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Circadian and seasonal changes in the expression of clock genes in the ovine pars tuberalis

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ABSTRACT. The pars tuberalis (PT) is part of the pituitary that expresses melatonin receptors, therefore it is suggested that PT mediates the effect of melatonin on neuroendocrine functions and may be involved in the photoperiodic regulation of pituitary gland processes. Decoding of the melatonin signalling in the PT is possible thanks to the expression of circadian clock genes in the PT cells. Therefore, the present study was designed to determine circadian and seasonal changes in the expression of biological clock genes in the ewe PT. Two analogical experiments were performed in different photoperiods: 1 month before the summer (long-day photoperiod; LD) and winter (short-day photoperiod; SD) solstice. In each photoperiod, ewes were euthanized after the last blood collection in the middle of the day (n = 6) or night (n = 6). The expression of the basic helix-loop-helix ARNT like 1 (*BMAL1*), casein kinase 1 epsilon (*CK1ε*), clock circadian regulator (*CLOCK*), cryptochrome circadian regulator 2 (*CRY2*) and period circadian regulator 1 (*PER1*) genes in the ovine PT was higher ($P < 0.05$) during the day compared to the night regardless of the season. Only the cryptochrome circadian regulator 1 (*CRY1*) gene was characterised by a higher ($P < 0.05$) nocturnal level of expression during both short and long day seasons. The expression of melatonin receptor *MTNR1A* gene was higher ($P < 0.05$) during the day in both photoperiodic conditions, and was higher ($P < 0.05$) during the SD season. It was demonstrated that the direction of circadian changes in the expression of clock genes was relatively constant regardless of the season analysed. However, both diurnal and nocturnal expression of clock genes was higher in the SD season than in the LD photoperiod. This suggests that the protein products of the expression of these genes may exert a more significant effect on the secretory activity of this part of the pituitary gland in the SD season, but elucidating this relationship requires further in-depth research.

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Introduction

Decoding the melatonin signal in the pars tuberalis (PT) is possible thanks to the expression of circadian clock genes in PT cells (Johnston and Skene, 2015). The proper functioning of the circadian clock depends on the rhythmic expression of the aforementioned genes, which, in a coordinated manner, form a molecular oscillator mechanism (Sen and Hoffmann, 2020). In the ovine PT,

the expression of seven clock genes has been identified: period circadian regulator 1 and 2 (*PER) 1* and *2,* clock circadian regulator *(CLOCK)* and basic helix-loop-helix ARNT like 1 (*BMAL1*), cryptochrome circadian regulator 1 and 2 (*CRY*) *1* and *2* and casein kinase 1 epsilon (*CK1ε*). All of these genes show a rhythmic pattern of expression, with *PER* reaching peak expression during the first half of the light phase and *CRY* at the onset of the dark phase (Lincoln et al., 2002). The significance of maintaining a well-functioning circadian clock within the PT is underscored by research conducted on transgenic mice with *PER1* and *MT1* gene knockouts, revealing reversed rhythm of the thyroid-stimulating hormone *β* gene expression or the switch off of its day-night oscillation (Barrett et al., 2007). This suggests that melatonin affects thyrotropic hormone homeostasis in the PT through melatonin receptor MTNR1A and PER1 proteins (Barrett et al., 2007). Some studies proposed that alterations in clock gene expression could also impact the secretion of gonadotropins. Research on mice showed that the deletion of *BMAL1* caused only a slight increase in luteinising hormone (LH) levels in prooestrus, and an increase in oestrus follicle-stimulating hormone concentration, contributing to minor abnormalities in oestrus cycles, with no effect on fertility (Chu et al., 2013). In addition, mice with a mutation in the *CLOCK* gene were characterised by a reduced rate of LH pulses (Sen and Hoffmann, 2020). These findings indicate the importance of clock gene expression in preserving the appropriate secretion of gonadotropins. The well-established connection between the circadian timing system and the hypothalamic-pituitary-gonadal (HPG) axis underscores its vital role in regulating the reproductive process (Sen and Hoffmann, 2020). Emerging evidence suggests that clock genes and their protein products may be directly involved in the daytime-dependent regulation and adaptation of hormone synthesis and release (Wunderer et al., 2013). The exclusive expression of melatonin receptors in the PT strongly implies that the PT mediates the effect of melatonin on neuroendocrine functions and may be involved in the photoperiodic regulation of processes occurring in the pituitary gland. Our previous *ex vivo* study showed that melatonin reduced LH release induced by gonadotropin-releasing hormone (GnRH) in the ovine PT explants (Wojtulewicz et al., 2017). In addition, this effect was also described in rodents (Nakazawa et al., 1991). Our previous study in a sheep model has also shown that GnRH/LH secretion is subjected to diurnal changes, which in turn are affected by seasonal photoperiod alterations (Kopycińska et al., 2022). Sheep exhibit seasonal breeding patterns, and are reproductively active during the short-day (SD) season, whereas the long-day (LD) season is a non-breeding phase for ewes marked by the occurrence of seasonal anoestrous. Regulation of seasonal reproduction in ewes is orchestrated by the annual cycle of the GnRH pulse generator's sensitivity to negative feedback from oestradiol (Lehman et al., 2010; Kumar et al., 2017). The differing lengths of light exposure and melatonin signalling between SD and LD seasons likely influence the processes occurring in the PT.

Hence, the objective of this study was to investigate the variations in the expression of biological clock genes within the PT of ewes considering both circadian and seasonal dynamics.

Material and methods

Animals and experimental design

All procedures were carried out with the approval of the Local Ethics Committee of Warsaw University of Life Sciences at SGGW (Warsaw, Poland), authorized under No. 23/2015 (approved on 21/05/2015) and No. WAW2/62/2017 (approved on 21/06/2017).

Two corresponding experiments were carried out at two different photoperiods. The trials were scheduled to take place approximately one month before the summer solstice LD photoperiod and winter solstice SD photoperiod. In the SD photoperiod, the oestrus cycle of 2–3year old ewes was synchronised using a Chronogest® controlled-release vaginal sponge for sheep (Merck Animal Health, Boxmeer, The Netherlands), following the methodology outlined in Przybył et al. (2021). The experiments were specifically conducted on the 10th day of the luteal phase, during oestradiol and progesterone plateau. For each experiment, 12 sheep were divided into two groups: day and night group with 6 animals in each group. The animals were fed *ad libitum* according to the recommendations of the National Research Institute of Animal Production for adult ewes (Strzetelski, 2009). To avoid isolation stress, the ewes had constant visual contact with each other. All animals were implanted with venous catheters into their jugular vein the day before the experiment. Before euthanasia, blood samples were collected into heparinised tubes and immediately centrifuged in an MPW 260RH centrifuge (MPW Med. Instruments, Warsaw, Poland) for 10 min at 1000 × g at 4 °C. Plasma was stored at

−20 °C until analysis. To prevent any stimulation of the retina and the potential disruption of nocturnal melatonin secretion, overnight experiments were performed in the dark using red light. The animals were euthanized after blood collection in the middle of the day or night, respectively. The brains were directly removed from the skulls, the PTs were dissected into two portions, both immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

Melatonin radioimmunoassay

Plasma melatonin concentration was measured using a modified direct RIA technique described by Fraser et al. (1983), with modifications of the Department of Histology and Embryology, University of Warmia and Mazury employing the G/S/704-6483 antibody and 3H-melatonin (Olsztyn, Poland). The sensitivity of the assay was established at 5 pg/ml. The intra- and interassay coefficients of variation in both radioimmunoassays were below 10%.

Relative gene expression analysis by real-time PCR

Total RNA was extracted from the PT fragments using the NucleoSpin® RNA kit (MACHEREY-NAGEL GmbH and Co., Düren, Germany). All isolation steps were conducted in accordance with the manufacturer's instruction. The purity and concentration of the isolated RNA samples were quantified spectrophotometrically using a NanoDrop 1000 instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). The integrity of the isolated RNA was confirmed by electrophoresis using 1% agarose gel stained with ethidium bromide. For cDNA synthesis, the Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA) was applied. Real-Time PCR was conducted in a Rotor-Gene 6000 instrument (Qiagen, Dusseldorf, Germany), using HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and specific oligonucleotide primers (Genomed, Warszawa, Poland). The primers applied in the analysis were selected based on our extensive experience or were originally designed (Table 1) using the Primer3 bioinformatic tool. Amplification specificity was confirmed by final melting curve analysis. Gene expression analysis was performed using the Rotor Gene Q Series Software (Qiagen, Dusseldorf, Germany). The expression of each clock gene was normalised to the mean expression of three reference genes, i.e. actin beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and beta-2 microglobulin (*B2M*). The results are presented in arbitrary units as the ratio of target gene expression to the mean expression of housekeeping genes.

Table 1. Oligonucleotide primer sequences for real-time PCR reactions

GenBank Acc. No.	Gene	Amplicon size, bp	Forward / reverse	Sequence 5'→ 3'	Reference
NM_001009284.2	*B2M* beta-2 microglobulin	119	Forward	CTTCTGTCCCACGCTGAGTT	Originally designed
			Reverse	GGTGCTGCTTAGAGGTCTCG	
U39357	*ACTB* actin beta	168	Forward	CTTCCTTCCTGGGCATGG	Szczepkowska et al., 2022
			Reverse	GGGCAGTGATCTCTTTCTGC	
NM_001034034	*GAPDH* glyceraldehyde- 3- phosphate dehydrogenase	134	Forward	AGAAGGCTGGGGCTCACT	Szczepkowska et al., 2022
			Reverse	GGCATTGCTGACAATCTTGA	
NM_001130932.1	*CLOCK* clock circadian regulator	115	Forward	CAGTCAGTCTCAAGGAAGCGT	Originally designed
			Reverse	GGTGTAGAGGAAGGGTCCGA	
NM_001129734.1	*BMAL1* basic helix-loop-helix ARNT like 1	92	Forward	CGGAGTCGGTGGTTCAGTTT	Originally designed
			Reverse	TCCAGGACGTTGGCTAAAACA	
NM_001129735.1	*CRY1* cryptochrome circadian regulator 1	150	Forward	TAGCAGCAGTGCAAGTTGT	Originally designed
			Reverse	TGCGTGTCCTCTTCCTGACTA	
NM_001129736.1	*CRY2* cryptochrome circadian regulator 2	139	Forward	GATTGCGGCTCCATGACAAC	Originally designed
			Reverse	GACTGAAGCAGGAACCTCCA	
XM_027974927.1	*PER1* period circadian regulator 1	138	Forward	GTCCCTGCTACTGGCACATT	Originally designed
			Reverse	GGAGCAACTGGAGGCTTCTT	
NM_001078109.1	*CK1ε* casein kinase 1 epsilon	143	Forward	CACCTGGGCATCGAGCAAA	Originally designed
			Reverse	TTCTTCTCGCTGATCCGCTC	
XM_614283.6	*MTNR1A* melatonin receptor	114	Forward	GCCTCCATCCTCATCTTCAC	Król et al., 2016
			Reverse	GCTCACCACAAACACATTCC	

Statistical analysis

Statistical analysis was performed using the STATISTICA 12 software (Stat Soft. Inc., Tulsa, OK, USA). Gene expression results were subjected to two-way analyses of variance (ANOVA) to identify significant effects of two parameters, namely day/night and season. Subsequently, Fisher's post-hoc test was conducted. The results are presented as mean ± SEM; statistical significance was set at $P < 0.05$.

Results

Effect of photoperiod on blood melatonin concentration

The data presented in Figure 1 demonstrates a statistically significant increase ($P < 0.05$) in circulating melatonin levels during the nocturnal period compared to daytime levels in ewes. Moreover, blood melatonin concentrations were higher during SD at night in comparison to LD at night.

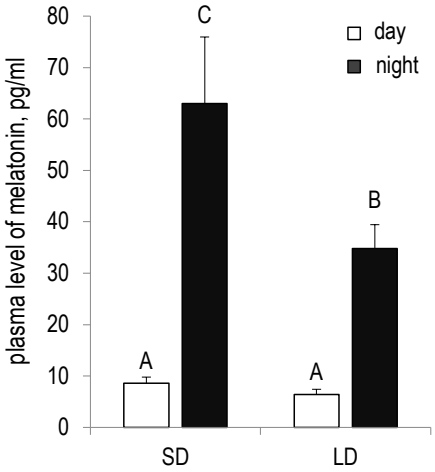

Figure 1. Effects of circadian and seasonal changes on ovine plasma melatonin concentrations in samples collected during the last hour of the experiment (12:00 / 24:00) in the short-day (SD) and long-day (LD) seasons

Data are presented as mean ± SEM; different capital letters indicate significant ($P < 0.05$) differences according to two-way ANOVA followed by Fisher's post-hoc test

Effect of photoperiod on mRNA expression of clock genes in the PT

It was found that mRNA expression of *BMAL1, CK1ε, CLOCK, CRY2,* and *PER1* in the ovine PT was higher ($P < 0.05$) during the day in comparison to the night (Figure 2). Moreover, this pattern of day/night alterations in clock gene expression was constitutive and did not depend on photoperiodic conditions. Only the *CRY1* gene was characterised by a higher ($P < 0.05$) nocturnal expression level during both SD and LD seasons.

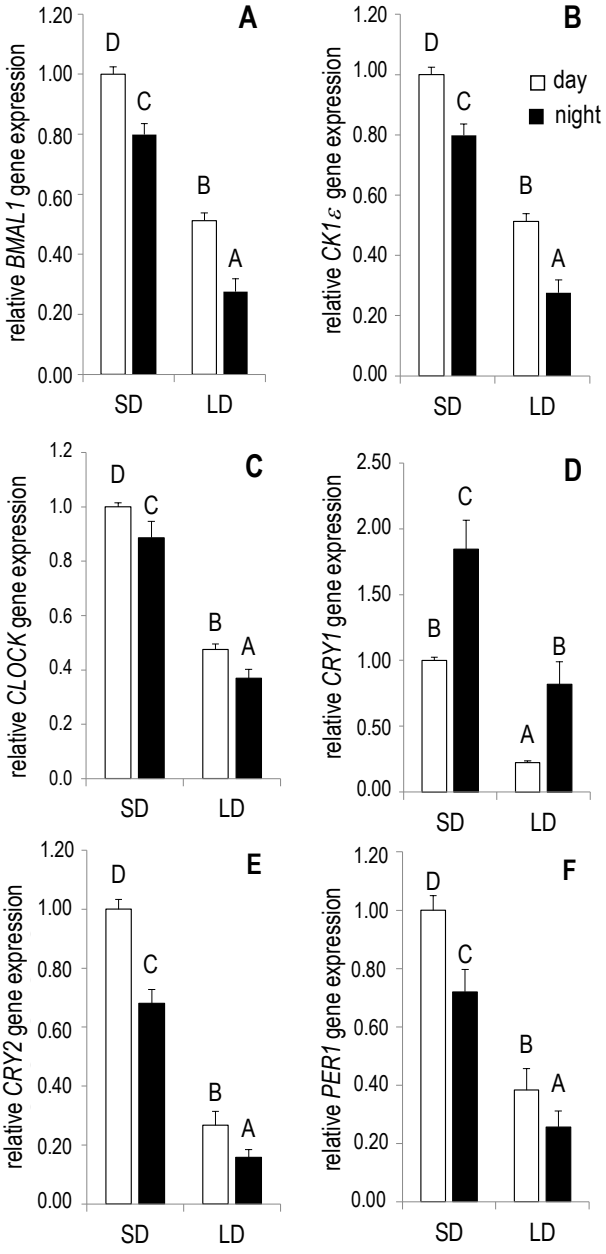

Figure 2. Effects of circadian and seasonal changes on the expression of basic helix-loop-helix ARNT like 1 (*BMAL1*) [A], casein kinase 1 epsilon (CK1ε) [B], clock circadian regulator (*CLOCK*) [C], cryptochrome cryptochrome circadian regulator 1 (*CRY*) 1 [D] and 2 [E] and period circadian regulator 1 (*PER1*) [F] genes during the short-day (SD) and long-day (LD) periods

Data are presented as mean ± SEM; different capital letters indicate significant ($P < 0.05$) differences according to two-way ANOVA followed by Fisher's post-hoc test

MTNR1A gene expression

The level of *MTNR1A* gene expression was higher ($P < 0.05$) during the day under both photoperiodic conditions (Figure 3). Moreover, this gene's expression was notably elevated ($P < 0.05$) during the SD season compared to the LD season, but only during a day.

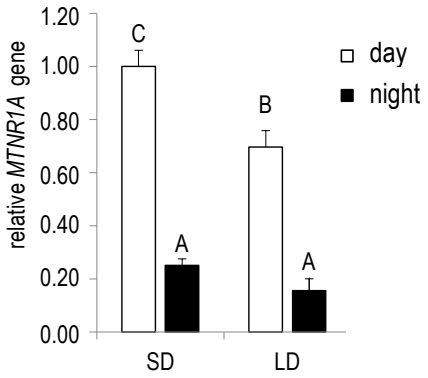

Figure 3. Effects of circadian and seasonal changes on melatonin receptor *MTNR1A* gene expression during the short-day (SD) and long-day (LD)

Data are presented as mean ± SEM; different capital letters indicate significant ($P < 0.05$) differences according to two-way ANOVA followed by a Fisher's post-hoc test

Discussion

Our study demonstrates that photoperiodic conditions can influence the expression of circadian clock genes in the ovine PT. It was found that both diurnal and nocturnal expression of all examined clock genes was lower in the LD photoperiod in comparison to the SD photoperiod. Our findings suggest that the expression of clock genes in the ovine PT is conservative and the observed day/night changes in the expression of these genes are similar regardless of the SD and LD season. Specifically, we noted that mRNA expression of the *BMAL1, CK1e, CLOCK, CRY2,* and *PER1* genes was lower in the middle of the night compared to the levels recorded in the middle of the day. On the other hand, *CRY1* gene expression was increased during the day compared to the night. It should be mentioned that although our study was conducted in sheep, an animal active during the day, the determined pattern of clock gene changes in the PT was surprisingly consistent with those observed in the rodents, i.e. primarily nocturnal animals. The findings of our work closely resemble those of a mouse study, which showed that the expression of *Bmal1*, *Clock,* and *Per1* clock genes in the suprachiasmatic nucleus (SCN) in females was lower during the middle of the dark phase compared to the light phase (Zhang et al., 2014). It is worth mentioning that the latter study also showed that the pattern of changes in clock gene expression was partly different in male mice; this gender effect suggests that gonadal hormones may affect the expression of these genes. A study in rats reported that the expression of the *Clock, Cry2*, and *Per1* genes was reduced in the middle of the dark phase, whereas *Cry1* gene expression was stimulated in submandibular gland cells in comparison to corresponding expression determined in the middle of the light phase (Satou et al., 2017). The latter results do not allow to draw unambiguous conclusions concerning the influence of light conditions on BMAL1 mRNA expression in rat submandibular gland cells. It is well known that the casein kinase 1 delta (*Ck1*δ)/ε gene plays an important role in clock functioning, and is believed to regulate *PER* and *CRY* stability and nuclear entry. However, the present findings suggest that the reduced expression of *CK1δ/ε* at night results in lower phosphorylation of these circadian clock components. It should be mentioned that published evidence indicates that diurnal rhythms in the expression of clock genes may differ within the same species, and could be affected by the analysed tissue and/or genetic differences. A study in high drinking in the dark-1 (HDID-1) mice (Grigsby et al., 2022), which revealed distinct diurnal circadian gene expression patterns in various brain regions, including the SCN, ventral tegmental area, and nucleus accumbens. These patterns diverged not only from each other but also from previous studies on mice, such as the work of Zhang et al. (2014). Genetic variations in clock genes are associated with phenotypic effects in a repertoire of biological processes, including diurnal preference, sleep, metabolism, mood regulation, addiction, and fertility (Zhang et al., 2013). The significant influence of genetic diversity, including animal breeds, on the expression of biological clock genes may at least partially explain the considerable differences between the results of our research and the previously published work by Lincoln et al. (2002). The latter authors described the expression and circadian changes of seven clock genes in the PT of female Soay sheep. They observed that *CRY1* and *CRY2* exhibited a nocturnal peak, while *PER1* and *PER2* displayed a diurnal peak of expression regardless of the season. In contrast, the *CLOCK* and *BMAL1* genes showed a nocturnal increase in their expression under SD conditions, but this peak shifted to the onset of the darkness during the LD photoperiod. The partly divergent pattern of photoperiodic changes in clock gene expression within the sheep pineal gland, as revealed in our study, might stem from differences in the experimental model. In Lincoln's study, photoperiodic conditions were artificially created to provide constant light intensity (Lincoln et al., 2002), but all experiments were performed in autumn and prepared for short days. One group remained under SD photoperiod (8:16), while other

group was transitioned to the LD photoperiod (16:8) for 6 weeks. Our study on the other hand was conducted using animals during the naturally occurring LD season (late spring/summer) and SD (late autumn/winter), which might have a significant impact on the physiology of animals, as well as affect the pattern of changes in the expression of clock genes. Photoperiodical changes in the rhythm of clock gene expressions observed in our study were significantly more similar to those observed in recent experiments on rodents than in Lincoln's study. This could be due to more accurate and sensitive methods (Real-Time PCR) applied in the present study, as opposed to *in situ* hybridisation technique previously used by Lincoln et al. (2002). It is important to emphasize that the expression of clock genes can also be significantly affected by diet. Other research conducted on mice has demonstrated that obesity induced by a high-fat diet alters the circadian clock system, and obesity and metabolic syndrome are highly correlated with the expressions of circadian clock genes and their downstream targets (Ando et al., 2006; Hsieh et al., 2010). It is well established that circadian clock exerts a significant influence on the immune system, and disruption of circadian rhythms has been linked to inflammatory pathologies (Abo and Layton, 2021). In particular, shift work is associated with impairment in immune function, and those alterations are sex-specific. The goal of this study is to better understand the mechanisms that explain the weakened immune system in shift workers. To achieve that goal, we have constructed a mathematical model of the mammalian pulmonary circadian clock coupled to an acute inflammation model in the male and female rats. Shift work was simulated by an 8h-phase advance of the circadian system with sex-specific modulation of clock genes. The model reproduces the clock gene expression in the lung and the immune response to various doses of lipopolysaccharide (LPS). In contrast, the effect of immune system activation on the circadian clock system remains less comprehensively explored. However, a study in male Wistar rats showed that inflammation induced by intravenous injection of LPS caused transient suppression of biological clock genes and suggested that the biological clock plays an important role in the response to systemic inflammatory stimulation (Okada et al., 2008). All this evidence suggests that the interaction between the immune and circadian-clock systems are complex and bidirectional.

We also found that although the direction of changes in biological clock gene expression in the ovine PT was not affected by the season, the relative expression of the studied genes was lower in all cases during the LD season compared to the SD season. These season-dependent differences in the expression of clock genes could result from differences in *MTNR1A* gene expression and the duration of melatonin release and blood concentration of this hormone in the LD and SD seasons. Our study showed that the nocturnal melatonin blood levels reached significantly higher values during the SD season than those observed in the LD season. These results are consistent with our previous studies in ewes, which showed the same seasonal dependence in melatonin secretion (Herman et al., 2015; 2017). Moreover, we determined that basal *MTNR1A* gene expression in the ovine PT was lower during the LD season compared to the SD season. Reduced expression of *MTNR1A* mRNA during the LD photoperiod may lead to lower sensitivity of this part of the pituitary to melatonin. The action of melatonin is very important for the proper functioning of the circadian clock. This molecule represents an important neuroendocrine output of the circadian clock, and it delivers the circadian message to the whole organism. Moreover, melatonin appears to be one of the agents synchronising the circadian system (Pfeffer et al., 2018). Studies in wild-type (WT) mice, melatonin 1 receptor knockout (MT1 ko), and melatonin 2 receptor knockout (MT2 ko) mice showed the importance of MTNR1A expression for melatonin function in the PT (Von Gall et al., 2005). It was found that the PT deprived of melatonin signal transmitted via MTNR1A receptor was characterised by a dramatic reduction in the expression of all clock genes, including *mPer, mCry, Clock*, and *Bmal1* compared to tissues collected from WT and MT2 KO mice. Based on this study, it can be concluded that similarly to mice, reduced expression of clock genes in the ovine PT during the LD photoperiod could result from lower nocturnal melatonin concentration and *MT1* gene expression in this part of the pituitary. It should be pointed out that the nocturnal increase in *CRY1* gene expression in the PT found in our study could also be attributed to the influence of melatonin. Previous studies showed that acute injection of melatonin caused a very strong, rapid and transient induction of *Cry1* in the PT of rats (Dardente, 2007), sheep (Hazlerigg et al., 2004; Johnston et al., 2006) and hamsters (Tournier et al., 2007; Wagner et al., 2008).

Furthermore, seasonal differences in clock gene expression may also result from the distinct reproductive status of animals during the SD and

LD photoperiods. Sheep are seasonal breeders, reproductively active under SD conditions, whereas seasonal anoestrous occurs during the LD photoperiod. Anoestrous entails a cessation of ovarian cyclicity, resulting in reduced levels of circulating ovarian steroids. This particularly affects progesterone, as its levels never exceed 1 ng/ml (Herman et al., 2010). In contrast, during the luteal phase, in which our experiment was performed, circulating progesterone levels in ewes are markedly elevated, reaching even several fold higher values (Bartlewski et al., 2001; Long et al., 2013). Previously published works have suggested that among the gonadal steroids, it is progesterone that has the strongest effect on the expression of clock genes (Nakamura et al., 2010). The latter study showed that progesterone upregulated *PER1, PER2*, and *BMAL1* expression, which may explain higher expression levels of clock genes in the PTs from animals in the SD season compared to the LD season observed in the present work.

Clock genes expression can also be modulated by ambient temperature. A study in zebrafish model showed temperature-dependent changes in the expression, phosphorylation, and function of the CLOCK (Lahiri et al., 2005). A more recent experiment in *Drosophila melanogaster* revealed a relationship between daily temperature cycles and peripheral clock gene expression. It was found that housing under cycling temperature conditions rescued the age-related decrease in the amplitude of almost all clock genes in male flies, but not female flies. This suggests that the effect of temperature on peripheral clock gene expression may be sex-specific (Goh et al., 2021). However, a study on warm-blooded animals – mice – did not show such a significant effect of environmental temperature conditions on the expression of clock genes in the liver of animals (Rabearivony et al., 2020). It should be mentioned that our experiment was performed in the experimental barn that provided relatively constant temperature conditions, thus the influence of temperature on the changes in the expression of clock genes was negligible.

Conclusions

Our study has revealed a consistent pattern of circadian changes in the expression of clock genes within the pars tuberalis (PT), irrespective of whether it was examined during the long-day (LD) or short-day (SD) season. Nevertheless, both diurnal and nocturnal expression of clock genes in the PT was higher in the SD season as compared to the LD photoperiod. This implies that the protein products resulting from the expression of these genes may exert a more pronounced effect on the secretory activity of this part of the pituitary gland in the SD season. However, a comprehensive understanding of this intricate relationship necessitates further detailed research.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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