression, but significantly reduced *SREBF2* (Figure 5). Expression of the milk proteins, *CSN2* and *LALBA*, were also significantly reduced (Figure 5). These changes in gene expression occurred without any significant changes in milk composition, but support the lower production of these components (total milk protein, fat and lactose) and decreased milk yield that occurred with short-term exposure to light-dark cycle shifts.

Our previous studies with rodents revealed that the expression of the molecular clock gene *Bmal1* increased in the mammary gland during the transition from pregnancy to lactation [31]. BMAL1 heterodimerizes with NPAS2 or CLOCK to form a transcription factor, and is a member of the bHLH transcription factor family. Many genes important to organ function are direct or indirect transcriptional targets of BMAL1: NPAS2/CLOCK heterodimers [32-34]. We hypothesize BMAL1:CLOCK component of the mammary clock regulates metabolic output of gland during lactation. Thus, the decrease in *BMAL1* expression following 7d of chronic 8h L:D shifts may be partly responsible for the reduced expression of *CSN2*, *LALBA*, *FASN* and *ACACA*. This hypothesis is supported by bioinformatics analysis of promoter regions of these genes that show presence of multiple canonical and non-canonical E-boxes, i.e. the cis-acting DNA regulatory binding sequence of bHLH transcription factors (Table 2). The relatively high number of E-Box sequences in

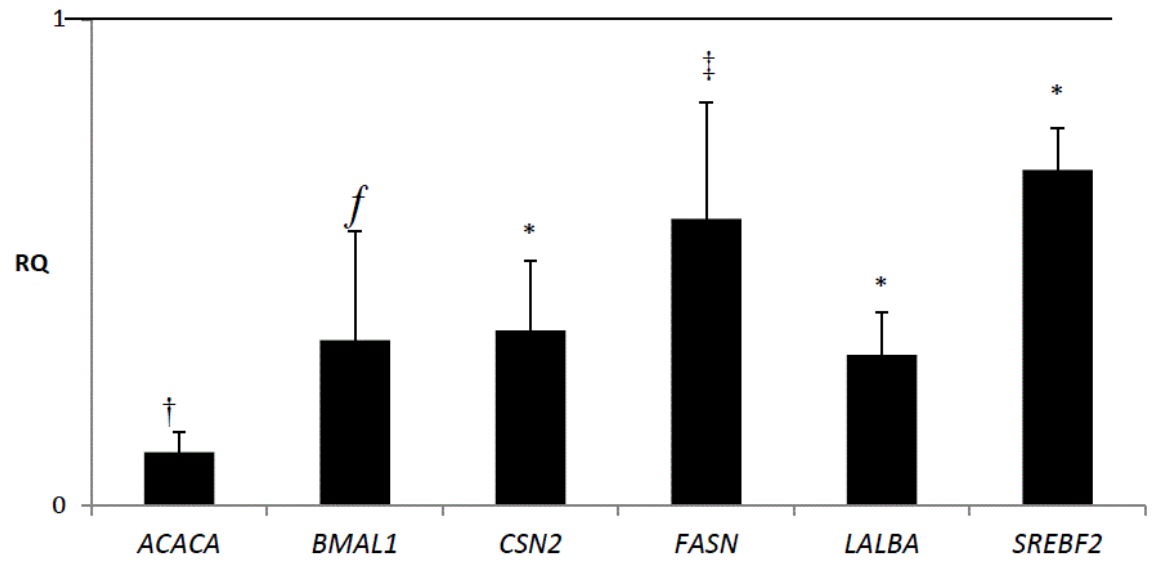

Figure 5: Mean mammary gene expression difference between Period 2 and Period 1 (Period 1 RQ=1). Gene expression was measured using Q-PCR analysis of total RNA isolated from the milk fat globules of samples collected at PM milking on last day of each treatment period. RQ=2- ΔΔCT with 18S as reference gene and mean ΔCT of Period 1 for each gene as normalizer for calculation of ΔΔCT. Values are mean RQ in Period 2±SEM; difference were analyzed using paired t-test with ‡P=0.1; *f*P=0.06; *P ≤ 0.05; †P ≤ 0.001

the promoter regions of these genes is indicative of the importance of integration of cues, including photoperiod and nutrient availability, in regulation of expression of genes involved in mammary metabolic output during lactation.

Previous studies on the impact of photoperiod on milk production reported measurable differences due to exposure to long day versus short day photoperiods after 4-6 weeks of acclimation [8]. Acclimation to thermal stress and photoperiod are homeorhetic processes [35]. Homeorhesis is long-term regulation that expresses the genetic potential of the animal within a given environment [36]. Here we report that the effects of continuously changing photoperiod (a model of chronic jet-lag) on milk production were measurable in a relatively short period of time (days rather than weeks). There is mounting evidence that circadian clocks are important in maintaining homeostasis [37,38]. Studies in humans and rodents showed abrupt

Nucleotide sequence	FASN	CSN2	LALBA	ACACA
CACGTG	-1807		-1078	
	-1047			
	-874			
	-336			
CAAGTG				-107
CAAATG			-281	
CAATTG		-1833		-156
CACATG				-918
CACTTG	-1685		-461	
			-328	
CAGGTG			-873	-735
CAGATG	-791		-1438	-1583
				-1376
CAGCTG			-857	
CAGTTG	-1229			
CATTTG	-966	-977		
CATATG		-1974	-422	-327
		-1907		
		-1522		
		-1436		
CATCTG	-1261		-976	-1463
CATGTG	-67	-658		

Table 2: Upstream location of canonical (CACGTG) and non-canonical (CANNTG)*E-box nucleotide sequences relative to FASN, CSN2, LALBA and ACACA transcription start sites

change in light:dark schedule has immediate effects on physiology and it takes approximately 3-5 days to reset central and peripheral rhythms to the new light-dark schedule. However, if light-dark cycle changes occur in succession, these signals continuously reset clocks, resulting in disrupted internal circadian rhythms (e.g., sleep and hormonal patterns) and impacts the animal's ability to maintain homeostasis. Although the experimental design does not reflect lighting schedules on modern dairy farms, it does reveal that abrupt changes in photoperiod impact milk production and may disrupt metabolism in dairy cows in the short term. Thus this study design provides a paradigm to study the effects of changing lighting schedules on animal production and a potential model for scientist to study effects of knocking out a functional circadian system on production efficiency.

Acknowledgements

We would like to thank Mike Grott and Heather Tucker for their technical assistance during this study.

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