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Photoperiod effects on milk production in goats: Are they mediated by the molecular clock in the mammary gland?

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ABSTRACT

US scientists, Dr. Theresa Casey and Dr. Karen Plaut, collaborated with Israeli scientists, Dr. Sameer Mabjeesh and Dr. Avi Shamay to conduct studies proposed in the BARD Project No. US-4715-14 *Photoperiod effects on milk production in goats: Are they mediated by the molecular clock in the mammary gland* over the last 3 years**.** CLOCK and BMAL1 are core components of the circadian clock and as heterodimers function as a transcription factor to drive circadian-rhythms of gene expression. Studies of CLOCK-mutant mice found impaired mammary development in late pregnancy was related to poor lactation performance post-partum. To gain a better understanding of role of clock in regulation of mammary development studies were conducted with the mammary epithelial cell line HC11. Decreasing *CLOCK* protein levels using shRNA resulted in increased mammary epithelial cell growth rate and impaired differentiation, with lower expression of differentiation markers including ad herens junction protein and fatty acid synthesis genes. When BMAL1 was knocked out using CRISPR-CAS mammary epithelial cells had greater growth rate, but reached stationary phase at a lower density, with FACS indicating cells were growing and dying at a faster rate. Beta-casein milk protein levels were significantly decreased in BMAL1 knockout cells. ChIP-seq analysis was conducted to identify BMAL1 target genes in mammary epithelial cells. Studies conducted in goats found that photoperiod duration and physiological state affected the dynamics of the mammary clock. Effects were likely independent of the photoperiod effects on prolactin levels. Interestingly, circadian rhythms of core body temperature, which functions as a key synchronizing cue sent out by the central clock in the hypothalamus, were profoundly affected by photoperiod and physiological state. Data support that the clock in the mammary gland regulates genes important to development of the gland and milk synthesis. We also found the clock in the mammary is responsive to changes in physiological state and photoperiod, and thus may serve as a mechanism to establish milk production levels in response to environmental cues.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Abstract - Poster	0	2	0
Reviewed	0	2	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Ph.D. Student	Suarez-Trujillo	Aridany	Purdue University	USA
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CONTRIBUTION of COLLABORATION

1. Investigator meetings:
    a. Video-conference meetings were held approximately three times per year to design experiments, discuss data, and plan next steps.
    b. In year 1- Dr. Mabjeesh visited the US scientists at Purdue University in West Lafayette, IN
    c. In year 2- all 4 PIs met at the American Dairy Science Association (ADSA) annual meeting in Orlando Florida to discuss study progress.
    d. In year 4- Dr. Mabjeesh and Dr. Casey met at the ADSA meeting in Knoxville, TN to discuss project results and plan for next steps, to include publishing final 2 manuscripts.
2. All investigators contributed to analysis of data and drafting of abstracts and manuscripts that resulted from this work.
3. Dr. Casey and Dr. Mabjeesh emailed throughout study to exchange data for analysis and interpretation of results.

ACHIEVEMENTS

1. Demonstrated that the CLOCK protein regulates mammary cell growth.
2. Showed that knock-out (removal) of the circadian clock gene, BMAL1 resulted in decreased beta-casein milk protein levels and decreased expression of CLOCK, decreased fatty acid synthesis expression, conclusively connecting mammary clock to regulation of milk synthesis.
3. Demonstrated that mammary clock is impacted by photoperiod duration (day length), and thus may be partly responsible for seasonal differences in milk production levels.
4. Demonstrated that circadian rhythms of core body temperature changed with physiological state and photoperiod duration, and thus may serve as a way of communicating across the body time of year and states of pregnancy and lactation.
5. Used ChIP-seq to identify genes regulated by mammary clock.

CHANGES TO ORIGINAL RESEARCH PLAN

There was one change to the research plan. We had proposed to send mammary biopsy tissue from goats to the United States from Israel for ChIP-qPCR analysis. However, due to import restrictions on goat tissue that are defined by the US Department of Agriculture, we found this was not allowable.

Publications for Project US-4715-14

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Abstract - Poster	*S. J. Mabjeesh*, A. Shamay, K. Plaut, C. Sabastian, and T. M. Casey*	Photoperiod manipulations during the dry period significantly impact mammary circadian clock in goats.	***Joint Annual Meeting; Salt Lake City, UT***	: 2016	Joint
Published	Abstract - Poster	*Aridany Suarez Trujillo, Jennifer Crodian, Avi Shamay, Sameer Mabjeesh, Karen Plaut and Theresa Casey*	Interactions among serotonin and circadian systems in the mammary gland	***Joint Annual Meeting; Salt Lake City, UT***	: 2016	Joint
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Published	Reviewed	*Theresa M. Casey, Karen Plaut, Mugagga Kalyesubula, Avi Shamay, Chris Sabastian, Yosi Wein, Enav Bar-Shira, Naama Reicher & Sameer. J. Mabjeesh*	Mammary core clock gene expression is impacted by photoperiod exposure during the dry period in goats	***Journal of Applied Animal Research***	46 : 1214-1219 2018	Joint

EFFECT OF PHOTOPERIOD ON THE GENES (*BMAL1, CLOCK, CRY1, CRY2,* AND *PER1*) CORRELATED TO THE CIRCADIAN RHYTHM IN DAIRY GOATS

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By

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I am greatly indebted to: my dad; Mr. Vincent Kiguli, my mum; Mrs. Josephine Nakachwa Kiguli, brothers, sisters, all family members and friends for all the social, moral and spiritual support during my studies and entire life. I give thanks and glory to God. May all who have assisted me in this course of my life be richly rewarded.

ABSTRACT

Goat milk consumption is on the rise because of its better digestibility, buffering capacity, alkalinity, and certain therapeutic advantages. Modulation of circadian rhythms in dairy animals affects milk yield. Circadian clocks control the rhythmic expression of many clock-controlled genes involved in metabolism in various tissues including the mammary gland. Photoperiod, which results from the light/dark cycle, is the most potent environmental cue that entrains circadian clocks in most organisms. Animals exposed to a short-day photoperiod (SDPP) during late pregnancy had a higher mammary growth and subsequently yielded more milk in the ensuing lactation period than those exposed to a long-day photoperiod (LDPP). The mechanism of how photoperiod effect increases milk yield is not yet fully understood. We hypothesized that photoperiod effects are mediated through the circadian clock proteins. The main goal of this study was to find out the effect of photoperiod on the mammary expression of core clock genes (*BMAL1*, *CLOCK*, *CRY1*, *CRY2,* and *PER1*) during late pregnancy and lactation. We also studied the effect of physiological state on mammary expression of these core clock genes. To study these effects, 12 Israeli Saanen goats were assigned to 2 treatments of 6 goats each at dry off (approximately 45 days prepartum) based on their body weight and previous milk production. They were assigned to either the long-day photoperiod group (LDPP) which had 16 hours of light and 8 hours of darkness or short-day photoperiod group (SDPP) which had 8 hours of light and 16 hours of darkness at a normothermic ambient temperature (23 ± 2°C) until 120 days postpartum. In the lactation period, goats were milked once daily, and the milk yield recorded automatically. Weekly milk samples were taken for milk composition analysis. Serial blood samples were taken to obtain plasma for prolactin analysis using Enzyme-Linked Immunosorbent Assay (ELISA). Mammary biopsies were taken at 3 weeks prepartum and 5 weeks postpartum and later, mRNA abundance of the studied core clock genes determined by real-time polymerase chain reaction (RT-PCR). Our results showed that, during the late gestation period, apparent dry matter intake (DMI) was similar between treatments and averaged at 1574.5 g/d/goat. However, during the lactation period, apparent dry matter intake was higher in SDPP than in LDPP (1699 ± 32.3 versus 1413 ± 24.1 g/d/goat). Animals in LDPP had a shorter gestation period than those in SDPP (144 ± 1.4 versus 151 ± 1.9 d). Milk yield was greater in SDPP than in LDPP (3.15 versus 2.7

kg/d). Likewise, milk protein content was higher in SDPP than LDPP (3.53 % versus 3.45%). Plasma prolactin concentration was affected by photoperiod being higher in LDPP than SDPP at 3 weeks prepartum (471.3 ± 40.3 versus 41.1 ± 5.75 ng/mL), 3 weeks postpartum (471.3 ± 40.3 versus 41.1 ± 5.75 ng/mL) and 5 weeks postpartum (365.4 ± 26.6 versus 20.9 ± 4.21 ng/mL). In both treatments, the physiological state of the animal affected the prolactin concentration ($P< 0.05$). Generally, the goats exhibited a diurnal body temperature variation with the highest temperature occurring during the light period and the lowest temperature occurring in the dark period. Time affected the temperature variation at 3 weeks prepartum ($P= 0.0286$) and 5 weeks postpartum ($P< 0.0001$). The body temperature of the goats was higher in LDPP than SDPP at 3 weeks prepartum (39.64 ± 0.06 versus 39.3 ± 0.1^{o}C) and 5 weeks postpartum (40.1 ± 0.15 versus 39.7 ± 0.1^{o}C); implying that temperature is one of the ways the differential photoperiodic information reached the peripheral clock in the mammary gland. The physiological state of the dairy goats affected their body temperature ($P< 0.05$) as well. The light/dark schedule affected the expression of *PER1* ($P= 0.0346$). In the SDPP treatment prepartum, *PER1* expression was higher in the light period than in the dark period ($P= 0.0026$). Comparing SDPP and LDPP indicated that, there was a higher gene expression of the core clock genes in SDPP than in LDPP. Photoperiod treatment affected the expression of *CLOCK* ($P= 0.0304$), *PER1* ($P= 0.0196$), *CRY1* ($P= 0.045$), *CRY2* ($P= 0.0286$) but not *BMAL1* which tended to be higher in SDPP ($P= 0.0812$). In the light phase of the prepartum period, *PER1* gene expression was higher ($P< 0.0001$) in the SDPP treatment than in the LDPP treatment. In the dark phase of the postpartum period, there was a higher expression of *CLOCK* ($P= 0.0078$) and *CRY2* ($P= 0.0493$) in the SDPP treatment than in the LDPP treatment. Our results revealed an effect of physiological state on the clock genes with a general increase in their expression from late pregnancy to lactation. Physiological state had an effect on the gene expression of *BMAL1* ($P= 0.0142$), *CLOCK* ($P< 0.0001$), *CRY1* ($P= 0.0087$) and *CRY2* ($P= 0.0001$). In the light phase of the LDPP treatment, there was an increase in gene expression of *BMAL1* ($P= 0.0147$) and *CLOCK* ($P= 0.0043$) in the postpartum versus prepartum period. In the light phase of the SDPP treatment, there was an increase in gene expression of *CLOCK* ($P= 0.0129$), *PER1* ($P= 0.002$) and *CRY2* ($P= 0.0196$) in the postpartum period versus prepartum period. In the dark phase of the SDPP treatment, there was an elevation in gene

expression of *CLOCK* (*P*= 0.0004) and *CRY2* (*P*= 0.0055) in the postpartum versus prepartum period. On the contrary, in the light phase of the SDPP treatment, *PER1* gene expression was higher prepartum than postpartum (*P*= 0.002). In addition, *PER1* had a treatment by physiological state interaction (*P*= 0.022). Our findings suggest that light is an excellent zeitgeber (time giver) for mammary proliferation. SDPP treatment caused a more robust circadian clock that may have given rise to a better mammary proliferation during late pregnancy and a greater metabolic output during the lactation period that resulted in a higher milk yield. Our study has provided the first report of the effect of photoperiod manipulation (LDPP versus SDPP) on the mammary clock gene expression during late pregnancy and during the lactation period. The molecular clock was affected by the physiological state and, it was upregulated in the lactation period to coordinate the metabolism needed for milk production. Thus, homeorhetic adaptations of the mammary gland to support lactation are mediated by the circadian clock system. Taken together, these results suggest that homeorhetic changes in response to photoperiod treatment are regulated by the circadian clock during late pregnancy and during the lactation period. Management strategies such as photoperiod manipulation that modify the circadian clock, are one of the ways to effectively improve animal production like increase in milk yield and also promote animal welfare.

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ABBREVIATIONS

ARNTL	Aryl Hydrocarbon Receptor Nuclear Translocator-like
BMAL1	Brain and Muscle ARNT-like 1
CLOCK	Circadian Locomotor Output Cycles Kaput
CRY1	Cryptochrome 1
CRY2	Cryptochrome 2
CSN2	Beta-casein
DMI	Dry Matter Intake
E-box	Enhancer box
IGF-1	Insulin-like Growth Factor-1
LALBA	Alpha-lactalbumin
LDPP	Long Day Photoperiod; 16h light and 8h dark
NPAS2	Neuronal PAS Domain Protein 2
PCR	Polymerase Chain Reaction
PER1	Period
Q-PCR	Quantitative Polymerase Chain Reaction
RORE	Retinoic acid-related Orphan Receptor Response Element
SCN	Suprachiasmatic nucleus
SDPP	Short Day Photoperiod; 8h light and 16h dark

1. INTRODUCTION

1.1. Farm Animals and Circadian Rhythms

Goat milk consumption is on the rise because of its better digestibility, buffering capacity, alkalinity, and certain therapeutic advantages compared to milk from other animals (Park et al., 2007). A number of factors can affect milk yield and production from dairy animals. One of these factors is modulation of their circadian rhythms.

Circadian rhythms are roughly 24 – hour cycles of biological processes that display entrainable oscillations. These oscillations can persist in a self-sustained manner even without environmental cues (Takahashi, 2015; Edgar et al., 2012). Almost all organisms including goats have these circadian rhythms. Circadian rhythms evolved as a means for animals to anticipate and adapt to both daily and seasonal fluctuations in environmental conditions (Malpaux et al., 2001; Partch et al., 2014). This ensures that animals modulate their physiology and metabolism in synchrony to their surroundings, according to the daily and seasonal changes in: climate, water, and food availability by modifying their: growth rate, energy balance, reproduction, and habitats. Adaptations are coordinated through circadian and circannual fluctuations in the levels of a number of body hormones, metabolites and neurotransmitters (Malpaux et al., 2001; Goldman, 2001; Suárez-Trujillo and Casey, 2016). The body modulates their cyclic nature by a complex network of interconnected biological processes. These circadian rhythms then manifest in animals as sleep-wake cycles, feeding-fasting cycles, physical activity levels, blood pressure changes, and body temperature fluctuations (Aguilar-Arnal and Sassone-Corsi, 2013; Takahashi, 2015).

Recent findings have also revealed several ways that affect circadian rhythms in farm animals and consequently affecting their yield. Examples of environmental cues in the farm that can modify the circadian rhythms of animals include photoperiod, temperature, and feed (Casey and Plaut, 2012; Dibner et al., 2010). It has been shown that offering different feed rations at different times of the day modifies diurnal rhythms in dairy animals. Feeding time itself can also affect digestibility, hormone levels and body metabolites in animals (Niu et al., 2014, 2017). Several experiments that modified the environment of dairy animals through photoperiod treatments

revealed profound effects on milk yield. Dairy animals exposed to a short day photoperiod at late gestation gave more milk than those exposed to a long day photoperiod (Mabjeesh et al., 2007; Mikolayunas et al., 2008). Likewise, health management of farm animals can affect their circadian rhythms. This is because, the commonly used medications to manage animal diseases have circadian targets and hence sensitive to the time of their administration (Zhang et al., 2014). These circadian events in mammals take place in a well-organized fashion.

1.2. Circadian clocks

In the first place, the organization of the mammalian circadian system has an input pathway through which environmental information is delivered to the master circadian clock; and also output pathways through which the master circadian clock delivers circadian information to synchronize circadian rhythms to peripheral clocks in the rest of the body (Lowrey and Takahashi, 2011). In mammals, the core of this biological network is found in the suprachiasmatic nucleus (SCN) of the hypothalamus of the brain. The SCN is composed of about 20,000 neurons which oscillate in synchrony and consequently serve as the master synchronizer for the entire body (Curtis and Fitzgerald, 2006; Aguilar-Arnal and Sassone-Corsi, 2013). To prove that the SCN is the master clock, its ablation resulted in loss of all hormonal and activity rhythms (Ralph et al., 1990).

The master clock is entrained and synchronized to daily light cycles by photoreceptors found in the retina of the eyes (Buhr and Takahashi, 2013; Kornmann et al., 2007). Rods, cones and most importantly; photosensitive retinal ganglionic cells (pRGCs) - that selectively express melanopsin - mediate this circadian photic entrainment. Subsequently, these intrinsically photosensitive retinal ganglionic cells (ipRGCs) transmit all the light information to the master clock through the retinohypothalamic tract (Brown and Robinson, 2004; Pilorz et al., 2016). The light information then adjusts the phase of the SCN oscillations and consequently synchronize the circadian clock of the body with geophysical time. The SCN then relays this phase information and directs the phase of oscillations found in the rest of the body through a combination of neural, hormonal, temperature and systemic signals (Aguilar-Arnal et al., 2013; Buhr and Takahashi, 2013).

All circadian clocks both central and peripheral are endogenous and share several fundamental features which include: self-sustained and their oscillations persist in constant conditions,

entrainable to environmental cues like light, temperature compensated and hence their endogenous period length is similar across the physiological range, cell autonomous, and genetically determined (Lowrey and Takahashi, 2011; Stratmann and Schibler, 2006).

1.3. Molecular Mechanism of the Circadian clock

The molecular basis of the mammalian circadian clock that produces physiological rhythms results from the interactions of mainly two interlocked transcriptional/translational feedback loops as shown in Figure 1. (Zhang et al., 2014; Chen and Yang, 2015). The primary feedback loop which forms the mammalian core oscillator consists of: BMAL1 (ARNTL) and CLOCK (or its ortholog NPAS2) which are bHLH-PAS (basic helix-loop-helix; PER-ARNT-Single-Minded) proteins that form the positive limb of this feedback circuit. BMAL1 and CLOCK proteins heterodimerize, translocate to the nucleus and initiate transcription from a number of genes which contain Enhancer box (E-box) cis-regulatory elements. Among the target genes are *PER* (*PER1* and *PER2*) and *CRY* (*CRY1* and *CRY2*) with E-boxes in their promoters and subsequently form the negative limb of this feedback loop upon translation (Lowrey and Takahashi, 2011; Buhr and Takahashi, 2013; Amaral and Johnston, 2012).

The PER and CRY proteins also heterodimerize, translocate and accumulate in the nucleus where they repress their own transcription by directly inhibiting CLOCK-BMAL1 transcriptional activities. When PER-CRY complex concentration is degraded below a required level of efficient repression, it relieves the inhibition of the CLOCK-BMAL1 heterodimer, which then initiates a new transcriptional cycle (Sato et al., 2006; Lowrey and Takahashi, 2011; Stratmann and Schibler, 2006).

In addition to the primary feedback loop above; CLOCK-BMAL1 heterodimers also initiate the transcription of a secondary feedback loop which acts in coordination with the primary loop. In the secondary loop, there is also E-box mediated transcription of the orphan nuclear receptor genes *Rev-Erbα/β* (reverse orientation c-erbA α/β) and *RORα/β* (retinoic acid receptor-related orphan receptor α/β). The resulting REV-ERB and ROR proteins then compete for the Retinoic acid-related Orphan Receptor Response Element (RORE) binding sites within the promoter of *BMAL1*. ROR proteins activate transcription of *BMAL1* while REV-ERB proteins repress its

transcription thereby stabilizing the clock mechanism (Amaral and Johnston, 2012; Buhr and Takahashi, 2013; Lowrey and Takahashi, 2011; Takahashi, 2016).

Transcriptional regulation of clock gene expression requires the rhythmic assembly and recruitment to chromatin of multiprotein complexes in a cyclic fashion. A combination of both clock specific and ubiquitous histone-modifying proteins remodel chromatin required for this cyclic transcription process. This has been observed in the acetylation and deacetylation of histones at a number of clock controlled genes (Aguilar-Arnal and Sassone-Corsi, 2013; Buhr and Takahashi, 2013; Etchegaray et al., 2003). Moreover, the CLOCK-BMAL1 heterodimer is recruited to the chromatin at the E-boxes in a circadian manner. The CLOCK protein itself has histone acetyl-transferase (HAT) activity and enzymatically influences chromatin remodeling including acetylating its own transcription partner BMAL1, which is essential for circadian rhythmicity (Doi et al., 2006; Aguilar-Arnal and Sassone-Corsi, 2013).

The stability and the rate at which PER and CRY are degraded also sets the period of the circadian clock. Casein Kinase 1ε and Casein Kinase 1δ enzymes phosphorylate PER proteins making them targets for ubiquitination by βTrCP which are eventually degraded by the 26S proteasome (Shirogane et al., 2005; Eide et al., 2005; Buhr and Takahashi, 2013). AMPK1 phosphorylates CRY1 while DYRK1A/GSK-3β cascade phosphorylates CRY2. FBXL3 then polyubiquinates the CRY proteins which leads to their being targeted for proteosomal degradation (Busino et al., 2007; Lamia et al., 2009; Buhr and Takahashi, 2013).

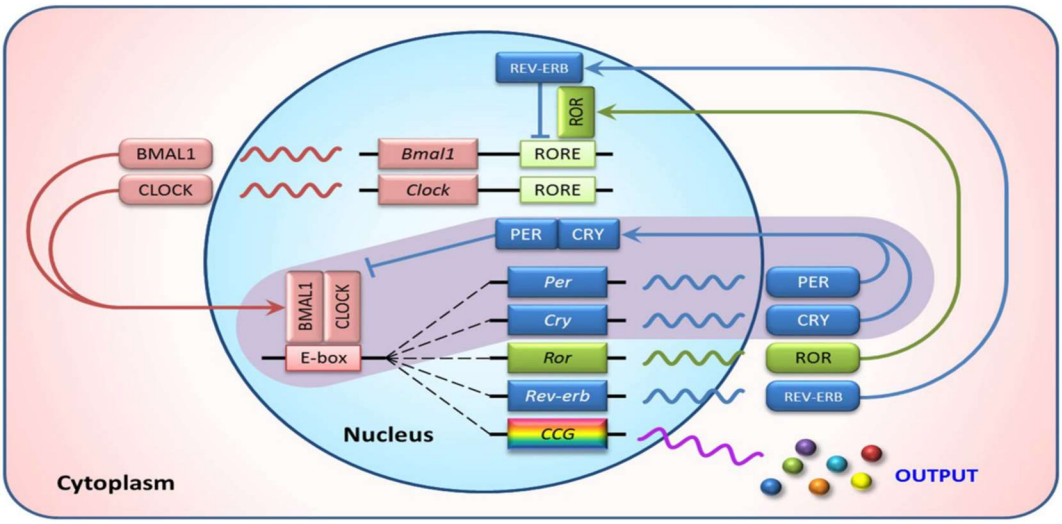

Figure 1: Mammalian Transcriptional Translational Feedback Loop: It consists of a primary feedback loop where CLOCK-BMAL1 heterodimer binds on the Enhancer-box (E-box) elements in the promoters of many clock controlled genes (CCG) including *PER* and *CRY* activating their transcription. PER and CRY proteins heterodimerize and translocate to the nucleus and inhibit their own transcription by interacting with CLOCK-BMAL1 heterodimer. ROR activates while REV-ERB represses Retinoic acid-related Orphan Receptor Response Element (RORE)-mediated transcription of *BMAL1* resulting into a secondary feedback loop (Adapted from Chen and Yang, 2015).

1.4. Peripheral clocks and Temperature

This transcription feedback loop not only occurs in the master clock, but also in peripheral clocks which are found in almost every mammalian tissue including the mammary tissue (Casey et al., 2014b; Wang et al., 2015). The clock mechanism then controls the rhythmic expression of many clock controlled genes in various tissues (Chen and Yang, 2015). Several factors are known to influence this mechanism and entrain clock gene expression in peripheral clocks. These include: neural, humoral, metabolic factors and body temperature fluctuations (Reppert and Weaver, 2002; Buhr and Takahashi, 2013).

Light received by an organism can influence its body temperature levels and rhythms (Gubin et al., 2017). Unlike the SCN which is resistant to cyclic temperature changes within the physiologic range, temperature changes are able to entrain and affect circadian rhythms in peripheral clocks

(Buhr et al., 2010). In addition to the entraining ability of temperature; an acknowledged property of circadian clocks is temperature compensation, whereby a period of a rhythm remains constant at several physiologically permissive temperatures. The temperature resistance of the SCN gives it an advantage as the master clock to be able to generate and unidirectionally control temperature levels in the peripheral clocks of the body (Buhr et al., 2010).

1.5. Circadian rhythms and metabolism

About 10% of the total genome is transcribed in a circadian manner including genes responsible for metabolism. In addition, 43% of all protein coding genes exhibit circadian rhythms somewhere in the body. (Buhr and Takahashi, 2013; Zhang et al., 2014). Some of the genes expressed in a circadian fashion including hormones such as: glucagon, corticosterone, leptin, and insulin; are involved in metabolism (Froy, 2010). Also, glucocorticoids and CRY control how the glucocorticoid-responsive elements (GREs) activate and repress transcription by the glucocorticoid receptor (Lamia et al., 2011; Bass, 2012). Additionally, glucocorticoids modulate the expression of REVE-ERB-α which is a negative regulator of *BMAL1* transcription (Preitner et al., 2002; Bass, 2012). PER2 can likewise interact with several nuclear hormone receptors and hence coregulating nuclear receptor mediated transcription and the nuclear receptors then direct many pathways that modulate metabolism and physiology (Schmutz et al., 2010). CLOCK also interacts with the estrogen receptor alpha (ERα), although estrogen seems to boost transcription directed by CLOCK and ERα through CLOCK-dependent sumoylation (Masri et al., 2015).

Additionally, the circadian clock has been shown to regulate body metabolism and energy balance in peripheral tissues. ATP and several metabolites like glucose, fatty acids circulate in a circadian fashion (Froy, 2010, 2012; Masri et al., 2015). On the other hand, it has been revealed that cellular energy levels influence circadian rhythms. Importantly, NAD+:NADH ratio regulates CLOCK-BMAL1 ability to bind to DNA (Froy, 2012; Buhr et al., 2010).

Appropriate body metabolism requires proper functioning of the circadian clocks and circadian rhythms to sustain it. An increasing number of studies have shown how disrupting the circadian rhythms leads to metabolic disorders. Furthermore, many metabolic diseases have been shown

to result from faulty circadian clocks. For example, pancreatic beta cells with a non-functional clock gene, *BMAL1*, leads to hypoinsulinaemia and diabetes (Marcheva et al., 2010; Buhr et al., 2010; Froy, 2012). Hence, the circadian clock is deeply implicated in the metabolic control of the body. Extensive cross-talk between circadian clock and the metabolic systems enables organisms to anticipate physiological requirements in advance of the daily light-dark cycle in order to adjust the phase of internal cycles according to environmental changes (Bass, 2012; Masri et al., 2015). In addition to regulating circadian rhythms, circadian clock genes together with circadian controlled genes control metabolism in several peripheral tissues including the liver and mammary gland (Wang et al., 2015). Therefore, the circadian clock is crucial in controlling metabolism in a metabolically demanding period of an mammal like lactation (Casey et al., 2014b)

1.6. Mammary gland and circadian clock

The mammary gland is an organ in mammals responsible for secreting milk. It is mainly composed of alveoli, a number of ducts with a stromal compartment (Hennighausen and Robinson, 2005). Mammary gland development begins during fetal life (Hennighausen and Robinson, 2005). During puberty, further development arises with accelerated ductal outgrowth and branching. During pregnancy, the mammary gland proliferates and the alveolar compartment matures accompanied by colostrogenesis towards parturition (Hennighausen and Robinson, 2005; Suárez-Trujillo and Casey, 2016). Upon delivery, the mammary gland is fully developed, it produces and secretes milk to nourish the young. When milk secretion is established, galactopoiesis ensues (Hennighausen and Robinson, 2005; Suárez-Trujillo and Casey, 2016). Control of metabolism in several body tissues during pregnancy and lactation involves a homeorhetic regulation besides homeostatic control in order, to ensure the dramatic mammary development and functioning during these demanding physiological states (Bauman and Currie, 1980). At the end of lactation, involution occurs with massive cell death, collapse of the alveoli and the epithelial compartment is remodeled (Hennighausen and Robinson, 2005).

The circadian clock highly influences this process of proliferation and differentiation in the mammary gland. It has been shown that the events of cell division in the mammary gland occur

in a timely fashion (Borst and Mahoney, 1982). This process of cell division is unidirectionally affected by the circadian clock (Matsuo et al., 2003). The differential expression of the core circadian genes during pregnancy and lactation has been revealed. Furthermore, it has been demonstrated that a genetic loss of a core circadian clock gene negatively affects mammary gland development (Casey et al., 2014b, 2016).

1.7. Photoperiod and the circadian clock in farm animals

Photoperiod - which embodies the light/dark cycle - is the most effective environmental cue for the entrainment of the circadian clocks in most organisms (Piccione et al., 2008; Buhr and Takahashi, 2013). Photoperiod involves the use of day length, to time changes in the body's physiology and behavior (Goldman, 2001). Environmental manipulation like photoperiod treatment is one of the ways to increase milk production in dairy animals like goats because it is non-toxic, non-invasive and effective (Mabjeesh et al., 2007).

Exposure of dairy goats, cows and ewes to a short day photoperiod during the dry period has resulted into a significant increase in milk yield compared to a long day photoperiod (Mabjeesh et al., 2013; Velasco et al., 2008; Mikolayunas et al., 2008). A number of factors have been attributed to this higher milk yield which include the inverse relationship between the prolactin hormone and the prolactin receptor in the mammary gland, lymphocytes and hepatic tissues. Animals exposed to a short day photoperiod have lower prolactin hormone levels but a higher expression of the prolactin receptor in the mammary gland, lymphocytes and hepatic tissues (Velasco et al., 2008; Auchtung et al., 2003). Likewise, IGF-1 is impacted by photoperiod treatment.

Furthermore, dairy animals exposed to a short-day photoperiod during late pregnancy have a better mammary growth than those exposed to a long day photoperiod (Crawford et al., 2015). They also tended to have lower apoptosis levels of their mammary epithelium (Wall et al., 2005; Crawford et al., 2015). It is generally accepted that mammary glands during pregnancy are experiencing cell proliferation and progress through various levels of the cell cycle. The beneficial effects of the short day photoperiod which increases mammary cell proliferation and decreased mammary cell death have been attributed to the indirect effects of the inverse relationship of

prolactin and the prolactin receptors in the mammary gland (Crawford et al., 2015). Another relationship has been suggested with IGF-II (Velasco et al., 2008). We propose that these are downstream effects of the circadian clock system which do not fully address the fundamental mechanism of how photoperiod impacts milk production and metabolic balance. Besides, 7% of the genes expressed in the mammary gland have circadian patterns of expression (Maningat et al., 2009).

The circadian clock has been shown to control several steps of the cell cycle (Matsuo et al., 2003). The core circadian clock can activate genes such as Cyclin D1, c-Myc and Wee1, that directly modulate progress in the cell cycle (Hunt and Sassone-corsi, 2007; Matsuo et al., 2003) Added to this, is the failure of animals without a functional core clock gene to sufficiently produce milk (Casey et al., 2016). Recent findings have also shown that; animals exposed to shifting light and dark cycles exhibit changes in the expression of a core clock gene: BMAL1 and genes whose products regulate fatty acid synthesis. Furthermore, expression of milk proteins *CSN2* and *LALBA* were significantly reduced (Casey et al., 2014a).

A few researchers have shown that different photoperiod treatments in farm animals differentially affect clock gene expression in some of tissues like the SCN, pituitary gland and liver (Lincoln et al., 2002; Andersson et al., 2005). However, the effect of photoperiod treatment during late pregnancy on the expression of the core clock genes in the mammary gland has not yet been established.

We hypothesized that photoperiod affects the mammary circadian clock which regulates genes important to mammary proliferation during late pregnancy and metabolic activity (milk synthesis) in the lactation period. Clock controlled gene expression may be impacted by environmental exposure and physiological state. Higher expression of clock genes leads to greater metabolic activity that increases milk production.

1.8. Significance of the study

There is an increase in the demand of goat milk and its products like yoghurt, cheese and milk powder because of its greater benefits in comparison with milk from other animals (Mabjeesh et al., 2007; Park et al., 2007). Besides, goats are a much greater source of milk than cows for people

in the developing world who are prone to malnutrition (Haenlein, 2004). Increasing importance of goat milk arises from its advantages such as providing a suitable alternative and treatment option for people who suffer from cow milk allergies and gastro-intestinal disorders (Haenlein, 2004). To increase this milk production, one of the effective and non-invasive means is by photoperiodic manipulation (Mabjeesh et al., 2007). However, the process of how increase in milk yield due to photoperiod treatment takes place is not fully understood. This study aimed at adding to that body of growing information in order to inform better strategies on how to increase milk production.

2. OBJECTIVES

The main goal of this study was to find out the effect of photoperiod on the mammary expression of core clock genes (*BMAL1*, *CLOCK*, *CRY1*, *CRY2* and *PER1*) during late pregnancy and lactation.

2.1. Specific Objectives

- To measure the impact of long day versus short day photoperiod exposure during the dry period on mammary abundance of *BMAL1*, *CLOCK*, *CRY1*, *CRY2* and *PER1* genes; at 3 weeks prepartum and 5 weeks postpartum.
- To evaluate the effect of physiological state (late pregnancy versus lactation) on the mammary expression of the Core clock genes: *BMAL1*, *CLOCK*, *CRY1*, *CRY2* and *PER1*.
- To measure the effect of photoperiod on the prolactin hormone profile during late pregnancy and lactation periods.
- To assess the effect of photoperiod on temporal body temperature under the different photoperiod regime.
- To analyze the effect of photoperiod on milk yield.
- To measure the effect of photoperiod on milk components (fat, protein, lactose, somatic cell count).

3. MATERIALS AND METHODS

3.1. Animal Studies and Experimental design

All procedures in this study involving animals were approved by the Hebrew University Institutional Animal Care and Use Committee (Rehovot, Israel). To test the photoperiod effect, a total of 12 Israeli Saanen goats were assigned to 2 treatments of 6 goats each at dry off (45 days prepartum) based on their body weight and previous milk production. They were assigned to either the long day photoperiod group (LDPP) which had 16 hours of light and 8 hours of darkness or short-day photoperiod group (SDPP) which had 8 hours of light and 16 hours of darkness at normothermic ambient temperature (23 ± 2°C). All goats were housed in two separate but identically environmentally controlled rooms which were attuned in accordance to the treatment (photo phase light intensity = 350 lux at eye level of goats). Goats were fed daily at 0700 – in the light phase - a total mixed ration (TMR) and pellets to meet their nutrition requirements for adequate body maintenance and also meet the demands of their physiological stage. 600g of pellets were offered per goat during pregnancy and 1200 – 1500 g of pellets were offered per goat during the lactation period depending on milk production and time postpartum. TMR was offered adlibitum. The dry matter intake was measured for each group and continuously adjusted to allow for 10% refusals. The TMR in the diet consisted of 12.5% CP, 48.2% NDF, 2.76% Crude fat and 2.3 MCal/kg DM. The pellets consisted of 26% CP, 3.63% Crude fat and 2.7 MCal/kd DM (Table 1).

Table 1. Total mixed ration (TMR) and pellets composition of goats (expressed as % DM).

Feeds	TMR	Pellets
Corn grains	13.9	10.4
Wheat bran	19.6	1.7
Gluten feed		5.3
Barley grain		11.4
Soybean meal		31.2
Sunflower meal	9.5	6.1
Wheat grain		11.3
DDGs		7.0
Vegetable oil		1.2
Grass hay	15.1	
Wheat silage	11.3	
Wheat straw	15.8	
Citrus pulps	2.5	
Soybean hulls	9.5	0.9
Lime stone	1.3	5.0
Salt	0.5	5.0
NH_4Cl		0.5
Na bicarbonate		1.0
Cu sulfate	0.02	
Vitamin mix*	0.9	1.9
Composition,		
Moisture, %	37.1	10.4
Crude protein, %	12.5	26.0
Crude fat, %	2.76	3.63
ME, Mcal/ kg DM	2.30	2.70
NDF, %	48.2	16.6
Ca, %	0.85	2.30
P, %	0.46	0.48
Cu, %	0.49	0.51
Vit A, IU	5,862	14,000

*Vitamin premix 3678®, Bar Magen LTD, Israel. Vit A 8MIU, Vit E 1.6MIU, Vit E 20KIU, Anilox 15g, Mn 30g, Zn 100g, Fe 20g, I 0.5g, Co 1g, Se 0.1g, NaCl 3Kg, Na_2SO_4 3kg, NH_4Cl 5kg, limestone 0.5kg.

Goats remained in the experimental rooms from dry off until the completion of the study at 120 days postpartum. For the LDPP; lights were switched on for 16 hours beginning at 0600 up to 2200. For the SDPP; lights were switched on for 8 hours beginning at 0700 up to 1500. Lights were already on for both SDPP and LDPP treatments by 0730 so that daily milking could be done in the light phase for both treatments. Goats were milked once a day. Daily milk production was

recorded by automatic milk meter (Free-flow, Afigoat system, Affikim, Israel) for 120 days postpartum to sufficiently detect differences according to treatment. In addition, milk samples were taken on a weekly basis and analyzed by infrared procedure (Milkoscan 605; Foss Electric, Hillerød, Denmark).

3.2. Body Temperature

Serial rectal temperature measurements were taken from each of the goats over a 24 – hour period at 3 weeks prepartum, 3 weeks postpartum and 5 weeks postpartum. On each sampling day, temperature was taken 6 times at a 4 hour-interval within a period of 24 hours (0200, 0600, 1000, 1400, 1800, 2200 hours) to capture circadian rhythms in degrees Celsius using a digital thermometer (PROCARE, Vega Technologies Inc., Taipei, Taiwan).

3.3. Prolactin hormone analysis

Blood samples of 5 mls were collected from each goat via venipuncture of the jugular vein into a sterile evacuated tube containing sodium heparin (Vacutainer; Becton Dickinson and Co., Franklin Lakes, NJ), and instantly placed on ice. Blood was sampled every 4 hours within a 24 – hour period (0200, 0600, 1000, 1400, 1800, 2200 hours); at 3 weeks prepartum, 3 weeks postpartum and 5 weeks postpartum to capture circadian rhythms. Plasma was harvested from the blood after centrifugation at 4°C, 3000 x g for 15 minutes.

A quantitative sandwich Enzyme Linked Immunosorbent Assay (ELISA) was performed to obtain prolactin concentration in plasma samples. ELISA plates (Nunc, Thermo, Denmark) were coated with 1µg /ml of Monoclonal mouse anti-prolactin IgG1 (Clone 6F11, Abnova, Taiwan) diluted in bicarbonate buffer, pH=9.6 (Thermo, IL, USA) and incubated in a humidified chamber at 4°C overnight. Coated plates were then blocked with 0.5% skim milk (BD, Difco, Sparks, MD, USA) in phosphate buffered saline (Thermo, IL, USA) at 37°C for 2 hours. Plasma samples (diluted 1:10 in blocking solution) and a serially diluted recombinant prolactin (Prospecbio, Ness-ziona, Israel) used for standard curve; were placed on plates and incubated in a humidified chamber at 4°C overnight. Rabbit anti ovine prolactin (Bio-Rad, Hercules, CA, USA) diluted 1:10,000 in blocking solution was added and plated; and then incubated in a humidified chamber at 37°C for 2 hours. Detection was done using HRP conjugated goat anti rabbit IgG (Jackson, PA, USA) diluted

1:10,000 in blocker at 37°C for 1 hour. TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was used as substrate. Optical absorbance was determined at 450nm using a Bio Tek microplate reader (Bio Tek, Winooski, VT, USA). Plasma prolactin concentrations were determined by comparing absorbance values to a standard curve.

3.4. Mammary tissue sample analysis

3.4.1. Mammary biopsies

Serial tissue biopsies were taken from the mammary gland after local anesthesia was administered into the area of incision as described (Mabjeesh et al., 2005). They were taken at 3 weeks prepartum and 5 weeks postpartum for all goats at the expected peak and trough of BMAL1 abundance in a circadian cycle (Casey et al., 2017, unpublished). For the SDPP group, mammary biopsies were taken at 1000 hours and 2200 hours while for the LDPP, mammary biopsies were taken at 1400 hours and at 0200 hours. At each time point, biopsies were taken from a different half of the udder. A small piece of the mammary biopsy (100-150 mg) was put in RNA Save (Biological Industries Israel Beit Haemek Ltd., Kibbutz Beit Hammek, Israel) kept overnight at 4°C and later stored in -80°C until mRNA extraction was performed according to manufacturer's instructions.

3.4.2. mRNA Isolation

Total mRNA was isolated from the mammary tissue sample using TRIzol-Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

3.4.3. mRNA abundance determination by Real Time Polymerase Chain Reaction

cDNA was synthesized from 1.0 µg total RNA with a Revert Aid RT-PCR Kit (Thermo Fisher Scientific, Tamar, Mevaseret-Zion, Israel) in a T100™ Bio-Rad Instrument. The PCR fragments were authenticated by carrying out electrophoresis on 1.5% agarose gel.

Q-PCR analysis was performed with SYBR green, with primers designed to measure expression of two reference genes (BACTIN and GAPDH) and the core clock genes: *ARNTL* (aka *BMAL1*), *CLOCK*, *PER1*, *PER2*, *CRY1* and *CRY2* (Table1). Real-time PCR was carried out using a Roche LightCycler®

96 instrument for all the samples. The PCR reaction was composed of: 3.0 µL of cDNA sample diluted at 1:25, 1 µL of each primer (4 µM) (Table 2) and 10 µL of Platinum SYBR Green qPCR supermix-UDG (Thermo Fisher Scientific, Rhenium, Israel) in a final volume of 20 µL. All the PCR reactions were then performed in duplicate in ABgene PCR plates (Thermo Fisher Scientific) closed with optically clear flat QPCR caps (Thermo Fisher Scientific) under the following conditions: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of 95°C for 30 seconds and 60°C for 1 minute.

To control for false positives, a non-template control was included for each template and primer pair. Cycle threshold (Ct) values for all samples were calculated using Roche LightCycler® 96 program. Relative gene expression was calculated using the delta-delta CT method (Livak and Schmittgen, 2001) with mean of SDPP treatment across all circadian time points as the normalizer.

Table 2: Primer sequences employed for real-time PCR analysis of core circadian clock gene expression in the mammary tissue of dairy goats.

Gene	Forward	Reverse
ARNTL (BMAL1)	GTGCAACGCGATGTCTAGCA	AAATCCATCTGCTGCCCTGA
CLOCK	GCCTGAAAGACACGAGAACT	TTGGCATCTTTCTTGGTGTAG
PER 1	GTACGTGCTCCAGGATCCAAT	GTTCCCTCCGCTGGTCCTC
CRY 1	AAGACCTCGAATGAATGCAA	ATCTGAACACAAATGCGGTTT
CRY 2	GCCGCCCCTTACCTACAAGC	CGTCTCTCCTCCCCGCCAGA
GAPDH	TTCCAGTATGATTCCACCCATG	GCCTTTCCATTGATGACGAG
BETA ACTIN	TGGACTTCGAGCAGGAGATGG	CACCGTGTTGGCGTAAAGGGTC

3.5. Statistical Analysis

A two-way analysis of variance (ANOVA) was used to estimate the statistical significance of the treatment (LDPP vs SDPP) and one of the two parameters: either time or physiological state as the fixed variables, goat as the random variable, and their interaction. Comparisons of means between treatments were performed using a Student's t-test. Data are summarized as least squares means ± standard error (SE). Statistical analysis was performed using JMP Pro software (version 13.0.0 SAS Institute Inc., Cary, NC, 1998 - 2014). A 0.05 significance level was used.

4. RESULTS

4.1. Performance of goats and milk yield

All the selected 12 goats were healthy throughout the experiment. Group dry matter intake was similar between treatments during the dry period averaging at 1574.5 g/d/goat (Table 3). On the other hand, in the lactation period, apparent dry matter intake was significantly higher in the SDPP treatment than LDPP averaging at 1699 ± 32.3 g/d/goat and 1413 ± 24.1 g/d/goat respectively (P< 0.001; Table 3). Animals in LDPP had a shorter gestation period than those in SDPP averaging 144 ± 1.4 d and 151 ± 1.9 d respectively (P< 0.0179; Figure 2).

During the first 12 weeks in lactation (WIL), goats exposed to the SDPP produced more milk than those in the LDPP averaging at 3.15 kg/d versus 2.7 kg/d respectively (Figure 3 and Table 4; P< 0.0001). Week in lactation also affected the milk yield (P= 0.0026). Milk production for SDPP peaked in week 7 while for LDPP: in week 10. Milk percentage protein was higher in the SDPP than LDPP (Figure 4A and Table 4; P = 0.0407). However, there were no significant differences between treatments in somatic cell count averaging at 1,932 x 10^3cells/ml. Percentages of fat and lactose were also similar between treatments averaging at 3.49% and 4.72% respectively (Table 4 and Figure 4).

Table 3: Group dry matter intake (g/d/goat) during late pregnancy and lactation period of goats exposed to the long day photoperiod (LDPP) or short-day photoperiod (SDPP).

	Photoperiod			
Physiological State	LDPP	SDPP	SEM	Effect (P-value)
Pregnancy	1499	1650	48.6	0.1602
Lactation	1413	1699	27.6	<0.0001

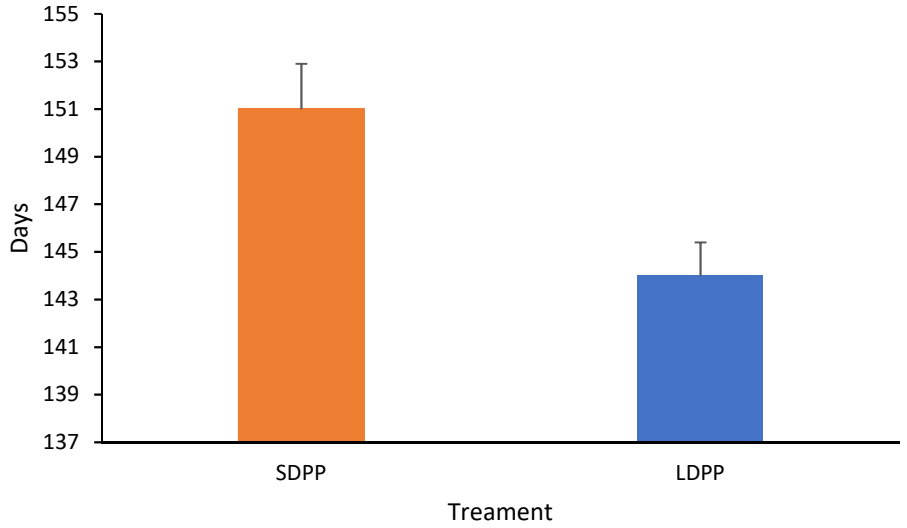

Figure 2: Mean gestation period ± SE between the SDPP and LDPP treatment. Effect of treatment (*P*= 0.0179).

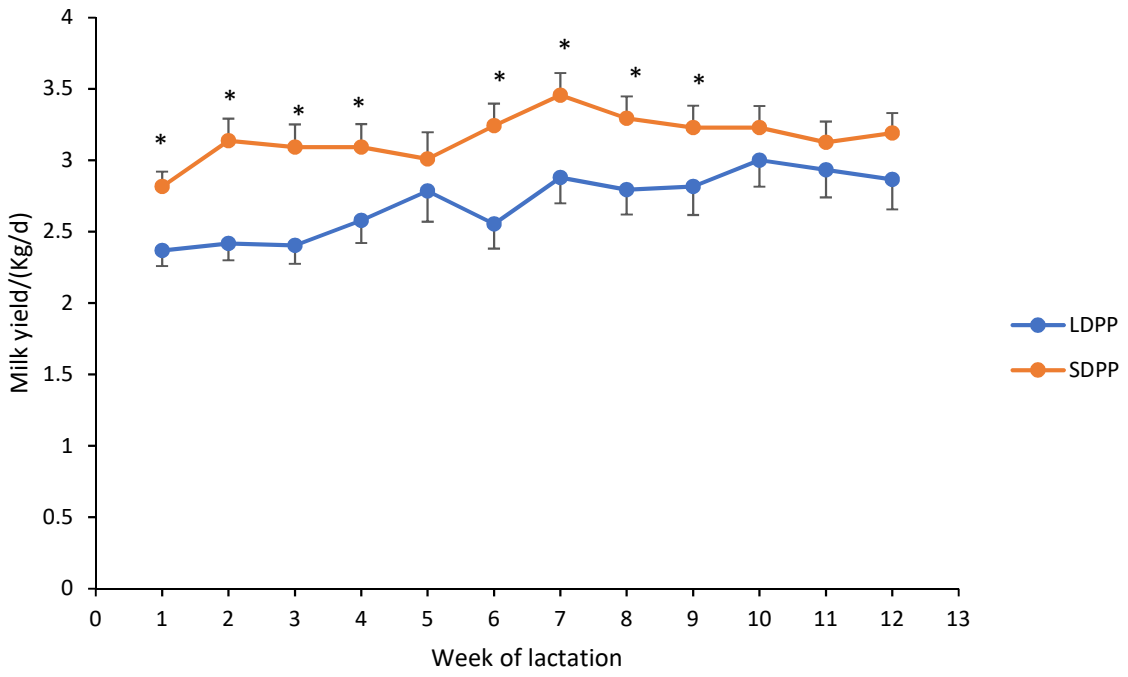

Figure 3. Milk yield of dairy goats exposed to long-day photoperiod (LDPP) or short-day photoperiod (SDPP) during late pregnancy and lactation period (SEM = 0.034; *P*< 0.0001 for Photoperiod treatment, *P*= 0.0026 for week in lactation).

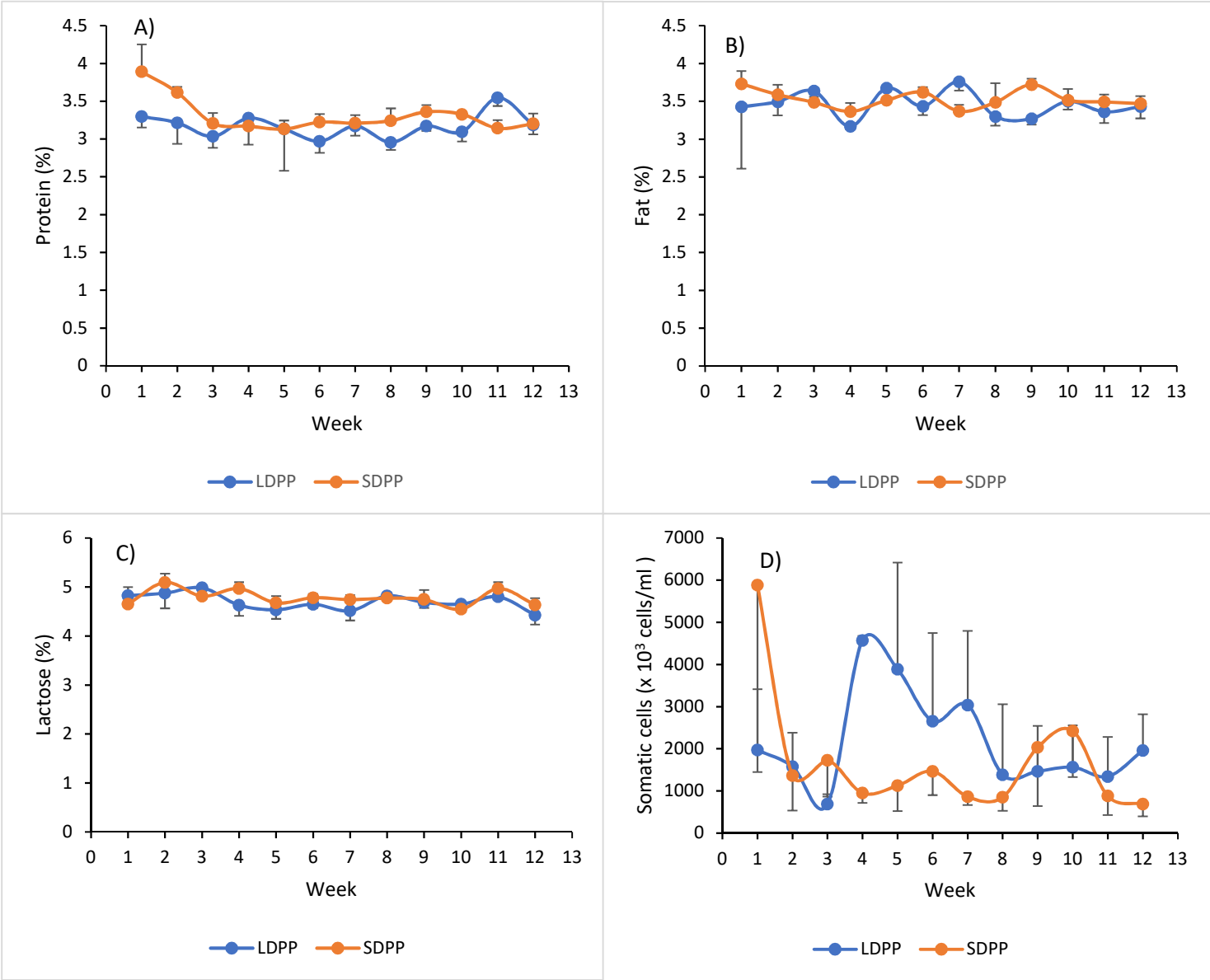

Figure 4. Percentage of milk protein (A), milk fat (B), milk lactose (C) and; the somatic cell count (D) from goats exposed to long-day photoperiod (LDPP) or short-day photoperiod (SDPP) during late pregnancy and the lactation periods.

Table 4. Milk yield and milk composition of dairy goats exposed to LDPP or SDPP during late gestation and lactation periods (12 WIL).

	Photoperiod			Effect (*P*-value)		
	LDPP	SDPP	SEM	Photoperiod	WIL	PP X WIL
Milk yield (kg/d)	2.70	3.15	0.034	<0.0001	0.0026	0.9147
Milk composition						
Fat (%)	3.45	3.53	0.026	0.1518	0.3228	0.1141
Protein (%)	3.17	3.31	0.034	0.0407	0.1578	0.2620
Lactose (%)	4.70	4.74	0.032	0.5004	0.2022	0.5958
Somatic Cells(x10^3cells/ml)	2175	1689	305.8	0.4597	0.9057	0.7109

PP - Photoperiod

WIL – Week in Lactation

4.2. Body temperature

Generally, the goats exhibited a diurnal rectal temperature variation with the highest temperature occurring during the light period and the lowest being recorded in the dark period. The temperature variation of the goats followed the lighting schedules of their respective treatments.

At 3 weeks prepartum, photoperiod affected the rectal temperature, being higher in the LDPP treatment than in the SDPP treatment averaging at 39.64 ± 0.06 and 39.3 ± 0.1^{o}C respectively (P< 0.0066; Figure 5 and Table 5). There was also a significant effect of time (P = 0.028). In the LDPP group, the highest mean temperature (39.9^{o}C) occurred towards the end of the light period at 18:00 hours and the lowest temperature (39.44^{o}C) an hour after lights were switched off at 22:00 hours (Figure 5). In the SDPP treatment, the mean highest rectal temperature (39.5^{o}C) occurred during the light period at 10:00 hours while the lowest temperature (39.25^{o}C) was obtained in the night at 2:00 hours. In both treatments, there was a notable rise in the rectal temperature towards the feeding time which took place at 7:00 hours (Figure 5).

At 3 weeks postpartum, the rectal temperature was similar between treatments averaging at 37 ± 0.08°C (Figure 6 and Table 5). Likewise, there was no effect of time (Table 5). For the LDPP treatment, the highest temperature occurred at 14:00 hours during the light period (39.9°C) while the lowest temperature (39.6°C) occurred at night at 22:00 hours (Figure 6). LDPP exhibited an increase in temperature towards lights on but decreased immediately upon switching on. SDPP showed an increase in temperature towards the feeding time which coincided with the lights on (Figure 6).

At 5 weeks postpartum, photoperiod affected the rectal temperature being higher in LDPP than SDPP averaging at 40.1 ± 0.15 and 39.7 ± 0.1°C respectively (P< 0.0272; Figure 7 and Table 5). There was also a significant effect of time (P< 0.0001; Table 5). For the LDPP group, the highest temperature (40.6°C) was recorded at 6:00 hours which coincided with the time for switching on the lights while the lowest temperature (39.8°C) was recorded in the dark at 22:00 hours (Figure 7). On the other hand, for the SDPP group, the highest temperature (40.1°C) was recorded a few hours after lights went off at 18:00 hours and the lowest temperature was also recorded in the dark at 2:00 hours (Figure 7). A sharp rise in the temperature for LDPP animals took place towards lights on followed by a drop. For the SDPP group, a gradual increase in temperature occurred towards the feeding time (Figure 7). There was no photoperiod by treatment interaction for all physiological states.

The physiological state affected the rectal temperature of the dairy goats in both the LDPP (P< 0.0065) and SDPP (P< 0.0478) treatments. In the LDPP treatment, the highest temperature occurred 5 weeks postpartum; followed by 3 weeks postpartum and the lowest at 3 weeks prepartum averaging at 40.2 ± 0.17, 39.9 ± 0.11 and 39.7 ± 0.06°C respectively (Figure 8). Likewise, in SDPP treatment, the highest temperature was obtained at 5 weeks postpartum followed by 3 weeks postpartum and the least at 3 weeks prepartum averaging at 39.9 ± 0.13, 39.8 ± 0.15 and 39.4 ± 0.1°C respectively (Figure 8).

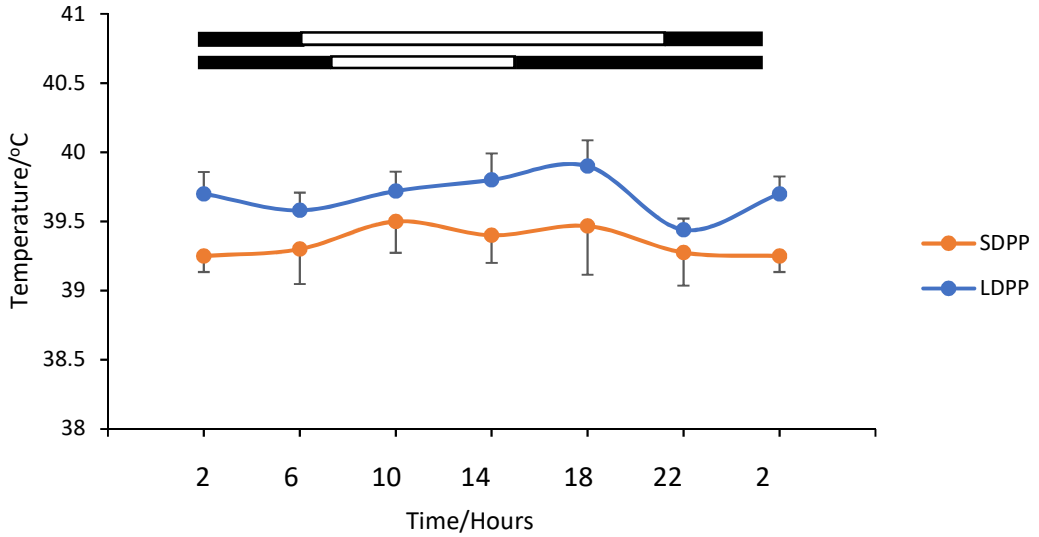

Figure 5: 24 – hour profile of mean rectal temperature variation of goats exposed to a long-day photoperiod (LDPP) or short-day photoperiod (SDPP) at 3 weeks prepartum. Average rectal temperature differed between photoperiod treatment (*P*= 0.0065) and time of sampling (*P*= 0.0286). No photoperiod by time interaction (*P*> 0.05). SEM = 0.06. White rectangles show light period while black rectangles show dark period.

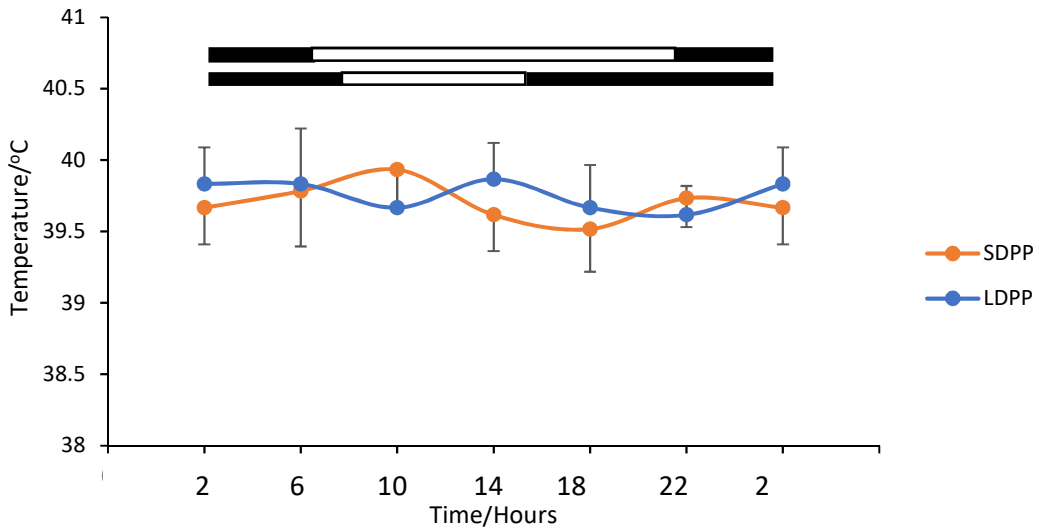

Figure 6: 24 – hour profile of mean rectal temperature variation of goats exposed to a long-day photoperiod (LDPP) or short-day photoperiod (SDPP) at 3 weeks postpartum. No significant effect of treatment, no significant effect of hour and no treatment-hour interaction (*P*> 0.05). SEM = 0.08. White rectangles show light period while black rectangles show dark period.

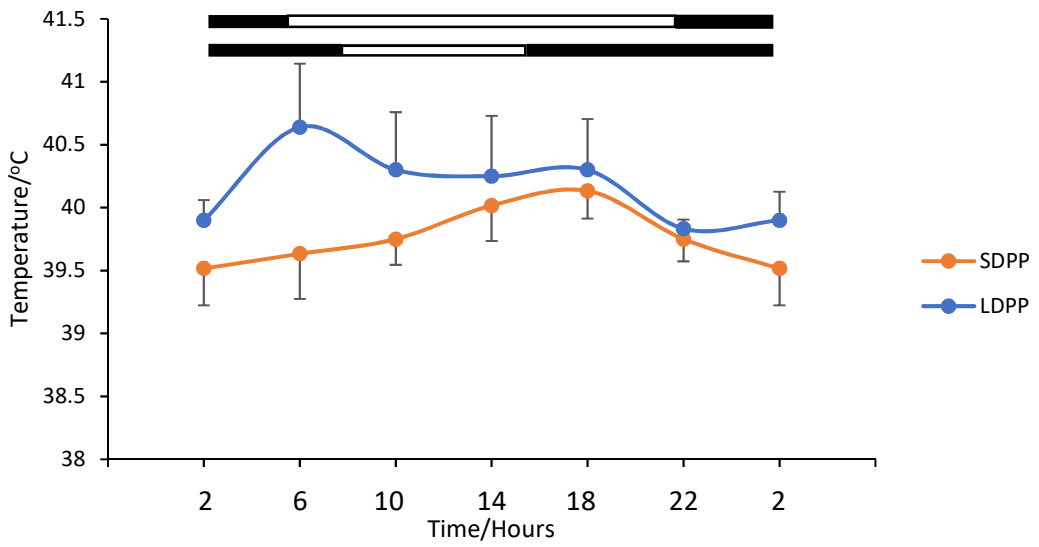

Figure 7: 24 – hour profile of mean temperature variation of goats exposed to a long-day photoperiod (LDPP) or short-day photoperiod (SDPP) at 5 weeks prepartum. Average rectal temperature differed between photoperiod treatment (*P*= 0.0272) and time of sampling (*P*< 0.0001). No photoperiod by time interaction (*P*> 0.05). SEM = 0.09. White rectangles show light period while black rectangles show dark period

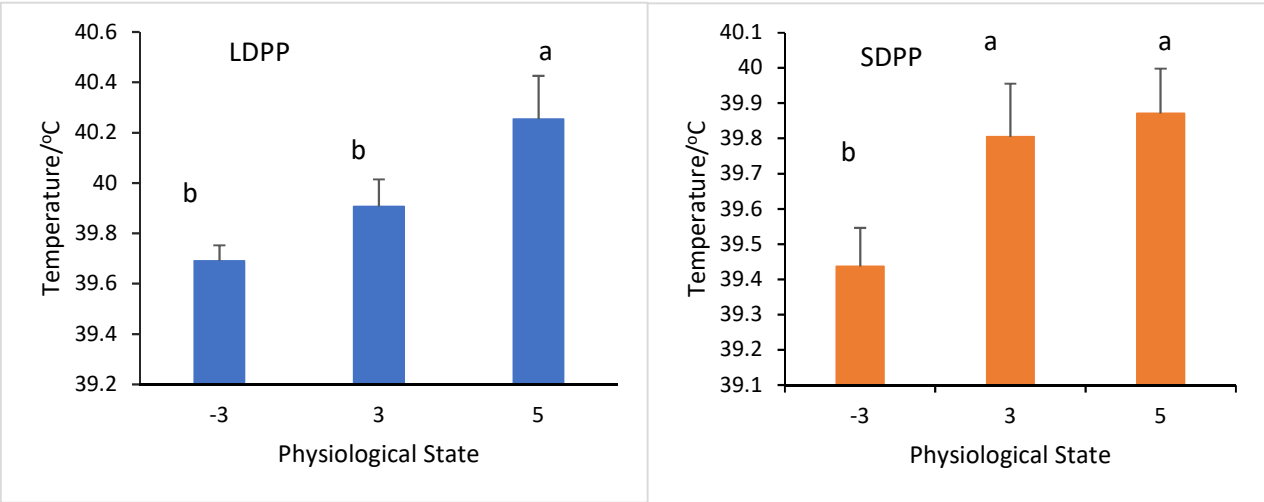

Figure 8: Rectal temperatures of dairy goats exposed to either long day photoperiod (LDPP) or short day photoperiod (SDPP) during late pregnancy and lactation sampled at 3 weeks prepartum (-3), 3 weeks postpartum (3) and 5 weeks postpartum (5). Letters (a, b) denote differences (P< 0.05) among the different production stages.

Table 5: Rectal temperature (°C) of dairy goats exposed to the long-day photoperiod (LDPP) or short-day photoperiod (SDPP) during late pregnancy, measured every 4 hours in a period of 24 hours.

	Photoperiod			Effect (*P*-value)		
Physiological state	LDPP	SDPP	SEM	Photoperiod	Time	PP x Time
3 weeks prepartum	39.6	39.3	0.06	0.0066	0.028	0.982
3 weeks postpartum	39.7	39.7	0.08	0.9178	0.8202	0.6853
5 weeks postpartum	40.1	39.7	0.09	0.0272	0.0001	0.0754

PP - Photoperiod

4.3. Plasma prolactin hormone

Plasma prolactin concentrations were measured at 3 weeks prepartum, 3 weeks postpartum and 5 weeks postpartum. On each sampling day, measurements were made every 4 hours in a period of 24 hours at 0200, 0600, 1000, 1400, 1800 and 2200 hours.

Plasma Prolactin concentrations were affected by treatment ($P< 0.001$), physiological state of the animal ($P< 0.001$) and time of sampling ($P= 0.002$; Table 6). There was also a time by treatment interaction ($P= 0.0015$), physiological state by treatment interaction ($P< 0.0001$).

On all sampling days and hours, plasma prolactin concentrations were significantly higher in the LDPP treatment as shown in Figure 9. At 3 weeks prepartum, plasma prolactin concentrations were significantly higher in LDPP than SDPP averaging at 823.4 ± 56 ng/mL and 40.2 ± 10.2 ng/mL respectively (Figure 9A and Table 6). The highest prolactin concentrations were recorded at 10:00 hours (Figure 9A). Prolactin concentrations were significantly affected by photoperiod ($P< 0.0001$) and time of sampling ($P< 0.0001$). There was also photoperiod by hour interaction ($P< 0.001$; Table 6).

At 3 weeks postpartum, prolactin concentrations were significantly higher in LDPP than SDPP averaging at 471.3 ± 40.3 ng/mL and 41.1 ± 5.75 ng/mL respectively (Figure 9B and Table 6). The highest prolactin concentrations in LDPP were recorded at 18:00 and 22:00 and the lowest at

14:00 hours (Figure 9B). There was a slight increase in prolactin concentrations in SDPP at 06:00 but were nearly the same for the rest of the hours. Prolactin concentration was significantly affected by Photoperiod ($P < 0.0001$) but not by hour of sampling (Table 6). There was a significant photoperiod by hour interaction ($P < 0.0444$; Table 6).

At 5 weeks postpartum, also prolactin concentrations were significantly higher in LDPP than SDPP averaging at 365.4 ± 26.6 ng/mL and 20.9 ± 4.21 ng/mL respectively (Figure 9C and Table 6). Highest concentrations were measured at 14:00 hours and lowest levels at 06:00 hours. In SDPP, prolactin concentrations were slightly high at 02:00 hours (Figure 9C). Prolactin concentrations were affected by photoperiod. The hour of sampling had not effect. There was no photoperiod by hour interaction.

In the LDPP treatment, prolactin concentrations were significantly higher at 3 weeks prepartum averaging at 820 ± 56 ng/mL (Figure 10). At 3 and 5 weeks postpartum, levels were similar averaging at 471.8 ± 40.3 ng/mL and 366 ± 26.6 ng/mL respectively (Figure 10). In the SDPP treatment, prolactin concentrations were similar at 3 weeks prepartum and 3 weeks postpartum averaging at 49.8 ± 7.59 ng/mL and 41.1 ± 5.73 ng/mL respectively while at 5 weeks postpartum, prolactin concentrations were significantly lower averaging at 20.9 ± 6.19 ng/mL (Figure 10).

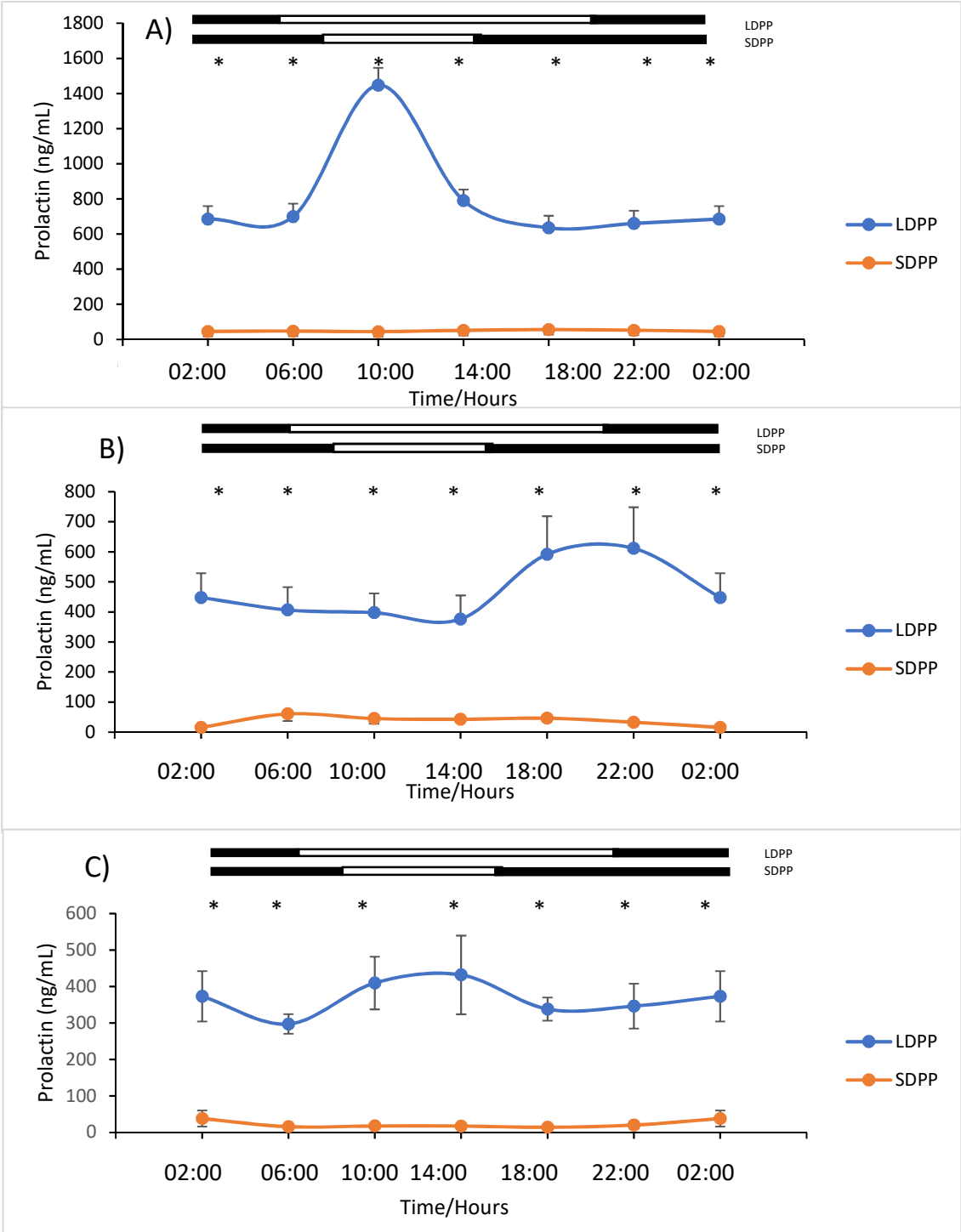

Figure 9. Mean plasma hormone concentration at 3 weeks prepartum (A), 3 weeks postpartum (B), and 5 weeks postpartum (C) of goats exposed to the long day photoperiod (LDPP) or short-day photoperiod (LDPP) during late pregnancy and lactation. (SEM = 59.51; *P*< 0.0001 for photoperiod, *P*= 0.020 for Hour, *P*= 0.0015 for Photoperiod – hour interaction) *Indicates a significant effect of photoperiod. White rectangles show light period while dark rectangles show dark period.

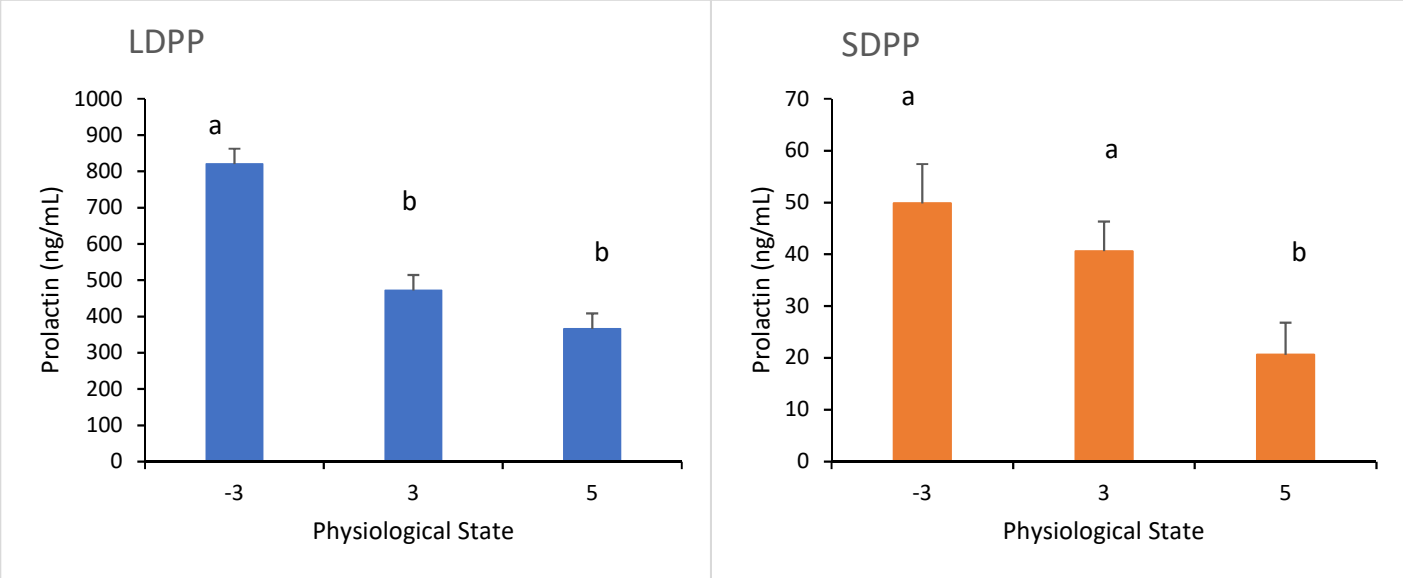

Figure 10: Mean plasma prolactin concentrations of goats exposed to either long day photoperiod (LDPP) or short day photoperiod (SDPP) during late pregnancy and lactation sampled at 3 weeks prepartum (-3), 3 weeks postpartum (3) and 5 weeks postpartum (5). Letters (a – b) denote differences (*P*< 0.05) in prolactin concentrations at the different Physiological states.

Table 6: Plasma prolactin concentrations (ng/mL) of dairy goats exposed to either long-day photoperiod (LDPP) or short-day photoperiod (SDPP) during late pregnancy.

	Photoperiod			Effect (*P*-value)		
Physiological state	LDPP	SDPP	SEM	Photoperiod	Hour	PP x hour
3 weeks prepartum	823.4	40.2	29.9	<0.0001	<0.0001	<0.001
3 weeks postpartum	471.3	41.1	45.03	<0.0001	0.0696	0.0444
5 weeks postpartum	365.5	20.9	27.27	<0.0001	0.6284	0.6784

PP = Photoperiod

4.4. Mammary clock gene expression

All the measured clock genes – *BMAL1, CLOCK, CRY1, CRY2,* and *PER1* – were expressed in the goat mammary gland. The goats were treated to a long-day photoperiod (16 hours of light and 8 hours of darkness) or short-day photoperiod (8 hours of light and 16 hours of darkness). This light/dark cycle affected only *PER1* gene expression (*P*= 0.0346; Table 7). There was a higher relative *PER1* gene expression in the light period than in the dark period in the prepartum phase of the SDPP treatment (*P*= 0.0026; Figure 11A).

To evaluate whether photoperiod affected the mammary circadian clock, we compared clock gene expression between SDPP treatment and LDPP treatment. Overall, there was a relatively higher gene expression in the SDPP treatment than in the LDPP treatment. Photoperiod treatment had an effect on the expression of *CLOCK* (*P*= 0.0304)*, PER1* (*P*= 0.0196)*, CRY1* (*P*= 0.045)*, CRY2* (*P*= 0.0286) but not on *BMAL1* that only tended to be significantly higher in SDPP (*P*= 0.0812; Table 7). In the light phase of the prepartum period, there was a higher gene expression of *PER1* (*P*< 0.0001) and *BMAL1* (*P*= 0.0223) in the SDPP treatment than in the LDPP treatment (Figure 12A). *CLOCK* gene expression tended to be higher in the SDPP treatment (*P*= 0.097). In the dark phase of the prepartum period, *CRY1* and *PER1* tended to be higher in the SDPP treatment than in the LDPP treatment (*P*= 0.0643, *P*= 0.1182 respectively; Figure 12A). In the light phase of the postpartum period, *CRY2* tended to be higher in the SDPP group (*P*= 0.0639). In the dark phase of the postpartum period, there was a higher expression of *CLOCK* (*P*= 0.0078) and *CRY2* (*P*= 0.0493) in the SDPP treatment than in the LDPP treatment (Figure 12B). *CRY1* tended to be higher in the SDPP treatment (*P*= 0.0725).

To determine the effect of the physiological state on the circadian clock, we compared clock gene expression between late pregnancy (prepartum) and lactation (postpartum). Generally, there was an increase in clock gene expression in the postpartum period versus prepartum period. The physiological state had an effect on the gene expression of *BMAL1* (*P*= 0.0142), *CLOCK* (*P*< 0.0001), *CRY1* (*P*= 0.0087), *CRY2* (*P*= 0.0001) but not on *PER1* (*P*> 0.05; Table 7). In the light phase of the LDPP treatment, there was an increase in gene expression of *BMAL1* (*P*= 0.0147) and *CLOCK* (*P*= 0.0043; Figure 13A) in the postpartum versus prepartum period. *CRY2* gene expression

tended to be higher in the postpartum period (*P*= 0.0793; Figure 13A). In the light phase of the SDPP treatment, there was an increase in gene expression of *CLOCK* (*P*= 0.0129), *PER1* (*P*= 0.002) and *CRY2* (*P*= 0.0196) in the postpartum period versus prepartum period (Figure 13B). In the dark phase of the SDPP treatment, there was elevation in gene expression of *CLOCK* (*P*= 0.0004) and *CRY2* (*P*= 0.0055) in the postpartum versus prepartum period (Figure 13B). *CRY1* tended to be higher postpartum (*P*= 0.0897; Figure 13B). On the contrary, in the light phase of the SDPP treatment, *PER1* gene expression was higher prepartum than postpartum (*P*= 0.002; Figure 13B). Additionally, *PER1* had a photoperiod-physiological state interaction (*P*= 0.022). This is because, the SDPP treatment had a higher PER1 gene expression in the prepartum than postpartum whereas the LDPP treatment had a higher PER1 gene expression in the postpartum than in the prepartum period.

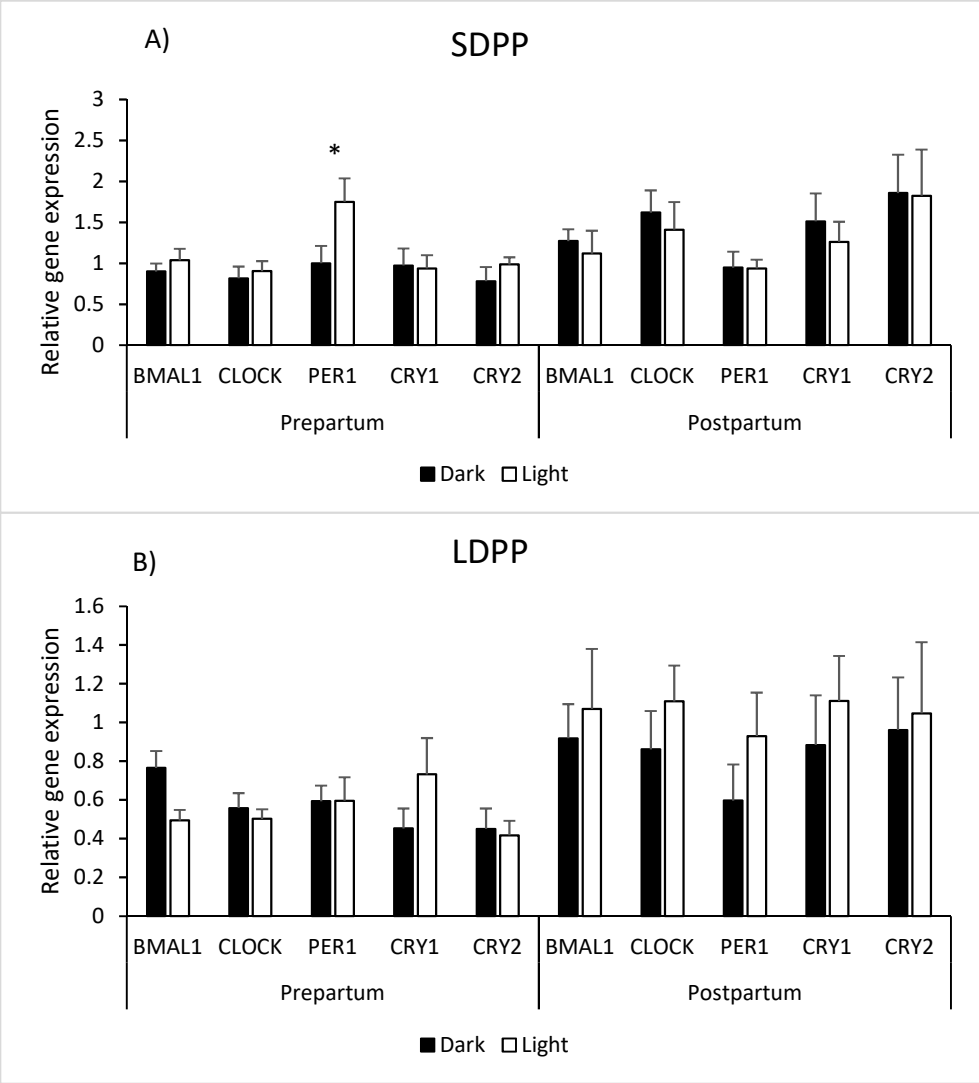

Figure 11: Mean mammary Clock gene expression between light and dark time periods in A) Short day photoperiod (SDPP) treatment and B) Long day photoperiod (LDPP) treatment. Values are presented as relative gene expression ± SE. *P< 0.05.

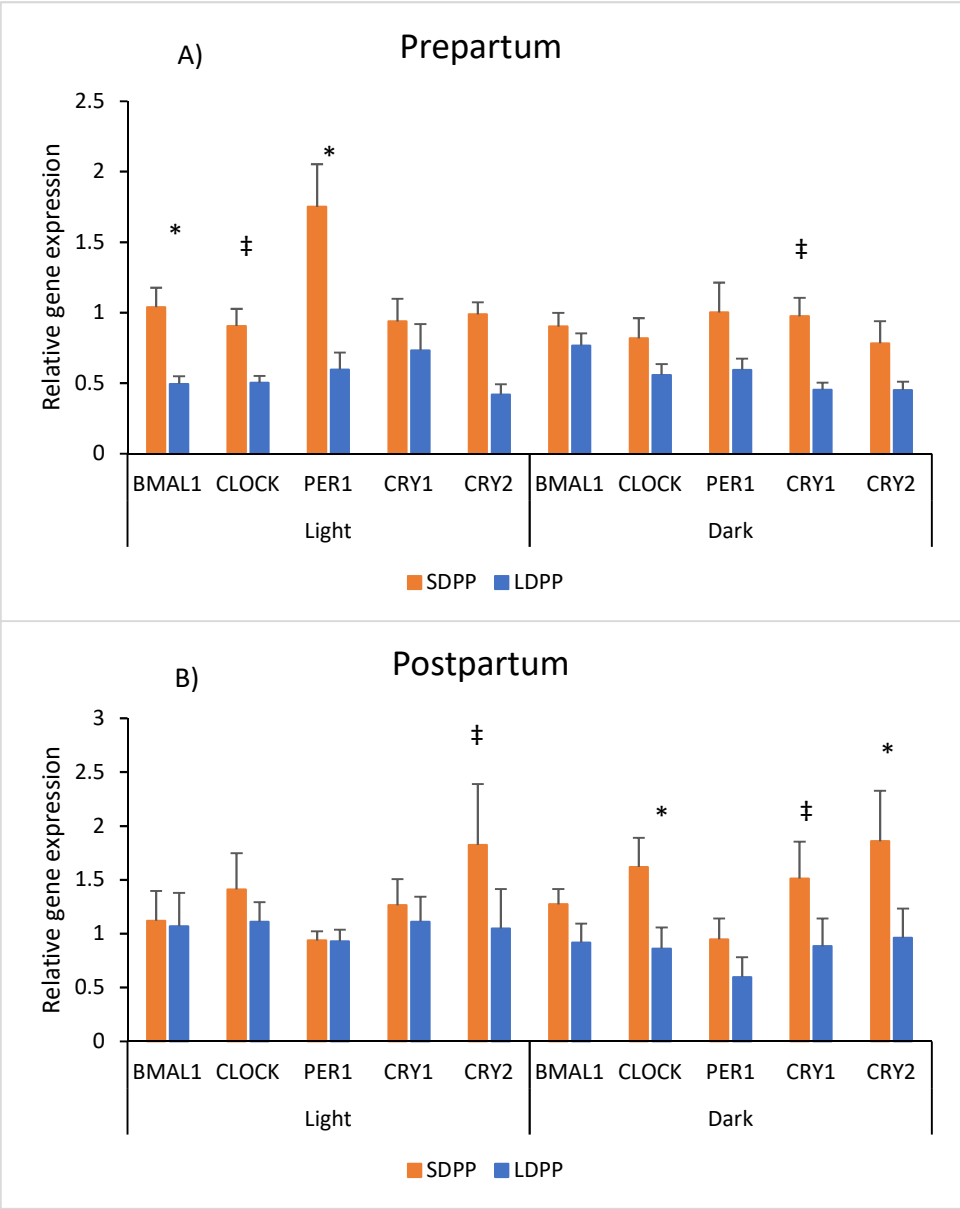

Figure 12: A) Mean mammary Clock gene expression between long-day photoperiod (LDPP) and short-day photoperiod (SDPP) during the prepartum period. B) Mean mammary gene expression between LDPP and SDPP during the postpartum period. Values are presented as Relative gene expression ± SE; with SDPP as the reference. *P< 0.05, ‡P< 0.1

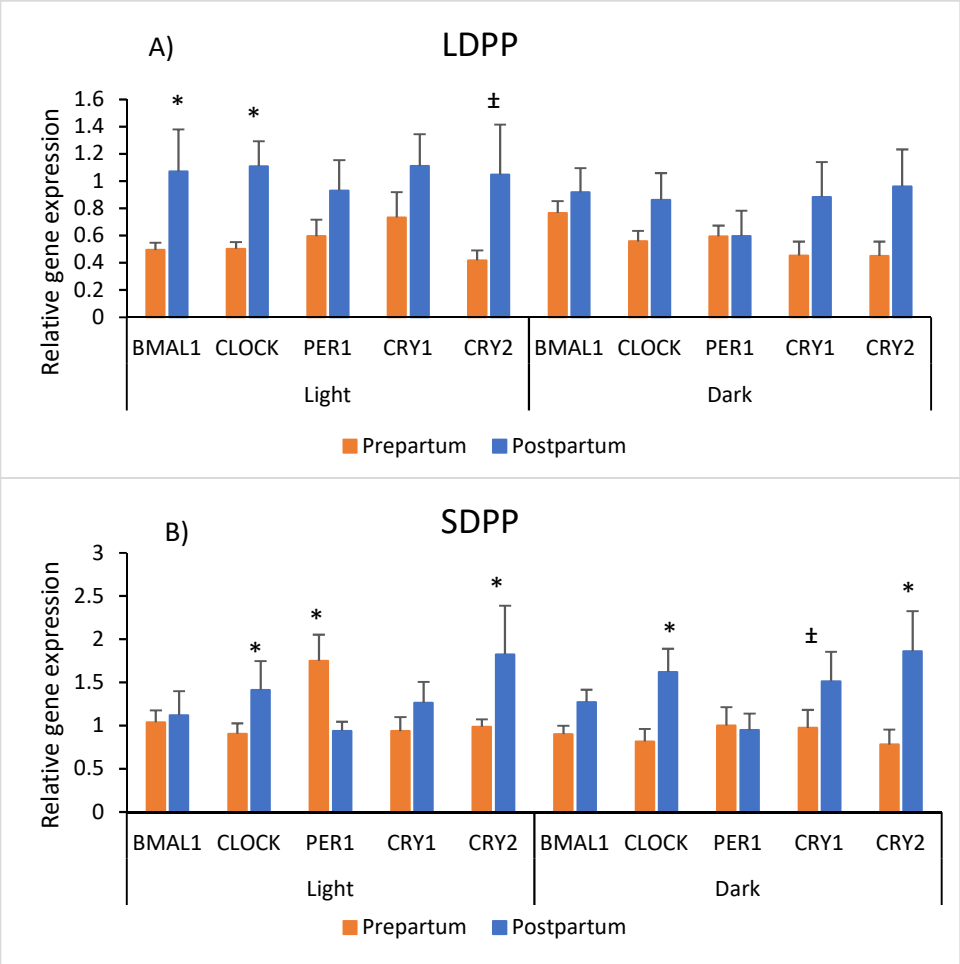

Figure 13: Mean mammary Clock gene expression between prepartum and postpartum in A) short-day photoperiod (SDPP) treatment and B) long-day photoperiod (LDPP) treatment. Values are presented as relative gene expression ± SE. *P< 0.05, ‡P< 0.1.

Table 7: Clock gene expression fixed effect of dairy goats exposed to the long-day photoperiod (LDPP) or short-day photoperiod (SDPP) during late pregnancy and lactation period.

Gene	Photoperiod	Time (Light/Dark cycle)	Physiological state	Photoperiod x Physiological state	Photoperiod x Time
BMAL1	0.0812	0.7665	0.0142	0.5534	0.8174
CLOCK	0.0304	0.8561	<0.0001	0.3298	0.4267
PER1	0.0196	0.0346	0.2985	0.022	0.4123
CRY1	0.045	0.7099	0.0087	0.9257	0.1893
CRY2	0.0286	0.7463	0.0001	0.284	0.8656

5. DISCUSSION

The present study was designed to test the hypothesis that photoperiod treatment during late pregnancy and lactation affects mammary circadian clock gene expression that subsequently influences the mammary metabolic activity during lactation. Importantly, we found out that photoperiod affected the expression of the mammary circadian clock genes. Generally, the core clock genes were more highly expressed in SDPP than in the LDPP both during late pregnancy and during lactation periods at the sampled two-time points assumed to be the maximum and minimum expression based on a previous experiment (Casey et al., unpublished). Our study has provided the first report of the effect of photoperiod on the mammary circadian clock genes in dairy goats during the dry and lactation periods. Similarly, the expression of the clock genes was affected by the physiological state of the animal in agreement with findings in mice (Casey et al., 2014b). Likewise, the time of sampling (light vs dark) affected the expression of the core clock genes consistent with findings in the Soay sheep (Lincoln et al., 2002). Our study has highlighted that dairy goats exposed to SDPP during the dry period produced more milk in the subsequent lactation than those exposed to LDPP in agreement with previous findings (Mabjeesh et al., 2007, 2013). We have further demonstrated that even in a photoperiod treatment that extended into the lactation phase, the SDPP treated animals yielded more milk.

5.1. Light/dark cycle and the mammary circadian clock

In this study, the light/dark periods significantly affected the expression of *PER1*. However, the light/dark cycle did not affect the rest of the clock genes probably due to species and tissue-specific transcription circadian rhythms in the goat mammary gland which: proliferates during pregnancy, differentiates during lactation and regresses during involution in recurring cycles (Zhang et al., 2014; Casey et al., 2014b; Andersson et al., 2005; Metz et al., 2006). *PER1* gene expression was higher during the light period than in the dark period of SDPP animals during late pregnancy in line with previous findings in mice and sheep (Lincoln et al., 2002; Metz et al., 2006; Matsuo et al., 2003). The higher expression of *PER1* in the light period may provide an explanation of how light/dark information reached the mammary gland, since *PER1* is known to be light inducible (Albrecht et al., 2001). This implies that *PER1* also has non-redundant roles of mediating

photic entrainment important for transducing photoperiod information even in the mammary gland of dairy goats (Albrecht et al., 2001; Steinlechner et al., 2002).

The effect of light/dark cycles on the mammary expression of clock genes such as *PER1* found out in this study, lends support to the timely DNA synthesis in proliferating mammary glands demonstrated in mice (Borst and Mahoney, 1980). Moreover, *PER1* was found to be light inducible in both diurnal animals like sheep (Lincoln et al., 2002) and nocturnal animals like mice (Albrecht et al., 2001). Studies in mice revealed a higher mammary gland synthesis at midnight (dark period) in comparison with the noon time (light period) (Borst and Mahoney, 1980). Further experiments showed that reversing the light schedule, also reversed the time of mammary gland synthesis whereby peak mammary synthesis corresponded to the middle of the dark period, and thus demonstrated that light is such an excellent zeitgeber (time giver) for mammary gland proliferation even in in-vivo systems (Borst and Mahoney, 1982). It is possible that the timing of periodic mammary gland synthesis according to light/dark schedules was gated by the circadian clock genes – such as PER1, which is responsive to light changes as demonstrated by this study – in line with previous studies in mice fibroblasts (Nagoshi et al., 2004).

The presence of light induces high expression of *PER1* which is associated with a high expression of wee1 that suppresses the entry into the mitotic-phase (Matsuo et al., 2003). Therefore, this could have resulted in the low mammary gland synthesis during the day (light period) because of the suppression of the mitotic phase; and the high mammary gland synthesis at night (dark period) when this suppression is relieved. With the SDPP treatment, the light period when mammary gland synthesis is suppressed is short (8 hours), whereas the dark period when the suppression is relieved is longer (16 hours). On the other hand, in the LDPP, the light period when mammary gland synthesis is suppressed is longer (16 hours), whereas the dark period when the suppression is relieved is shorter (8 hours). This response to light/dark cycles by the mammary clock genes may partly explain the greater mammary proliferation and subsequent higher milk production found in SDPP animals that have a longer dark period for mammary gland synthesis than LDPP animals shown to occur in dairy cows (Crawford et al., 2015; Velasco et al., 2008). The disparity in mammary proliferation could also be mediated by the differential circadian clock gene expression between the SDPP and LDPP treatments.

5.2. Photoperiod effect on the mammary circadian clock

Photoperiod affected the expression of the core clock genes being elevated in the SDPP treatment versus the LDPP treatment. In the prepartum period, this greater clock gene expression in SDPP may have caused the greater mammary proliferation in SDPP than in LDPP observed in dairy cows in a number of studies (Velasco et al., 2008; Crawford et al., 2015). Several studies have demonstrated the effect of the core clock genes we measured in this study on proliferation. To begin with, findings in rats showed that; the gene expression of *BMAL1* was higher in late pregnant rats that had greater mammary proliferation than in virgin rats with no mammary proliferation (Metz et al., 2006). This may be explained by the detected positive correlation between *BMAL1* and circadian clock-controlled genes, such as cyclin D1 (promotes G1-S transition) and c-Myc (involved in the G0-G1 transition) which regulate the progress in the cell cycle that occurred during pregnancy (Metz et al., 2006; Cardone and Sassone-Corsi, 2003; Walisser and Bradfield, 2006). A similar association could have happened in SDPP during late pregnancy which had higher *BMAL1* expression levels than LDPP in the light period and therefore; may have had higher levels of cell cycle genes such as: cyclin D1 and c-Myc that contributed to a greater mammary proliferation in SDPP animals than in LDPP animals.

Similarly, *CLOCK* – a heterodimeric partner of *BMAL1* – may have affected mammary proliferation since findings in a Clock mutant mouse showed a deficiency in cell proliferation (Miller et al., 2007). This resulted from the upregulation of genes that inhibit the cell-cycle such as: p21, p27, Chk1, Chk2 and Atr1; and downregulation of the proproliferation genes such as: epidermal growth factor (Egfr), estradiol receptor α (ER), and the kinases ERK1 and PI3K (Miller et al., 2007). In the same manner, the LDPP which tended to have lower *CLOCK* gene expression levels in the light period during late pregnancy, may have led to the upregulation of genes that inhibit the cell cycle and downregulation of proproliferative genes that resulted in the lower mammary proliferation that occurs in dairy animals in LDPP (Wall et al., 2005).

Additionally, findings in dairy cows revealed that, LDPP animals have higher mammary apoptotic levels than SDPP animals during late pregnancy (Wall et al., 2005). This could have been partly mediated by *CLOCK* since findings in *Clock*/*Clock* mutant mice revealed an upregulation of a

number of important pro-apoptotic genes (Antoch et al., 2008). Therefore, *CLOCK* gene expression which tended to be lower during late gestation in LDPP animals in their light period, may have caused greater apoptosis that negatively affected their mammary proliferation that eventually led to the lower mammary gland proliferation in LDPP than in SDPP animals.

Likewise, *CRY1* gene expression may have affected goat mammary proliferation since findings in *CRY* deficient mice demonstrated lower rates of mitosis coupled with slow liver regeneration after partial hepatectomy (Matsuo et al., 2003; Walisser and Bradfield, 2006). Our study showed that LDPP goats tended to have lower expression levels of *CRY1* gene that may have caused lower rates of mitosis in their mammary gland that could partly explain the observed lower mammary proliferation levels in LDPP dairy animals in comparison with SDPP dairy animals (Velasco et al., 2008). Therefore, the elevated expression of the core clock genes in SDPP versus LDPP in the prepartum period revealed by the current study, is a likely explanation for the higher mammary proliferation shown to occur in SDPP treated dairy animals (Velasco et al., 2008; Wall et al., 2005).

Regarding the postpartum period, the higher expression of the clock genes in the SDPP than in LDPP possibly led to the greater mammary differentiation and consequently more milk production in SDPP animals as found out in this study. In the first place, SDPP animals had a greater mammary proliferation prepartum as observed in dairy cows (Wall et al., 2005), hence had more mammary tissue to differentiate. During the postpartum phase, mammary cell differentiation takes place and is positively correlated with the expression of circadian clock genes. (Metz et al., 2006). Postpartum, SDPP goats had a higher gene expression of *CLOCK* in the dark period than LDPP goats. Studies in mice revealed that, a higher *CLOCK* gene expression caused a higher mammary differentiation (Casey et al., 2016). Therefore, greater mammary circadian clock gene expression such as *CLOCK* in SDPP animals may have caused greater mammary differentiation in SDPP than in LDPP goats in this study.

5.3. Photoperiod and milk yield

SDPP treated goats which had better mammary proliferation and differentiation subsequently had a higher milk yield than the LDPP treated goats in agreement with previous findings

(Mabjeesh et al., 2013). Photoperiod effect on metabolic output may be mediated by the circadian clock as demonstrated by continuously shifting light cycles in dairy cattle, that resulted in a lower expression of a core clock gene, *BMAL1,* in addition to lower levels of; *Beta casein, alpha-lactalbumin, fatty acid synthase,* and *Acetyl CoA-carboxylase* in the mammary gland and ultimately reduced milk yield (Casey et al., 2014a). This could partly be responsible for the lower milk yield in LDPP that had a lower gene expression of clock genes but a higher milk yield in SDPP that had a higher gene expression of clock genes. A more robust circadian clock gene expression in SDPP that was revealed in this study, may have affected the expression of several other clock-controlled genes, some of which are involved in mammary metabolism and milk synthesis since 7% of the genes in the lactating mammary gland have circadian patterns of expression (Maningat et al., 2009). Moreover, loss of a circadian clock gene like *CLOCK* in mice resulted into poor milk production (Casey et al., 2016; Dolatshad et al., 2006). SDPP animals in our study had higher expression of *CLOCK* in the dark phase during the lactation period than LDPP animals. Therefore, the higher circadian gene expression in SDPP could have resulted in a higher metabolic activity that led to the higher milk yield observed in SDPP animals in this study and several previous ones (Mikolayunas et al., 2008; Mabjeesh et al., 2013; Velasco et al., 2008).

Milk fat and lactose content were not affected by photoperiod in agreement with previous findings (Mabjeesh et al., 2007). SDPP had a higher percentage milk protein than LDPP. This finding is contrary to previous studies in which the percentage milk protein was similar between the two treatments (Mabjeesh et al., 2007; Velasco et al., 2008). A possible explanation for this is that, in our study, the photoperiod treatment extended into the lactation period while in the previous studies the treatment stopped at parturition. Another possible explanation for this is that, *PER1* levels were elevated in SDPP versus LDPP. *PER1* is positively associated with the expression of a gene that encodes the primary milk protein beta-casein (Metz et al., 2006). Hence, SDPP with higher *PER 1* gene levels could also have had a higher expression of beta-casein that caused the observed significantly higher percentage protein levels in the milk of SDPP animals.

5.4. Physiological state and mammary circadian clock

Mammary clock gene expression was affected by physiological state. Generally, there was an increase in the expression of mammary clock gene levels from late pregnancy to lactation. *BMAL1* levels were elevated during lactation versus pregnancy consistent to what was observed in the mouse mammary gland (Metz et al., 2006). *CLOCK* gene expression was higher in lactation than in pregnancy in agreement to what was observed in the differentiated mouse mammary HC11 cells and mouse liver in vivo (Casey et al., 2014b). Our findings of a higher clock gene expression during the lactation period concur well with previous findings in mouse HC-11 cells, in which the expression of clock genes such as *BMAL1* was elevated in differentiated cells compared with non-differentiated cells (Metz et al., 2006). Our results have revealed the plasticity of the mammary circadian clock in dairy goats as already seen in mice that transpires to prepare them for the metabolically demanding lactation period in order to sufficiently coordinate metabolism to meet the lactational demands of the young (Casey et al., 2014b; Bell and Bauman, 1997).

The transition from pregnancy to lactation after parturition is accompanied by great metabolic changes. During this period, the rate of hepatic gluconeogenesis doubles to meet the glucose demands of the mammary gland (Bell and Bauman, 1997). One of the ways this homeorhetic process is mediated in the liver is by an increase in the amplitude of the core clock genes in the lactation period (Casey et al., 2014b). Moreover, gluconeogenesis is abolished by loss of *BMAL1* and depressed by *CLOCK* mutation as found out in mice (Rudic et al., 2004). This indicates that the circadian clock system is very important in ruminants since almost their glucose needs are principally met by hepatic gluconeogenesis (Bell and Bauman, 1997). The rapid demand of glucose is required by the mammary gland for lactose synthesis, which is the most important solute in milk (Bell and Bauman, 1997). The milk is needed to nourish the new born whose glucose demands are 2.5 times that of the uterus in late pregnancy (Bell, 1995). Results from our study revealed a generally higher expression of the core clock genes in the mammary gland during lactation. Therefore, one of the mechanisms that mediate the homeorhetic adaptation of the

mammary gland required to meet the milk demands during the lactation period, is by the upregulation of the mammary circadian clock system.

5.5. Photoperiod and gestation period

With regard to the gestation period, goats in the LDPP treatment had a shorter gestation period than those in the SDPP treatment, in agreement with previous findings in mares (Hodge et al., 1982). The effect of light on pregnancy is getting more attention since it has been shown that pregnant mothers under shift work and night schedules have a shorter gestation length and have an increased risk of preterm delivery (Nurminen, 1989; Mozurkewich et al., 2000; Gamble et al., 2013). This highlights the effect of light which could have played part in this study that resulted in the differences in the gestation lengths between SDPP and LDPP. The fetal SCN and several fetal tissues have been shown to possess a circadian clock and circadian rhythms in a number of mammals like the monkey and sheep, especially in late gestation (Seron-Ferre et al., 2007). The conceptus fits itself to live and develop in the rich circadian environment given by its mother to which it adapts so that it can prepare itself for the new and different environment postpartum (Seron-Ferre et al., 2007). As shown in seasonal animals like the sheep, the fetus senses the time of the day and year and hence programs itself for the environment after delivery (Herbosa et al., 1994). During pregnancy, the mother also communicates with environmental cues to the fetus – in various ways such as: the body temperature, several metabolic pathways and hormones like melatonin which are capable of crossing the placenta without being altered – hence communicating photoperiod length to the fetus and influencing its development (Reppert and Weaver, 1996; Seron-Ferre et al., 2007). Therefore, the difference in the communicated photoperiod length likely resulted in a shorter gestation period in LDPP than in SDPP. Temperature, which is one of the ways photoperiod information could have been communicated to the placenta, differed between treatments in the current study.

5.6. Effect of photoperiod on body temperature

Temperature levels were higher in the LDPP than SDPP at all physiological levels that is: 3 weeks prepartum, 3 weeks postpartum and 5 weeks postpartum. This is in agreement with previous findings in goats where the temperature was higher in LDPP than SDPP (Mabjeesh et al., 2013).

Ambient light can affect the body temperatures of mammals just as observed in human female subjects where darkness induced a reduction in body temperature (Gubin et al., 2017). This supports the results obtained in our study. Notably, the temperature levels in LDPP started falling later during the 24 h period than those in SDPP because the lights in LDPP were switched off several hours later demonstrating how light causes a delay in the evening decrease of body temperature similar to what was found in humans (Gubin et al., 2017). Body temperature exhibited a diurnal pattern and was affected by time. This matches well with previous findings in goats and other diurnal animals like horses, humans, and sheep whose trough forms at sunrise and the peak forms at sunset (Murray et al., 2002; Piccione et al., 2005, 2002).

Temperature control is under the central control of the SCN. Body temperature is one of the ways circadian information is sent from the SCN to the rest of the body (Buhr and Takahashi, 2013). When light is perceived by the eyes, a light signal is transmitted to the SCN, the SCN, in turn, provides its output to the dorsal subparaventricular zone responsible for controlling the circadian rhythms of body temperature via its projections to the medial preoptic region (Saper et al., 2005; Froy, 2007). Temperature rhythms which are set by the SCN - which is resistant to cyclic temperature changes within the normal range - are capable of entraining peripheral rhythms (Buhr et al., 2010). One of the known ways of how temperature entrains the peripheral clocks is by mechanisms related to the control of transcription of the heat-shock factor protein 1 (Buhr et al., 2010; Bass, 2012). This demonstrates one of the ways the differential photoperiod information perceived by the eyes managed to reach the mammary gland and other body tissues to bring about the observed differences in clock gene expression in this study. Additionally, LDPP had a higher body temperature that is associated with a lower milk yield, observed in this study and previous ones as well (Kendall et al., 2006; Her et al., 1988; Igono et al., 1992).

For both treatments, week 5 had the highest temperature and this may be attributed to the very high metabolic state of lactation the goats were going through bearing in mind that the environmental conditions were similar during pregnancy and lactation periods. Goats produced more milk in week 5 postpartum than week 3 postpartum which followed with the second highest body temperature. Body temperature measured during week 3 prepartum was lowest in comparison with the weeks postpartum (lactation period). Higher body temperatures were

recorded during lactation because of the higher metabolism during this period because the lactation period is one the most energetically demanding physiological periods in the life of an adult female (Casey et al., 2014b). The difference in temperature between the prepartum and postpartum could be another factor responsible for the different entrainment of the clocks in the mammary glands that resulted in the differential expression of several clock genes between prepartum and postpartum. Generally, towards 07:00 hours there was an increase in the body temperature. This is because the time for feed provision was restricted to 07:00 hours and the time for feed provision is known to give a rise to an anticipatory increase in body temperature (Kaur et al., 2008).

5.7. Photoperiod effect on prolactin concentration

In this study, prolactin concentration was generally affected by treatment, physiological state and time. In LDPP treatment during the prepartum period, prolactin concentration peaked at 1400. The circadian clock system could be responsible for this prolactin pulsatile activity since core circadian clock genes can bind on the non-canonical E-boxes of the prolactin promoter and affect prolactin mRNA expression (Leclerc and Boockfor, 2005; Bose and Boockfor, 2010). In LDPP postpartum, the lack of effect of time on prolactin concentrations could have resulted from the lack of difference in the clock gene expression between the light and dark period.

Prolactin concentration was higher in LDPP than SDPP. This observation is consistent with previous findings in dairy goats and cows (Mabjeesh et al., 2013; Velasco et al., 2008; Crawford et al., 2015; Lincoln et al., 2002). In both treatments, the concentration of prolactin was highest prepartum because, towards parturition, ruminants experience a steady increase in prolactin concentration resulting into a prolactin surge (Koprowski and Tucker, 1973). The prolactin periparturient surge is critical since it is required for the mammary gland to successfully differentiate, which is crucial for the initiation of lactogenesis that takes place during this time period (Akers et al., 1981; Knight, 2001). Prolactin concentrations were lowest 5 weeks postpartum matching those observed in earlier studies in dairy goats (Mabjeesh et al., 2007, 2013). At 5 weeks postpartum the milk secretion was fully established and therefore, at this stage, prolactin secretion has no limitation on the secretion of milk (Tucker, 2000).

A number of studies have revealed an inverse relationship between prolactin concentrations and the prolactin receptor expression in the mammary gland and other tissues like the liver and lymphocytes (Velasco et al., 2008; Auchtung et al., 2003). The lower prolactin concentrations in SDPP coupled with the higher prolactin receptor expression may partly explain the higher milk yield recorded for SDPP in this study. Furthermore, supplementing SDPP dairy animals with extra prolactin reduces the beneficial effects provided by the SDPP revealed through the lower milk yield than SDPP animals without prolactin supplementation. However, SDPP supplemented with prolactin still had a higher milk yield than LDPP despite having similar prolactin plasma concentrations (Crawford et al., 2015). This implies that photoperiod effects are mediated by other processes besides the influence of prolactin. Moreover, Lincoln et al. (2002) showed that photoperiod treatment affected circadian gene expression of the pars tuberalis of the pituitary gland which regulates prolactin secretion through a paracrine mechanism. Thus, the observed prolactin differences between the SDPP and LDPP likely resulted from the underlying effects of the central circadian clock mechanism.

5.8. Feed intake and photoperiod

During the prepartum period, the DMI was similar between treatments in agreement with previous findings in dairy goats (Mabjeesh et al., 2007). This indicates that during this period, photoperiod effects were not mediated by feed but by other means like the circadian clock system as suggested by this study. Besides, it has already been observed that during the periparturient period, there is a dramatic increase in glucose production without an increase in feed intake, although the increase in voluntary feed intake takes place later in the lactation period (Bell and Bauman, 1997). In the postpartum period, there was a greater DMI in the SDPP. This is likely to offer a supportive role to meet the greater metabolic output of the milk yield in the SDPP already mediated by the circadian clock gene network as found out in this study. The higher feed intake in SDPP could have been mediated by the orexigenic hormone ghrelin and anorectic hormone leptin which influence feed intake and are under circadian control given their circadian rhythms (Kirsz et al., 2012; Froy, 2007; Sugino et al., 2004). Moreover, it was shown that deletion of BMAL1 abolishes circadian rhythmicity of ghrelin expression together with the circadian rhythm of feed intake (Laermans et al., 2015). Leptin is affected by photoperiod, and

previous studies in sheep indicated that SDPP had lower leptin levels than LDPP (Chilliard et al., 2001; Kirsz et al., 2012). Since a decrease in leptin levels stimulates feeding behavior, lower leptin levels could have resulted in the higher feed intake observed during the lactation phase in SDPP in this study (Chilliard et al., 2001).

5.9. Conclusion

The main goal of the current research was to determine the effect of photoperiod on the mammary expression of the core clock genes (*BMAL1, CLOCK, CRY1, CRY2,* and *PER1*) during late pregnancy and lactation. The second major goal of this study was to investigate the effect of the physiological state on the mammary expression of the same core clock genes. We also aimed to assess the effect of photoperiod on: prolactin hormone concentration, body temperature, milk yield and milk components.

Our main finding from this research was that; photoperiod affected the mammary expression of the core circadian clock genes (*BMAL1, CLOCK, CRY1, CRY2,* and *PER1*) during late pregnancy and lactation. To the best of our knowledge, our study has provided the first report that photoperiod manipulations (LDPP vs SDPP) affect the mammary expression of the core clock genes both during the late pregnancy and lactation periods. The second major finding was that the physiological state affected the expression of the core circadian clock genes. This study further confirmed that SDPP leads to a higher milk yield than LDPP. For the milk composition, protein content was higher in SDPP than LDPP. Other milk components were not affected by photoperiod treatment. We also found that, the animals in the LDPP treatment had higher body temperature and prolactin hormone concentration.

Although this study was limited to 2 mammary biopsy samplings from each of the goats in a 24-hour period, we successfully captured mammary circadian clock differences. Our sampling technique (each one of the 2 mammary samples from the goats was obtained from a different half of the udder on the sampling day) prevented potential carry-over effects and minimized trauma, that ensured the integrity of the udder that allowed us to obtain milk yield data from the same animals as well.

The evidence from this study suggests that photoperiod effects on mammary gland development and milk yield are downstream effects of the photoperiod modulation of the circadian clock genes. Overall, this study strengthens the awareness that photoperiod is one of the non-invasive interventions to effectively improve animal production like increase in milk yield. This research extends our knowledge of how the environment (dark/light cycle) can affect the production of farm animals through an environmental gene interaction. Although light can be used to improve production exemplified by SDPP treatment both in late pregnancy and in the lactation periods that had a higher milk yield, it could also be a potential environmental pollutant in farm animals that can negatively affect production exemplified by LDPP treatment both in late pregnancy and in the lactation periods that had a lower milk yield. This indicates that the lighting schedules on farms ought to be managed. The results of this research highlight the importance of environmental management and manipulation such as photoperiod to modify circadian rhythms as one of the ways to effectively improve and optimize milk production from dairy goats and also promote animal welfare since it is non-invasive.

Our research findings suggest that homeorhetic adaptations of the mammary gland to support lactation are mediated by the circadian clock system. Taken together, these results suggest that homeorhetic changes in response to photoperiod treatment are regulated by the circadian clock during late pregnancy and during the lactation periods. Further research is required to establish photoperiod effects on the mammary circadian clock-controlled genes especially those involved in metabolism and the cell cycle in dairy animals.

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תקציר

בשנים האחרונות גדל הביקוש לחלב עיזים בגלל סיבות שונות ביניהם נעכלות טובה יותר, יכולת התרסה גבוה יחסית לחלב פרות וסגולות רפואיות. שינויים בשעון הביולוגי (צירקאדי) של יונקים משפיעים על תנובות החלב. השעון הצירקאדי מווסת מגוון רחב של גנים המעורבים במטבוליזם ברקמות שונות בגוף כולל רקמת העטין של מעלי גירה. אורך יום ולילה (פוטופריודה) מהווים גורם סביבתי משמעותי המשפיע על השעון הצירקאדי של בעלי חיים. למשל, מעלי גירה חולבות שנחשפו ליום קצר במהלך סוף תקופת ההיריון הניבו יותר חלב בתחלובה העוקבת כנראה בגין עליה בגדילת רקמת העטין. המנגנונים שעומדים מאחורי השפעה זו לא פוענחו עד תומם. לכן, במחקר זו הנחנו שהשפעות פוטופריודה הנ"ל על ייצור החלב קשורות ומושפעות משינויים בשעון הצירקאדי. השעון הצירקאדי מורכב ממספר חלבונים שיש להם השפעה חיובית או שלילית על ביטוי חלבונים הקשורים במטבוליזם של רקמות בפרט ומטבוליזם כלל הגוף. מטרות העבודה הנוכחית היו לחקור השפעת השינויים בפוטופריודה על ביטוי גנים המקדדים לחלבוני השעון הצירקאדי (*BMAL1*, *CLOCK*, *CRY1*, *CRY2*, P*ER1*) במהלך תקופת ההיריון המאוחרת ובתחלובה בעזים חולבות. לצורך הניסוי 12 עיזים (זאנן מקומי) שימשו לניסוי במתכונת של שני טיפולים (6 עיזים בכל טיפול). הטיפולים היו חשיפת העיזים החל מ-45 ימים לפני ההמלטה ועד 120 ימים בתחלובה ליום קצר (8 שעות אור ו-16 שעות חושך) או ליום ארוך (16 שעות אור ו-8 שעות חושך). העיזים שוכנו בשני חדרים מטבולים; יום קצר ויום ארוך ושאר התאנים הסביבתיים היו דומים (23 מ"צ טמפרטורת סביבה). במהלך התחלובה העיזים נחלבו פעם ביום במכון חליבה עם רישום אוטומטי של התנובות. המזון הוגש לעיזים פעם ביום בעשה 0700. במהלך התחלובה דגימות חלב נלקחו פעם בשבוע לאנליזה מלאה של הרכב החלב ונשלחו למעבדה המרכזית בקיסריה. במהלך הניסוי, דגימות דם נלקחו מוריד הצוואר למדידת ריכוז הפרולקטין בשיטת ELISA. כמו כן, רקמת העטין נדגמה באמצעות ביופסיה שלושה שבועות לפני מועד ההמלטה המשוער וחמישה שבועות במהלך התחלובה. מהרקמות הופק mRNA ובוצעו אנליזות באמצעות Real time PCR לביטוי הגנים של השעון הצירקאדי. תוצאות הניסוי מראות שצריכת המזון הקבוצתית (על בסיס חומר יבש) היתה דומה בין הטיפולים בתקופת היובש והיתה 1,574 גר' ליום לעז ואילו בתקופת התחלובה העיזים בטיפול היום הקצר צרכו יותר מזון מקטיפול היום הארוך (1,700 לעומת 1,400 גר' ליום לעז). עזים בטיפול יום ארוך, תקופת ההיריון שלהן היתה קצרה יותר בהשוואה ליום קצר (144 לעומת 151 ימים, בהתאמה). תנובות החלב של העיזים בטיפול יום קצר היתה גבוהה יותר מאלו ביום הארוך (3.15 לעומת 2.7 ק"ג ליום לעז). כמו כן, תכולת החלבון בחלב היתה גובה יותר בטיפול יום קצר בהשוואה טיפול יום ארוך (3.53% לעומת 3.45%, בהתאמה). ריכוזי פרולקטין בפלזמה הושפעו כצפוי מאורך היום והיו גבוהים יותר בטיפול יום ארוך בכל מועדי הדגימות. ביום ארוך, בשבוע השלישי לפני המלטה ריכוז הפרולקטין בעזי יום הארוך היה 471.3±26.6 בהשוואה ל-41.1±5.5 נ"ג/מ"ל בעיזים בטיפול יום קצר. בשבוע השלישי והחמישי לתחולבה הריכוזים היו 471±40.3 ו- 365.4±26.6 נ"ג/מ"ל ביום ארוך בהשוואה ל- 41.1±5.75 ו-20.9±4.1 נ"ג/מ"ל ביום קצר. טמפרטורת הגוף במשך היום הושפעה מטיפול הפוטופריודה ובאופן כללי והיתה גובה יותר בטיפול יום האורך בהשוואה ליום הקצר.

מחזוריות יום ולילה השפיעה על ביטוי הגן PER1 ברקמת העטין. ביום קצר, לפני ההמלטה ביטוי הגן PER1 היה גבוה יותר בתקופת היום מאשר במהלך החושך. באופן כללי טיפול יום קשר גרם לביטוי מוגבר יותר של הגנים המעורבים בשעון הצירקאדי. יום קצר הגביר את הביטוי שך CLOCK (*P*= 0.0304) , PER1 (*P*=0.0304) , CYR1 (*P*=0.045) ו-CYR2 (*P*= 0.028) אבל לא ביטוי הגן BMAL1. בנוסף לך, תוצאות המחקר מראות שהסטטוס הפיזיולוגי של העיזים גם השפיע על ביטוי הגנים של השעון הצירקאדי והיה גבוהה יותר באופן יחסי בתחלובה בהשוואה לתקופת ההריון המאוחרת.

תוצאות המחקר הנוכחי מראות שלאורך היום יש השפעה מובהקת על תהליכי שגשוג רקמת העטין ביום קצר בתקופת ההיריון המאוחרת וכנראה גם בתחלובה. טיפול ביום קצר הביא להגברת ביטוי הגנים של השעון הצירקאדי שהשפיעו באופן ישיר או עקיף על רקמת העטין לשגשג בתקופת ההיריון המאוחרת שהביאה לייצור מוגבר של חלב וחלבון החלב. השפעת הגנים של השעון הצירקאדי הגבירה תהליכים מטבולים לייצור וסנתזה. מחקר זה הנו ראשון שמדווח על השפעות שינויים באורך היום על ביטוי גנים של השעון הצירקאדי שמביאים להגדלת ייצור חלב במהלך התחלובה. אסטרטגית השימוש בשינויים סביבתיים להגדלת היצרנות של עזים חולבות בתקופת היובש, שנחשבת לתקופה נוחה לטיפול בבעלי חיים, מהווה צעד לקראת חקלאות מודרנית ושמירה על רווחת בעלי חיים.

השפעת פוטופריודה על הגנים (*BMAL1, CLOCK, CRY1, CRY2, PER1*) אשר במתאם לשעון הצירקאדי בעיזים חולבות

עבודת-גמר

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לשם קבלת תואר

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