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 (±2), 7, and 21 d relative to parturition. A day × 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 MG 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 (peripartal period) in ruminants and monogastrics are well known (McNamara, 1994; Loor 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 peripartal 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
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 metabolic 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 throughout 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 postpartum biopsies were performed sequentially before 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name (National Center for Biotechnology Information)</th>
</tr>
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<tbody>
<tr>
<td>ARNTL</td>
<td>Aryl hydrocarbon receptor nuclear translocator-like</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Clock</td>
</tr>
<tr>
<td>CRY1</td>
<td>Cryptochromes</td>
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<tr>
<td>CRY2</td>
<td>Cryptochromes</td>
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<tr>
<td>PER1</td>
<td>Period 1</td>
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<tr>
<td>PER2</td>
<td>Period 2</td>
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<tr>
<td>PER3</td>
<td>Period 3</td>
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<tr>
<td>RORA</td>
<td>RAR-related orphan receptor A</td>
</tr>
<tr>
<td>NCO1</td>
<td>Nuclear receptor corepressor 1</td>
</tr>
<tr>
<td>NPAS2</td>
<td>Neuronal PAS domain-containing protein 2</td>
</tr>
<tr>
<td>NR1D1</td>
<td>Nuclear receptor subfamily 1 group D member 1</td>
</tr>
<tr>
<td>NR2F2</td>
<td>Nuclear receptor subfamily 2</td>
</tr>
<tr>
<td>ALAS1</td>
<td>Aminolevulinate, delta-, synthase 1</td>
</tr>
<tr>
<td>FBYW11</td>
<td>Beta-transducin repeat containing</td>
</tr>
<tr>
<td>CCRN4L</td>
<td>Carbon catabolite repression 4 like protein</td>
</tr>
<tr>
<td>CPT1A</td>
<td>Carnitine palmitoyltransferase 1a</td>
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<tr>
<td>CSNK1D</td>
<td>Casein kinase I delta isoform</td>
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<tr>
<td>FEOH</td>
<td>Ferrochelatase</td>
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<tr>
<td>HDAC3</td>
<td>Histone deacetylase 3</td>
</tr>
<tr>
<td>LEP</td>
<td>Leptin (obese encoding)</td>
</tr>
<tr>
<td>PPARGA</td>
<td>Peroxisome proliferator-activated receptor α</td>
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<tr>
<td>PPARGC1A</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-α</td>
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<tr>
<td>PPARG</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
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<tr>
<td>FGF21</td>
<td>Fibroblast growth factor 21</td>
</tr>
</tbody>
</table>
Tissue Differences

means at the time points examined were evaluated using a mixed 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 PDIF11 statement in SAS.

Statistical Analysis

Normalized RNA expression data were log<sub>2</sub>-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 PDIF11 statement in SAS.

RESULTS

Tissue Differences

No interaction (day × 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 FGFl21 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 CCRN4L had a day × tissue interaction (P < 0.05; Figure 2). Although expression of ARNTL among tissues was similar prepartum, the expression of CRY1, PER2, and CCRN4L 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 CCRN4L 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 CCRN4L 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 NRAS was greatest (P < 0.05) in MG, and that of PPARG was greatest in LIV compared with AT and MG (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 NRAS increased (P < 0.05) markedly in LIV compared with AT and MG, 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 NRAS 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 FGFl21 had a change in expression due to day around parturition (Figure 3). In fact, the expression of NPAS2, NR1D1, PPARGC1A, PPARA, and FGFl21 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.
sion decreased to prepartal levels on the last day of sampling.

**DISCUSSION**

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

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**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.
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 (Mazzoccoli 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 peripartal period in dairy cattle.

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.
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 peripartal 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, with lower blood glucose concentrations and improved insulin sensitivity in db/db mice (a model of obesity, diabetes, and dyslipidemia). They suggested that CRY1 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 (Kharitonenkov 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 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 (Loor 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 (Bonnefont 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 (PPARs) 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 PPARα gene responds to circulating fatty acids as well as their metabolites. Notably, PPARα is under circadian control and can modulate circadian CLOCK gene transcription in nonruminants. As transcription of PPARα 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 PPARα expression postpartum. The relatively high mRNA abundance of PPARα 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 behavior. 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 PPARγ 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 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 PPARγ 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 posttranslational 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 proteosomal 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-α (**NR1D1**) transcriptional activity represses and **ROR** NR1D1 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-Aminolevulinate 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 **NCO1** 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 **βTrCP**) 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**, **ALAS1**, **PPARG**, **PER1**, and **PER2**. 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-α (**NR1D1**), 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, a potential 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 transcriptional activity 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-Aminolevulinate 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 **NCO1** 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.
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, PPARγ 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 non-ruminants (Alenghat et al., 2008). Research in rodents has shown that circadian clocks participate in the regulation of transcription factors such as PPARγ. For instance, the work of Kawai and Rosen (2010) demonstrated that PPARγ 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 PPARγ 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 Loo, 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 PPARγ and promote PPARγ Ser-273 phosphorylation to create a constitutively inactive PPARγ (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 PPARγ.

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 peripartal 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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REFERENCES


