Clock circadian regulator (CLOCK) gene network expression patterns in bovine adipose, liver, and mammary gland at 3 time points during the transition from pregnancy into lactation

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ABSTRACT

The transition from late gestation to early lactation is the most critical phase of the lactation cycle for mammals. Research in rodents has revealed changes in the clock circadian regulator (CLOCK) gene network expression around parturition. However, their expression profiles and putative functions during the periparturient period in ruminants remain to be determined. The present study aimed to investigate the expression pattern of the CLOCK network and selected metabolic genes simultaneously in mammary gland (MG), liver (LIV), and subcutaneous adipose tissue (AT). Seven dairy cows were biopsied at $-10 (\pm 2)$, 7, and 21 d relative to parturition. A day $\times$ tissue interaction was observed for *ARNTL*, *CRY1*, and *PER2* due to upregulation at 7 and 21 d postpartum, with their expression being greater in AT and MG compared with LIV. No interaction was detected for *CLOCK*, *CRY2*, *PER1*, and *PER3*. In general, the expression of *NPAS2*, *NR1D1*, *NR2F2*, *ALAS1*, *FECH*, *FBXW11*, *CCRN4L*, *PPARA*, *PPARGC1A*, and *FGF21* was lower at -10 d but increased postpartum in all tissues. The interaction detected for *CSNK1D* was associated with increased expression postpartum in AT and MG but not LIV. The interaction detected for *CPT1A* was due to upregulation in AT and LIV postpartum without a change in MG. In contrast, the interaction for *PPARG* was due to upregulation in AT and MG postpartum but a downregulation in LIV. Leptin was barely detectable in LIV, but there was an interaction effect in AT and MG associated with upregulation postpartum in MG and downregulation in AT. Together, these results suggest that the control of metabolic adaptations in LIV, MG, and AT around parturition might be partly regulated through the CLOCK gene network. Although the present study did not specifically address rhythmic control

of tissue metabolism via the CLOCK gene network, the difference in expression of genes studied among tissues confirms that the behavior of circadian-controlled metabolic genes around parturition differs by tissue and, as such, is closely associated with the metabolic function of the organ.

Key words: circadian genes, dairy cow, lactation

INTRODUCTION

The metabolic adaptations during the transition from pregnancy to lactation (periparturient period) in ruminants and monogastrics are well known (McNamara, 1994; Loo et al., 2013). Recent data from rats have demonstrated that changes in clock circadian regulator (CLOCK) expression and its associated gene networks occur in multiple tissues during the periparturient period. The rat mammary gland (MG) expresses *CLOCK* and other circadian rhythm genes (Plaut and Casey, 2012) and, in rodents, it is estimated that approximately 7% of the genes expressed in MG during lactation have a circadian pattern that includes the core *CLOCK* network and several metabolic genes (Plaut and Casey, 2012). Those data suggest that the circadian molecular system participates in coordinating the physiological changes in the dam needed to support lactation (Casey and Plaut, 2012).

The CLOCK and circadian rhythm gene network has been well studied in rodent liver (LIV), where it was demonstrated that the network helps to control a wide array of metabolic and physiologic functions with a periodicity of approximately 24 h (Feng and Lazar, 2012). Both epigenetics and transcriptional changes are part of regulatory mechanisms of circadian rhythms. As an example, Yang et al. (2012) reported that *PPARG* ablation led to suppression of circadian variation in oxygen consumption, CO₂ production, food and water intake, and locomotor activity, all of which were accompanied by impaired rhythmicity of the canonical *CLOCK* genes in adipose tissue (AT) and liver (LIV). Those data support an essential role of *PPARG* in the

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coordinated control of rodent circadian clocks involved in energy metabolism.

Ruminant animals such as dairy cattle have a well-defined daily pattern of feed intake and milk synthesis (Harvatine, 2012). For instance, a large survey of the milking-to-milking variation in milk yield and composition revealed that milk yield and milk fat concentration have a clear, repeated daily pattern over a 5-d sampling period (Quist et al., 2008). Others reported that dairy cows in confinement systems have a clear diurnal pattern of intake but reduce the number of daily meals as early as 7 to 10 d prepartum (Grant and Albright, 1995). This is considered a “normal” response of the animal, likely driven in part by hormonal and metabolite changes associated with the time of parturition.

Our general hypothesis was that the behavior of *CLOCK* and metabolic genes around parturition differs across tissues and is closely associated with the metabolic function of the organ. The main goal of the present study was to examine the behavior of the circadian system in bovine MG, LIV, and AT via transcript profiling of *CLOCK* and key metabolism-related genes that have been previously identified in these tissues (Table 1).

MATERIALS AND METHODS

Animals and Biopsies

All procedures involving animals received approval from the Institutional Animal Care and Use Committee at the University of Illinois, Urbana. Seven multiparous Holstein cows from the University of Illinois dairy herd were used for biopsy at -10 (±2), 7, and 21 d relative to parturition. Cows were housed in a ventilated, enclosed barn during the dry period (8 h light:16 h dark photoperiod) and had access to sand-bedded freestalls until 3 d before expected parturition, when they were moved to individual maternity pens bedded with straw until parturition. After parturition, cows were housed in a tiestall barn under ambient photoperiod conditions through the end of the study. Liver and AT were harvested under local anesthesia either through puncture biopsy (LIV) or blunt dissection from the tail-head region (AT; Ji et al., 2012). For MG, the cows received a small dose of a general anesthetic (xylazine) before applying a local anesthetic (lidocaine-hydrochloride) on the midsection of the right or left rear quarter; that is, biopsies were performed on alternate sites each subsequent time. Mammary tissue was harvested using a biopsy tool attached to a cordless drill (Bionaz and Loor, 2011). Tissue was frozen immediately in liquid N and later used for total RNA extraction. Pre- and postpartal biopsies were performed sequentially before

Table 1. Clock circadian regulator (CLOCK) network genes selected for transcript profiling

Gene symbol	Gene name (National Center for Biotechnology Information)
<i>ARNTL</i>	Aryl hydrocarbon receptor nuclear translocator-like
<i>CLOCK</i>	Clock
<i>CRY1</i>	Cryptochromes
<i>CRY2</i>	Cryptochromes
<i>PER1</i>	Period 1
<i>PER2</i>	Period 2
<i>PER3</i>	Period 3
<i>RORA</i>	RAR-related orphan receptor A
<i>NCOR1</i>	Nuclear receptor corepressor 1
<i>NPAS2</i>	Neuronal PAS domain-containing protein 2
<i>NR1D1</i>	Nuclear receptor subfamily 1 group D member 1
<i>NR2F2</i>	Nuclear receptor subfamily 2
<i>ALAS1</i>	Aminolevulinate, delta-, synthase 1
<i>FBXW11</i>	Beta-transducin repeat containing
<i>CCRN4L</i>	Carbon catabolite repression 4 like protein
<i>CPT1A</i>	Carnitine palmitoyltransferase 1a
<i>CSNK1D</i>	Casein kinase I delta isoform
<i>FECH</i>	Ferrochelatase
<i>HDAC3</i>	Histone deacetylase 3
<i>LEP</i>	Leptin (obese encoding)
<i>PPARA</i>	Peroxisome proliferator-activated receptor α
<i>PPARGC1A</i>	Peroxisome proliferator-activated receptor gamma coactivator 1-α
<i>PPARG</i>	Peroxisome proliferator-activated receptor gamma
<i>FGF21</i>	Fibroblast growth factor 21

the morning feeding. All cows had ad libitum access to a TMR balanced to meet the nutrient requirements of the cows during late pregnancy and early lactation. Fresh water was available to cows at all times during the day. Cows were fed individually once daily pre- and postpartum at 0630 h.

RNA Isolation and cDNA Synthesis

These procedures have been thoroughly described by Bionaz and Loor (2011) and Ji et al. (2012). Briefly, tissue was homogenized in TRIzol reagent (cat. no. 15596018; ThermoFisher, Waltham, MA) using a Tissue-Tearor (BioSpec Products, Bartlesville, OK) homogenizer to isolate total RNA. The RNA samples were purified using an RNeasy Mini Kit (cat. no. 74104; Qiagen Sciences Inc., Germantown, MD) before cDNA synthesis. Purity and integrity of the extracted RNA was determined using a Nanodrop spectrophotometer (ThermoFisher) and an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA). Total RNA was reverse-transcribed using SuperScript III First Strand Synthesis kit (ThermoFisher). Primers were designed and evaluated as previously described (Bionaz and Loor, 2011). Briefly, primers were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) with minimum amplicon size of 80 bp (whenever possible, amplicons of 100–120 bp were chosen) and limited

3' G+C percentage. Primer pair sequences for genes tested are reported in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2015-9430>). Before quantitative real-time (q)PCR was performed, primer products were verified according to Ji et al. (2012). The final data for each tissue type were normalized using the geometric mean of *ACTB*, *GAPD*, and *UXT*.

Statistical Analysis

Normalized RNA expression data were $\log_2$ -transformed before statistical analysis using repeated-measures ANOVA with the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) to determine the overall effect of stage of lactation and differences in expression among the 3 tissues studied. The fixed effects considered in the model were day relative to parturition and tissue, and cow represented the random effect. Differences between means at the time points examined were evaluated using the PDIFF statement in SAS.

RESULTS

Tissue Differences

No interaction (day $\times$ tissue; $P = 0.57$) or day ($P = 0.15$) effect was detected for *CLOCK* mRNA expression, although AT had greater expression ($P < 0.05$) overall compared with MG tissue (Figure 1). The expression of *CRY2*, *CRY3*, *PER1*, *PER2*, *NR1D1*, *NR2F2*, and *CCRN4L* was greatest ($P < 0.05$) in AT compared with both LIV and MG. However, expression of *CRY3* and *CCRN4L* was similar ($P > 0.05$) between LIV and AT. The expression of *CRY2*, *PER1*, *PER2*, *NR1D1*, and *CCRN4L* was greater ($P < 0.05$) in LIV compared with MG. Among all genes analyzed, only expression of *FBXW11* was greater ($P < 0.05$) in both AT and MG compared with LIV. In contrast, LIV had greater ($P < 0.05$) expression of *ALAS1*, *FECH*, *PPARA*, *PPARGC1A*, and *FGF21* compared with both AT and MG. Among these, the expression of *PPARGC1A* was greater ($P < 0.05$) in AT compared with MG.

Interaction Between Tissue Type and Day Around Parturition

Among the genes studied that form part of the core CLOCK signaling pathway, the expression of *ARNTL*, *CRY1*, *PER2*, and *CSNK1D* had a day $\times$ tissue interaction ($P < 0.05$; Figure 2). Although expression of *ARNTL* among tissues was similar prepartum, the expression of *CRY1*, *PER2*, and *CSNK1D* was lowest ($P < 0.05$) in LIV compared with AT and MG. Expression of *PER2* was greatest ($P < 0.05$) in AT and

that of *CRY1* greatest in MG, whereas expression of *CSNK1D* was similar ($P > 0.05$) in AT and MG. In the early postpartal period, the expression of *ARNTL* and *CRY1* was greater ($P < 0.05$) in AT and MG than in LIV, in which expression decreased markedly from the prepartal period. This pattern of expression remained the same at the last sampling time point (Figure 2). The temporal expression of *PER2* and *CSNK1D* in MG increased ($P < 0.05$) from prepartum to a peak level on the last sampling time point, whereas the temporal expression of both genes in LIV did not change.

Among the nuclear receptors and co-regulators studied, the prepartal expression of *RORA* was greatest ($P < 0.05$) in LIV, and that of *PPARG* was greatest in AT compared with other tissues (Figure 2). The expression of *NCOR1* was greatest ($P < 0.05$) in AT, intermediate in MG, and lowest in LIV. After parturition, the expression of *RORA* increased ($P < 0.05$) markedly in MG compared with AT and LIV, such that the early postpartal expression was similar between AT and MG, and MG had greater expression than AT on the last sampling time. Expression of *RORA* increased modestly in LIV after parturition but remained lower overall than in other tissues. The expression of *PPARG* in AT remained similar ($P > 0.05$) after parturition but increased in MG. No change in *PPARG* around parturition was observed for LIV, which had the lowest expression among tissues. The postpartal expression pattern of *NCOR1* did not differ ($P > 0.05$) from prepartal values and remained greatest in AT compared with LIV.

Other genes with a significant interaction ($P < 0.05$) included the enzyme *CPT1A* and the adipokine/cytokine *LEP*. In the case of *CPT1A*, LIV tissue had greater ($P < 0.05$) expression than AT and MG at all times examined. Furthermore, its expression in LIV and AT increased after parturition. Expression of *CPT1A* overall in AT was substantially greater ($P < 0.05$) than that in MG. The expression of *LEP* was not detectable in LIV. We detected substantial variation in *LEP* expression within AT and MG, but its expression decreased ($P < 0.05$) after parturition in AT, whereas it increased in MG.

Parturition and Lactation Effect

Of all genes studied without an interaction effect ($P > 0.05$), only *NPAS2*, *NR1D1*, *PPARGC1A*, *CCRN4L*, *PPARA*, and *FGF21* had a change in expression due to day around parturition (Figure 3). In fact, the expression of *NPAS2*, *NR1D1*, *PPARGC1A*, *PPARA*, and *FGF21* was greater ($P < 0.05$) postpartum compared with prepartum. The expression of *CCRN4L* also increased in the early postpartal period, but its expres-

Figure 1. Expression profile of genes with a significant tissue effect ($P < 0.05$). Gene expression was performed in tissue biopsies of adipose (AT), liver (LIV), and mammary gland (MG) during the transition from late pregnancy to lactation. Genes are listed in Table 1.

Figure 2. Expression profile of genes with a significant day × tissue effect ($P < 0.05$). Gene expression was performed in tissue biopsies of adipose (AT), liver (LIV), and mammary gland (MG) during the transition from late pregnancy to lactation. Letters (a-c) denote differences among tissues at a specific time point; *denotes differences relative to d -10 within a given tissue; #denotes differences relative to d 7 within a given tissue. Genes are listed in Table 1.

sion decreased to prepartal levels on the last day of sampling.

DISCUSSION

During the transition from late gestation to early lactation, mammals experience tremendous physiologi-

cal changes. Regulation of these changes is essential for ensuring a successful transition; for example, minimal incidence of metabolic and infectious disorders and optimal nutrient utilization for milk synthesis and nourishment of the young. Changes in mRNA expression during this transition period exert some control on the adaptations of adipose, liver, and mammary gland

Figure 3. Expression profile of genes with a significant time effect ($P < 0.05$). Gene expression was performed in tissue biopsies of adipose, liver, and mammary gland during the transition from late pregnancy to lactation. Genes are listed in Table 1. Letters (a,b) denote differences ($P < 0.05$) among tissues at different time points.

(Sumner-Thomson and McNamara, 2011; Loor et al., 2013).

Circadian rhythms generated by molecular circadian clocks located in the hypothalamus (the master clock) and peripherally in organs with key metabolic functions are important in regulating a wide range of cellular, metabolic, physiologic, and behavioral activities (Yan et al., 2008). Recent studies with rodents and using single-time-of-day samples have reported gene expression changes in circadian clock genes around parturition. For example, *ARNTL* and *CLOCK*, which control the core molecular clock genes, are upregulated during the transition from pregnancy to lactation in rats. In contrast, *NR1D1*, *DBP*, *BHLHB2*, *OPN4*, and *HTR7*,

which are negative regulators of intracellular circadian rhythms, are downregulated (Casey et al., 2009).

Apart from regulating circadian rhythms, circadian *CLOCK* genes and *CLOCK*-controlled genes are actively involved in the regulation of metabolism in multiple peripheral tissues including LIV, MG, and AT (Mazzocchi et al., 2012). By rhythmically altering *CLOCK*-controlled gene expression, circadian clocks can regulate metabolic pathway activity or flux. Although a single-time-of-day sample was evaluated, the changes observed for *CLOCK* genes and *CLOCK*-controlled genes in the present study indicate a possible regulatory role of these pathways during the periparturition period in dairy cattle.

Circadian CLOCK and Liver Metabolism

Liver is a major metabolic organ and several CLOCK-controlled genes play an important role in this tissue in nonruminants (Asher and Schibler, 2011), including control of gluconeogenesis. In the context of periparturient dairy cows, gluconeogenesis in liver is vital to meet glucose requirements because little glucose can be absorbed directly from the gastrointestinal tract. Studies in mice have revealed a regulatory role of circadian CLOCK genes on glucose metabolism (Zhang et al., 2010). The *CRY1* gene encodes a flavin adenine dinucleotide-binding protein that is a key component of the circadian core oscillator complex, which regulates the circadian CLOCK. Zhang et al. (2010) reported that the hepatic overexpression of *CRY1* was associated with lower blood glucose concentrations and improved insulin sensitivity in db/db mice (a model of obesity, diabetes, and dyslipidemia). They suggested that *CRY* mediates circadian regulation of hepatic gluconeogenesis by blocking the glucagon-mediated increase in intracellular cAMP concentrations and phosphorylation of Creb (Zhang et al., 2010). In the present study, although a single-time-of-day sample was evaluated, the gradual decrease in expression of *CRY1* in LIV compared with AT and MG tissue during the transition from pregnancy to lactation might have been one factor associated with the increase in gluconeogenesis typically observed during this time (Aschenbach et al., 2010). However, the fact that relative mRNA abundance of *CRY1* in LIV was quite low (data not shown) raises uncertainty as to its direct role in bovine hepatic gluconeogenesis.

More recently, Sun et al. (2015) reported that temporal signals of fasting and refeeding regulate the transcription of *Bmal1* (also known as *ARNTL*), the key transcription activator of molecular CLOCK, in murine hepatocytes. During fasting, glucagon activates the CREB/CRTC2 transcriptional complex and is then recruited to the *Bmal1* promoter to induce its expression, leading to activation of gluconeogenesis (Sun et al., 2015). In the present study, using a single-time-of-day sample for evaluation, the marked decrease of *ARNTL* expression in cow LIV after parturition suggests that it may not be important for gluconeogenesis regulation; that is, this transcription regulator might work differently or have a different function depending on species. This idea is supported by the fact that ruminant animals are in a constant gluconeogenic state because of extensive fermentation of carbohydrate in the rumen.

The fact that blood glucagon increases markedly after parturition (Herbein et al., 1985) and remains elevated for several months during lactation is thought

to be part of the mechanism enhancing gluconeogenesis in liver. Thus, glucagon is important for hepatic gluconeogenesis in LIV after parturition but likely does not induce its effects via *ARNTL*. In contrast, the upregulation of *ARNTL* in AT after parturition is in line with data generated in lactating rats, demonstrating greater sensitivity of AT to the lipolytic action of glucagon (Zammit, 1988). Thus, it would appear that *ARNTL* in bovine AT might be responsive to systemic glucagon and play a role in the hormone's actions that helps coordinate homeorhetic adaptations to lactation. It remains to be established if the much greater relative abundance of *PER1* in AT, LIV, and MG has a mechanistic role in controlling expression of *ARNTL*.

The hepatokine *FGF21* is a member of the fibroblast growth factor (FGF) family (Kharitonov et al., 2005). The expression of *FGF21* (and relative mRNA abundance; data not shown) was comparably high in LIV, but barely detectable in AT and MG, in agreement with data from Schoenberg et al. (2011), who reported that LIV was the major source of plasma *FGF21* in early lactation, with little or no contribution by white AT, skeletal muscle, or MG. The greater expression of *FGF21* in LIV could also have contributed to the gluconeogenic response. In fact, *FGF21* plays an important role in regulating both gluconeogenesis and hepatic oxidation of fatty acids in nonruminants (Fisher et al., 2011; Khan et al., 2014). Acute *FGF21* treatment in mice induced hepatic expression of key regulators of gluconeogenesis, lipid metabolism, and ketogenesis including glucose-6-phosphatase, phosphoenol pyruvate carboxykinase, 3-hydroxybutyrate dehydrogenase type 1, and *CPT1A* (Fisher et al., 2011). The response of *FGF21* expression suggests a similar role in hepatic gluconeogenesis and fatty acid oxidation to fulfill the energy demands of the cow after parturition.

In nonruminants, a large number of nuclear receptors and key enzymes involved in fatty acid metabolism are oscillating clock-controlled genes (Asher and Schibler, 2011). In the liver of dairy cows, fatty acids can be oxidized completely to carbon dioxide, partially oxidized to ketone bodies, or re-esterified to triglycerides (Lor et al., 2013). The amount of fatty acids entering each pathway is closely controlled. For instance, *CPT1A* mediates the transport of long-chain fatty acids across the outer mitochondrial membrane, and its deficiency results in a decreased rate of fatty acid β -oxidation (Bonnetfont et al., 2004). Although a single-time-of-day sample was evaluated in the current study, the increase in expression of *CPT1A* in AT and LIV postpartum suggests an increase in fatty acid oxidation capacity, which has been reported previously for LIV (Dann and Drackley, 2005). The results for LIV agree with

previous reports showing that cows in negative energy balance have greater hepatic *CPT1A* expression (McCarthy et al., 2010; Loor et al., 2013). The upregulation of *CPT1A* in AT after parturition, however, has not previously been reported but could represent an adaptation of the tissue to generate energy in the face of greater lipolytic stimuli.

The peroxisome proliferator-activated receptors (PPAR) are a group of nuclear receptor proteins that function as transcription factors regulating gene expression. Three types of PPAR have been identified: α , γ , and delta (formerly β) in both nonruminants and ruminants (Bionaz et al., 2013). The *PPARA* gene responds to circulating fatty acids as well as their metabolites. Notably, *PPARA* is under circadian control and can modulate circadian CLOCK gene transcription in nonruminants. As transcription of *PPARA* is induced by *CLOCK* and *BMAL1* and repressed by *PER2* and *CRY1* (Hayashida et al., 2010), the postpartal decrease of *CRY1* in LIV (although evaluated at a single time of day) may contribute to the observed increase in *PPARA* expression postpartum. The relatively high mRNA abundance of *PPARA* in MG has not, to our knowledge, been reported previously and is puzzling, given that bovine MG does not oxidize long-chain fatty acids to a quantitative extent (Bionaz and Loor, 2008; Bionaz et al., 2013).

Circadian CLOCK and Metabolism in Mammary Gland and Adipose

Global gene expression analysis (microarrays) results revealed that 7% of genes expressed in lactating human breast have a circadian pattern (Maningat et al., 2009), which underscores an important role of the circadian CLOCK in MG development and lactation. The *PER1* and *PER2* genes are necessary for normal mammary ductal development and the maintenance of mammary epithelial cell polarity (Plaut and Casey, 2012). Using single-time-of-day samples, Metz et al. (2006) reported that *Per2* expression was higher in proliferating virgin and early pregnant mouse MG compared with lactating MG. Its expression was positively correlated with *Cyclin D1* expression in early pregnancy, on d 1 and 7 of lactation, and on day of involution. Although the expression of *Per1* did not change significantly between early pregnancy and d 3 of involution, *Bmal1* and *Csn2* expression increased between early pregnancy and d 3 of lactation, with *Csn2* reaching peak expression on d 7 of lactation. It was suggested that *Per2* might play a role in mouse MG development and differentiation.

The *PER2* gene is a member of the Period family of genes and encodes components of the circadian rhythm controlling locomotor activity, metabolism, and be-

havior. In the rat suprachiasmatic nucleus, expression of *PER2* was high throughout the entire day, and a significant daily rhythm of this gene was observed on postnatal d 6 (Shimomura et al., 2001), where it might have had a stimulatory effect on the timing of corticosteroid secretion (Sellix, 2013). Research has shown that *PER2* regulates adipocyte differentiation by inhibiting *PPARG* directly (Gurnell, 2003). Indeed, lack of *PER2* results in enhanced adipocyte differentiation of cultured fibroblasts (Grimaldi et al., 2010).

In the present study, although a single-time-of-day sample was evaluated, the lack of change in *PER1* expression around parturition agrees with mouse data. However, the fact that *PER2* expression was greatest in AT and was the fourth-most-abundant gene in AT (data not shown) but did not change after parturition suggests a potential “housekeeping” role to prevent complete loss of AT mass. Such a role would be important because of the well-established catabolic state of AT after parturition (McNamara, 2012). In contrast, the increase in *PER2* expression postpartum in MG in the present study might have been related to the increase in *PPARG* expression, both of which would help control milk fat synthesis (Bionaz and Loor, 2008). Unlike mouse MG (Metz et al., 2006), the postpartal increase of *PER2* and not *PER1* in MG could be functionally related to β -casein synthesis.

Data from mouse MG and the present study suggest that *PER1* and *PER2* might play different roles in rodents than in ruminants. Their relative mRNA abundances in AT, LIV, and MG were vastly different, with *PER1* being one of the most abundant genes measured, especially in AT and MG (data not shown). Alternatively, the changes observed for both genes in mouse MG after parturition could be due to fat pad lipolysis. Whereas adult ruminant MG is close to 70% epithelial cells, the rodent mammary gland accumulates a substantial amount of adipose tissue during pregnancy (Capuco et al., 2001). Further research encompassing more than one sample during the day to delineate *PER* function in ruminant tissues is warranted.

Regulation of the Circadian Rhythm

Transcriptional, posttranscriptional, and post-translational mechanisms have been reported to control circadian rhythm in nonruminants (Kojima et al., 2011). Although we did not study rhythmicity per se, the results from the present study utilizing a single-time-of-day sample also indicated a possible regulatory mechanism of circadian rhythms in dairy cows during the peripartal period. For instance, casein kinase 1 delta (*CSNK1D*) and *CSNK1E* are key kinases acting on *PER* (and *CRY*). The enzyme encoded by

CSNK1D controls cytoplasmic and nuclear processes, including DNA replication and repair. The phosphorylated *PER:CRY*:kinase complex is translocated into the nucleus where it also participates in the control of the circadian rhythm (Meng et al., 2008). One of the roles for the phosphorylation of *CLOCK* proteins is to target them for polyubiquitination and degradation via the 26S proteasomal pathway (Takahashi et al., 2008). In the present study, the gradual increase of *CSNK1D* in AT and MG and the decrease in LIV just after parturition agreed with the results of *PER2* and *CRY1* and suggested that they might serve to control circadian rhythms.

It was noteworthy that *NPAS2*, *CCRN4L*, and *NR1D1* had higher mRNA expression postpartum, but the lack of an interaction effect indicated that circadian rhythms might have been enhanced overall in all tissues evaluated. The *CCRN4L* gene encodes a circadian rhythm deadenylase that acts downstream of *CLOCK* and *BMAL1* to stabilize circadian-related mRNA transcripts (Garbarino-Pico et al., 2007). In the mouse, it has been shown to exhibit circadian rhythmicity, which suggests a role of the protein in clock function or as a circadian *CLOCK* effector. Although *NR1D1* is considered a negative regulator of gene transcription, the upregulation of *NR1D1* postpartum and particularly in AT could serve to control adipogenesis alongside *PPARG* (Kumar et al., 2010) and *ARNTL* (Shimba et al., 2005). The *NPAS2* protein is a transcriptional activator that forms a core component of the circadian *CLOCK* (Debruyne, 2008). These responses underscore the role of the circadian *CLOCK* in the control of metabolic adaptations across tissues. The fact that their relative mRNA abundance (data not shown) was greater in MG suggests they may play a more important role in that tissue; for example, helping to coordinate adaptations to onset of milk synthesis.

The *BMAL1* protein has an important role in maintaining a stable oscillating *CLOCK*. By heterodimerizing with *CLOCK*, *BMAL1* participates in the control of target *CLOCK*-controlled gene expression. The nuclear receptor Rev-erb (*NR1D1*) represses and *ROR* activates *BMAL1* transcription. In fact, Guillaumond et al. (2005) reported that competition between these 2 nuclear receptor families determines the transcription of *BMAL1*. The orphan nuclear receptor *RORA* binds to the consensus sequence (Giguère et al., 1994) and regulates *BMAL1* gene expression (Sato et al., 2004; Guillaumond et al., 2005). In the present study involving a sample from a single time of day, an interaction effect was detected for *RORA* due to the increase in expression only in MG after parturition, suggesting a potential local role in postpartal regulation of circadian clocks at the onset of milk synthesis. The high relative

mRNA abundance of this gene in MG (data not shown) relative to that in AT and LIV supports this idea. The distinct longitudinal patterns of *RORA* expression across LIV, AT, and MG that we observed agree with previous data from rodents showing distinct expression patterns of *ROR* genes across tissues (Guillaumond et al., 2005).

Heme may act as a ligand to regulate nuclear receptor Rev-erb- α (*NR1D1*) transcriptional activity (Raghuram et al., 2007). δ -Aminolevulinic synthase (*ALAS*) is the first and rate-limiting enzyme in the mammalian heme biosynthetic pathway, and it catalyzes the condensation of glycine with succinyl-CoA to form δ -aminolevulinic acid (Bishop et al., 1990). Ferrochelatase (*FECH*), the last enzyme of the heme biosynthetic pathway, is a target for hypoxia-inducible factor 1 (HIF-1; Liu et al., 2004) and catalyzes the insertion of iron into protoporphyrin to form heme. The greater expression of *ALAS1* and *FECH* in LIV coupled with the lack of change in expression around parturition indicates that this organ is central for the synthesis of heme. Furthermore, the fact that *ALAS1* accounted for ~86% of total mRNA measured (data not shown) underscores its importance in adaptations of LIV around parturition. Whether intrahepatic heme has a role in coordinating gene transcription via *NR1D1* after parturition is unclear, particularly because AT had the highest expression of *NR1D1* but the lowest expression of *ALAS1*. The greater expression of *NCOR1* in AT (and relative mRNA abundance; data not shown) compared with LIV seems to agree with a link between *NR1D1* and heme as an important negative regulator of gene transcription (Raghuram et al., 2007). However, the marked decrease in expression of *ARNTL* in LIV after parturition seems to support a role of heme in the negative regulation of an essential component of the circadian oscillator.

In addition to the role of transcriptional activators and repressors, posttranslational modifications and degradation of circadian *CLOCK* proteins are crucial steps controlling circadian periodicity. In that context, and even though only a single-time-of-day sample was evaluated, the upregulation of *FBXW11* (also known as β -*TrCP*) expression in response to genotoxic stress contributes to decreasing the activity of *CDK1* by mediating the degradation of *CDC25A* in coordination with *Chk1* (Busino et al., 2003), thereby preventing cell cycle progression before the completion of DNA repair (Peschiaroli et al., 2006). The β -*TrCP1* and *FBXL3 E3* ubiquitin ligase complexes have been implicated in targeting *PER* and *CRY* proteins for degradation (Reischl et al., 2007). In the present study, the higher expression of *FBXW11* in AT and MG and the lower expression in LIV agreed with the results for *CSNK1D*,

which encodes an enzyme controlling DNA replication and repair. This suggested that they co-regulate the circadian rhythm by potentially degrading circadian CLOCK proteins.

As the master transcription factor of adipogenesis, *PPARG* regulates fatty acid storage and adipocyte glucose metabolism (Feige et al., 2006; Bionaz et al., 2013). Alenghat et al. (2008) proposed that circadian regulation of metabolism via transcription factors is critical for maintaining normal energy balance. In non-ruminants, the nuclear receptor co-repressor 1 (*NCOR1*) recruits histone deacetylases to DNA promoter regions (Hörlein et al., 1995; Wang et al., 1998), and the activation of histone deacetylase 3 (*HDAC3*) by *NCOR1* is a nodal point in the epigenetic regulation of circadian and metabolic physiology in nonruminants (Alenghat et al., 2008). Research in rodents has shown that circadian clocks participate in the regulation of transcription factors such as *PPARG*. For instance, the work of Kawai and Rosen (2010) demonstrated that *PPARG* exhibits a circadian expression pattern in AT that is magnified by consumption of a high-fat diet. In the present study, the interaction effect that we detected for *PPARG* was due to the increase in expression in MG after parturition. Such an increase was reported previously using a single-time-of-day sampling approach (Bionaz and Loor, 2008), and recent studies have confirmed the role of this nuclear receptor in coordinating lipid synthesis in lactating mammary gland. The dominant functions of adipocyte *NCOR* are to trans-repress *PPARG* and promote *PPARG* Ser-273 phosphorylation to create a constitutively inactive *PPARG* (Li et al., 2011). In the present study, *NCOR1* increased significantly after parturition in AT tissue, suggesting its role in maintaining body condition of cows under the negative energy balance postpartum by inhibiting *PPARG*.

CONCLUSIONS

Although we utilized a single-time-of-day sampling approach, the data indicate that the expression pattern of circadian CLOCK genes differs across tissues during the periparturient period. By altering CLOCK-controlled gene expression, the circadian clock could regulate key metabolic pathways in dairy cows, including gluconeogenesis and fatty acid oxidation during the transition from gestation to lactation. Changes in core CLOCK gene mRNA expression in liver, mammary gland, and adipose tissue during the periparturient period suggest that metabolic adaptations in multiple tissues may be coordinated by changes in molecular clocks. Regulation of circadian rhythm may be important to ensure a smooth transition of dairy cows from pregnancy to lactation. Future work should be performed

to study whether environmental cues (e.g., nutrition) could entrain the daily rhythms in these key organs, and whether the effects are at the transcriptional, translational, posttranslational, or even epigenetic level. Such work should encompass several time points during the day.

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