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Comparison of Expression Patterns of Six Canonical Clock Genes in Year-Round Estrous and Seasonal Estrous Rams

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This study was designed by WC, ZT and MC, WC and ZT conducted the experiments and analyzed the data. WC drafted the manuscript. WS, SG, LM and MC helped in preparation of revised manuscript.

Key words
Ram, Circadian rhythm, Seasonal estrous, Clock genes, Tissue expression

ABSTRACT

Circadian rhythm is a biological rhythm that is related closely to the rhythmic expression of a series of clock genes. A number of studies have revealed the role of circadian rhythms in the estrous mode of mammals. In the present study, the expression patterns of six canonical clock genes (*Clock, BMAL1, Cry1, Cry2, Per1* and *Per2*) were analyzed in year-round estrous rams (Small Tail Han sheep, STH) and seasonal estrous rams (Sunite sheep, SNT). The result showed that all six genes were expressed in brain, cerebellum, hypothalamus, pituitary, testis, epididymis, vas deferens and adrenal gland tissues in both breeds. The expression level of *Clock* and *BMAL1* showed similar trends in the brain, cerebellum, hypothalamus, pituitary, testis and epididymis in both breeds. The expression levels of *Clock, BMAL1,* and *Cry1* were significantly higher in the pituitary tissue of STH rams than in that of SNT rams, whereas the expression level of *Cry2* showed the opposite pattern. We speculate that *Cry1* and *Cry2* may have opposite roles in the circadian rhythm of rams. Moreover, the expression patterns of *Cry1/2* and *Per1/2* in the pituitary suggested that the CRY and PER proteins may function in the circadian rhythm either as a complex or as individual, Therefore, we concluded that circadian rhythmicity may regulate the estrous mode of rams via clock genes within transcription/translation feedback/feedforward loops. This is the first study to systematically analyze the expression patterns of clock genes in rams.

INTRODUCTION

Circadian rhythm, which is one of the basic characteristics of life, refers to biological processes that oscillate with a period of 24 h. Almost all creatures respond to cyclical changes in the external environment such as darkness, light and temperature through signal transduction processes, thus producing their own internal rhythms (Coulon *et al*., 2016; Lewis *et al*., 2018). It is widely accepted that the molecular mechanism of circadian rhythm is a feedback-loop model based on transcriptional translation, involving two transcriptional activators (BMAL1 and Clock) and two transcriptional repressors (period (PER) and Cryptochrome (CRY) proteins) (Leloup and Goldbeter, 2003; Preitner *et al*., 2002). The particular transcriptional feedback loops that are believed to control circadian rhythm are dependent on a small number of canonical clock genes, the expression levels of which are closely related to cycles in behavior and physiology (Janich *et al*., 2011; Marcheva *et al*., 2010; Nam *et al*., 2016).

To date, more than 10 genes have been identified that form the basis of cellular rhythmicity in mammals. These include two clock/cycle-related genes, *Clock* and *BMAL1,* two *CRY* genes, *Cry1* and *Cry2*, and two *PER* genes, *Per1* and *Per2*. (Angelousi *et al*., 2019; Moraes *et al*., 2017; Ye *et al*., 2018). Recent studies have suggested that *Clock* and *BMAL1* play key roles in the formation of circadian rhythm (Chen *et al*., 2016; Trott and Menet, 2018). *Clock* was the first mammalian circadian clock

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gene to be discovered (King *et al*., 1997). *BMAL1* encodes a functional chaperone of *Clock*; and their protein products form a dimer that binds to a specific motif, CACGTG (also known as "E-box"), in the promoter regions of *Cry1*, *Cry2*, *Per1* and *Per2*. (Zheng *et al*., 2019).

In mammals, seasonal estrous is one of the biological activities that is regulated by circadian rhythm. In the hypothalamic-pituitary-gonadal axis, seasonal changes in light and temperature are translated into nerve impulses that act on the pineal gland via the suprachiasmatic nucleus (Hastings *et al*., 2000). This leads to the release of melatonin, which in turn regulates the release of gonadotropin-releasing hormone (Chappell *et al*., 2003; von Gall *et al*., 2000), ultimately affecting reproduction.

Sheep (*Ovis aries*) are a typical seasonal estrous species (Tang *et al*., 2018). However, in the current research into the molecular mechanism of seasonal estrous mode in sheep, researchers tend to focus mainly on ewes but ignore rams, even though, there also are strong seasonal rhythms related to reproduction in males of many animal species, most notably hamsters (Reiter, 1980) and sheep (Lincoln, 2002). Studies have shown that rams can identify seasonal changes in light, and translate them into molecular signals that affect gonadal function by regulating the secretion of melatonin (Kennaway, 2005). Considering the effects of the clock genes on seasonal estrous in ewes, it is important to explore their potential roles in ram reproduction.

Small Tail Han sheep (STH) and Sunite sheep (SNT) are two Chinese local sheep (*Ovis aries*) breeds with different estrous modes, year-round estrous and seasonal estrous (mainly in winter and spring), respectively. Both are known for their excellent meat production performance (Tang *et al*., 2018). Huge difference between the two sheep breeds in estrous modes have resulted in increasing interest in the tissue expression profiles of clock genes in these sheep. In the present study, we compared the tissue expression profiles and mRNA expression levels of six canonical clock genes in eight prolificacy-related tissues between STH and seasonal estrous SNT rams. Our study paves the way for in-depth study of the seasonal estrous mode of rams.

MATERIAL AND METHODS

Selection of experimental sheep and sample collection

Three STH and three SNT rams were supplied by the Yuncheng Breeding Sheep Farm (Yuncheng County, China) and the Sheep and Goat Breeding Farm of Tianjin Institute of Animal Sciences (Tianjin, China), respectively. The six rams were healthy, approximately 2.5 years old and were kept in a sheltered outdoor paddock. Alfalfa hay and concentrate were provided and clear water was available ad libitum. Eight tissues (brain, cerebellum, hypothalamus, pituitary, testis, epididymis, vas deferens and adrenal gland) were collected from each animal. All tissues were snap frozen in liquid nitrogen and then stored at−80 °C to be used for RNA extraction.

All experimental procedures used in the present study were approved by the Science Research Department (in charge of animal welfare issues) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China). Ethical approval for the study and all of its protocols was provided by the animal ethics committee of IAS-CAAS (No. IASCAAS-AE-03, 12 December, 2016), which ensured that all efforts were taken to minimize pain and discomfort to the animals while conducting these experiments.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the eight collected tissues using a total RNA extraction kit for animal tissue (Tiangen, Beijing, China). Trizol (Invitrogen Inc., Carlsbad, CA, USA) was used to dissolve the tissues. Each tissue was homogenized and 50–100 mg samples were used for RNA extraction. The quantity and quality of total RNA were monitored using 1.5% agarose gel electrophoresis (U = 150 V;10 min) and ultraviolet spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan), respectively. The A260/280 ratios (1.8–2.0) of the RNA samples were all 1.9 to 2.0, which showed that the extracted total RNA was of acceptable purity with no contamination or degradation. Therefore, the RNA preparations were deemed fit for use in the follow-up experiments, and so were stored at −80 °C until use.

First strand of cDNA was prepared using a PrimeScript™ RT Reagent Kit according to the manufacturer's instructions (TaKaRa Bio Inc., Dalian, China). The PCR thermocycler program was as follows: 37 °C for 15 min, followed by 85 °C for 5 s. The reaction mixture contained 1.0 µL Prime Script RT Enzyme, 1.0 µL random 6-mers, 4.0 µL 5 × Prime Script Buffer (for Real Time), 1.0 µL total RNA and 13 µL RNase-free ddH_2O (total volume, 20 µL). Prior to storage at −80 °C, the standard working concentration of cDNA was 200 ng/µL. The quality of cDNA was evaluated by housekeeping gene (*RPL-19*) amplification, and cDNA were stored at −20 °C until use.

Primer design

Using the Primer Premier software (version 5.0, PREMIER Biosoft Co., Palo Alto, CA, USA), a total of seven primers were designed to amplify different fragments of the ovine *Clock, BMAL1, Cry1, Cry2, Per1, Per2* and *RPL-19* genes, based on their assembled

Table I. Primers used in this study to amplify the six canonical clock genes.

Gene Names	Primer Sequence (5′→3′)	Length (bp)	Tm (°C)	Accession No.
Clock	F: 5′-CAACGCACACATAGGCCTTC-3′ R: 5′-CTATTATGGGTGGTGCCCTGT-3′	181	60	NM_001130932.1
BMAL1	F: 5′-ATTGCAACCGGAAACGCAAG-3′ R: 5′-TGGTGGCACCTCGTAATGTT-3′	288	62	NM_001129734.1
Cry1	F: 5′-ACAGGTGGCGATTTTTGCTT-3′ R: 5′-TCCAGCTTCAGTTGCCAGTT-3′	215	61	NM_001129735.1
Cry2	F: 5′-AGGCTGTTCAAGGAATGGGG-3′ R: 5′-CGTAGGTCTCATCGTGGCTC-3	316	61	NM_001129736.1
Per1	F: 5′- GCCAGACAACCCTTCTACCAGT-3′ R: 5′- GGCTTGCACCTGCTTGACACA-3′	187	61	XM_027974931.1
Per2	F: 5′-TTACGACCACACATTCGCCA-3′ R: 5′-CCCCAGACTGCACGATCTTC-3′	171	61	XM_027967088.1
RPL-19	F: 5′-ATCGCCAATGCCAACTC-3′ R: 5′-CCTTTCGCTTACCTATACC-3′	154	60	XM_012186026.1

Abbreviations: bp, base pairs; Tm, melting temperature.

sequences in GenBank. All primers were synthesized by Beijing Tianyi Biotechnology Co., Ltd. (Beijing, China). The housekeeping gene (*RPL-19*, Genbank: XM_012186026.1) was used as an internal control to normalize the threshold cycle (Ct) values. Primers details are given in Table I.

Quantitative polymerase chain reaction

The expression levels of *Clock, BMAL1, Cry1, Cry2, Per1* and *Per2* in eight tissues (brain, cerebellum, hypothalamus, pituitary, testis, epididymis, vas deferens and adrenal gland) from SNT and STH rams were measured by quantitative polymerase chain reaction (qPCR).

The qPCR protocol used 20 µL of reaction mixture that contained 10 µL SYBR Premix EX Taq II (TaKaRa Bio Inc., Dalian, China), 0.8 µL each of forward and reverse primer (10 pmol/ul), 6.4 µL RNase-free ddH_2O and 2 µL cDNA. Amplifications were performed in triplicate wells using the following PCR thermocycler program: denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The dissociation curve was analyzed after amplification. The melting temperature (Tm) peak observed at 85 °C±0.8 on the dissociation curve was used to determine the specificity of the PCR amplification.

Statistical analysis

The $2^{-\Delta Ct}$ method (Livak, *et al*., 2001) was used to process the real-time PCR results. Statistical analyses were carried out using SPSS 19.0 software (IBM, Armonk, NY, USA). Levels of gene expression were analyzed for significant differences by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test as a multiple comparison test. All experimental data are presented as mean ± standard error of the mean (SEM). A probability of $p \leq 0.05$ was considered statistically significant, and a probability of $p \leq 0.01$ was considered to be highly statistically significant.

RESULTS

Expression levels of clock

As shown in Figure 1, Clock was expressed in all the tissues. Brain was found to have the highest *Clock* expression level, followed by cerebellum and hypothalamic. Expression levels in the hypothalamus, pituitary and epididymis were significantly higher in STH samples compared with SNT samples ($p < 0.05$).

Expression levels of BMAL1

As shown in Figure 2, *BMAL1* was expressed in all the tissues, The highest expression level was found in cerebellum, followed by brain and testis. Expression of *BMAL1* in the brain, cerebellum, hypothalamus, pituitary, testis and epididymis were significantly higher in STH samples compared with SNT samples ($p < 0.01$).

Expression levels of Cry1

As shown in Figure 3, *Cry1* was expressed in all the tissues. The highest expression level was found in testis, followed by cerebellum and hypothalamus. Expression levels of *Cry1* in the pituitary ($p < 0.05$), testis ($p < 0.01$) and adrenal gland ($p < 0.05$) were significantly higher in SNT samples compared with STH samples.

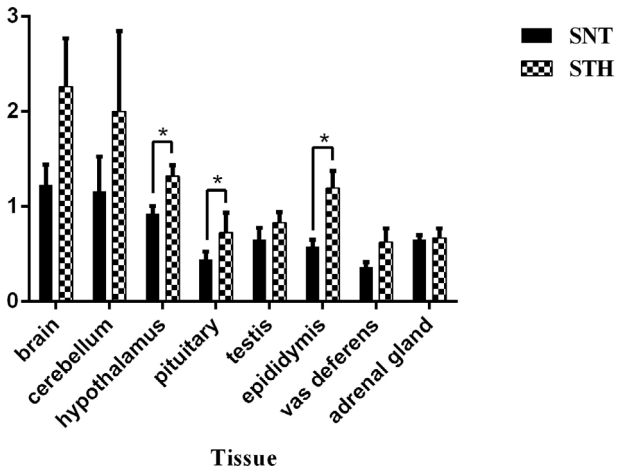

Fig. 1. Comparison of mean expression levels of *Clock* in eight tissues between seasonal estrous and year-round estrous rams. Asterisks indicate significantly different means. Key: * $p < 0.05$. Error bars show the standard error of the mean.

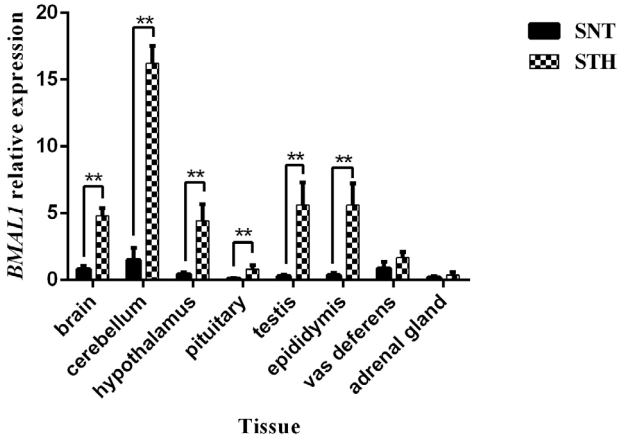

Fig. 2. Comparison of the mean expression of *BMAL1* in eight tissues between seasonal estrous and year-round estrous rams. Asterisks indicate significantly different means. Key: **$p < 0.01$. Error bars show the standard error of the mean.

Expression levels of Cry2

As shown in Figure 4, *Cry2* was expressed in all the tissues. The highest expression level was detected in cerebellum, followed by brain and hypothalamus. Expression levels of *Cry2* in the brain ($p < 0.05$), cerebellum ($p < 0.01$), hypothalamus ($p < 0.01$), pituitary ($p < 0.05$) and adrenal gland ($p < 0.05$) were significantly higher in STH samples compared with SNT samples.

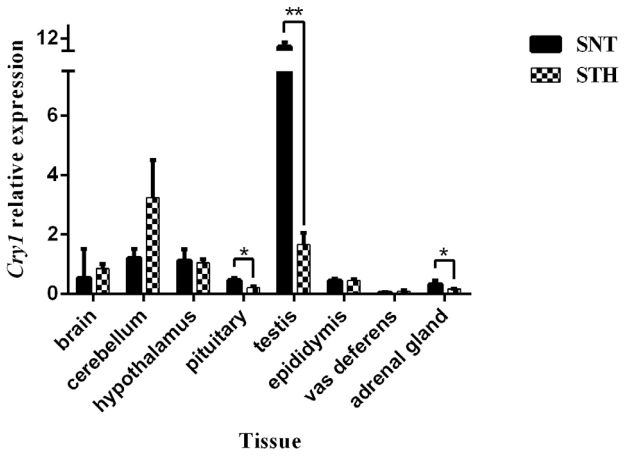

Fig. 3. Comparison of the mean expression of *Cry1* in eight tissues between seasonal estrous and year-round estrous rams. Asterisks indicate significantly different means. Key: * $p < 0.05$, **$p < 0.01$. Error bars show the standard error of the mean.

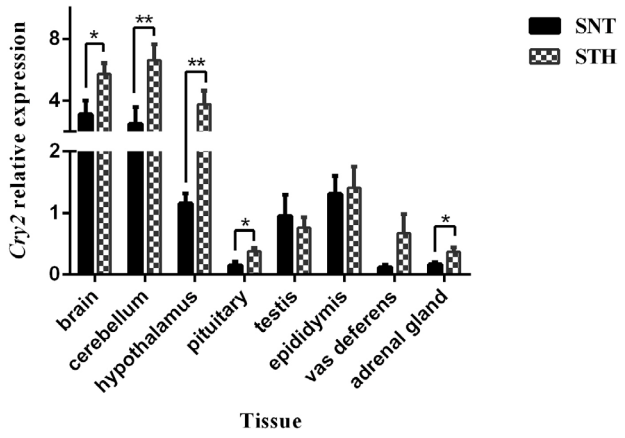

Fig. 4. Comparison of the mean expression of *Cry2* in eight tissues between seasonal estrous and year-round estrous rams. Asterisks indicate significantly different means. Key: * $p < 0.05$, **$p < 0.01$. Error bars show the standard error of the mean.

Expression levels of Per1

As shown in Figure 5, *Per1* was expressed in all the tissues. The highest expression level was found in the brain, followed by adrenal gland and hypothalamus. Expression levels of *Per1* in the pituitary and epididymis were significantly higher in SNT samples compare with STH samples ($p < 0.01$). The expression level of *Per1* in the testis was significantly higher in STH samples compare with SNT samples ($p < 0.01$).

Expression levels of Per2

As shown in Figure 6, *Per2* was expressed in all the

tissues. The highest expression level was in the brain, followed by cerebellum and hypothalamus. Expression levels of *Per2* in the brain, hypothalamus, pituitary, testis and epididymis were significantly higher in SNT samples compared with STH samples ($p < 0.05$, $p < 0.01$), whereas the expression levels of *Per2* in the vans deferens and adrenal gland were significantly higher in STH samples compared with SNT samples ($p < 0.05$ and $p < 0.01$, respectively).

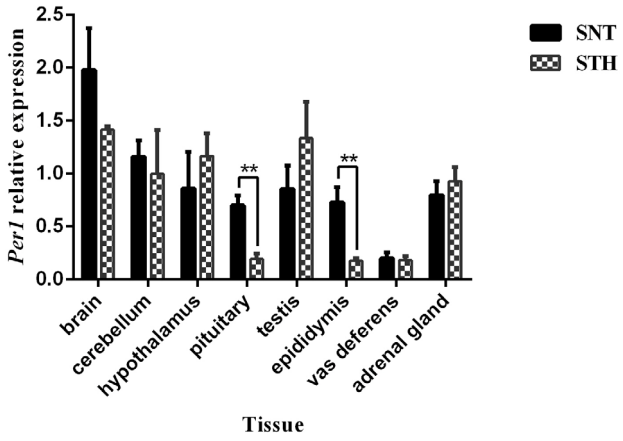

Fig. 5. Comparison of the mean expression of *Per1* in eight tissues between seasonal estrous and year-round estrous rams. Asterisks indicate significantly different means. Key: **$p < 0.01$. Error bars show the standard error of the mean.

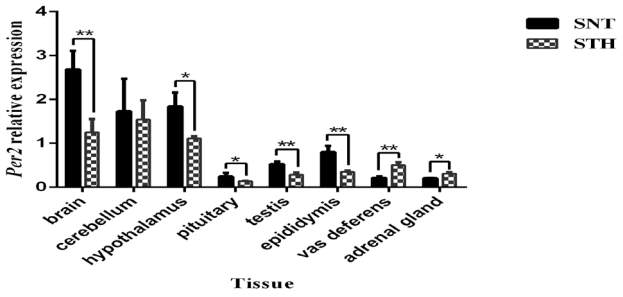

Fig. 6. Comparison of the mean expression of *Per2* in eight tissues between seasonal estrous and year-round estrous rams. Asterisks indicate significantly different means. Key: * $p < 0.05$, **$p < 0.01$. Error bars show the standard error of the mean.

DISCUSSION

Clock and BMAL1

Circadian rhythm is known to be crucial to the estrous mode of male animals, and members of the canonical clock gene family-*Clock* and *BMAL1*-are involved in male reproduction (Peruquetti *et al*., 2012). Alvarez *et al*. (2003) reported that down-regulation of *Clock* in mouse testis leads to a decrease in the fertilization capacity of the corresponding sperm, which highlights the critical function of *Clock* in spermatogenesis. *BMAL1* knock-out male mice are rarely able to successfully fertilize normal female mice. Although the mechanism underlying this effect is not known, it may be related to the influence of *BMAL1* on the secretion of testosterone and other steroid hormones (Alvarez *et al*., 2008). Furthermore, *Clock* and *BMAL1* are involved in the regulation of energy metabolism, lipid metabolism (through a vital role in the differentiation and maturation of fat cells), glucose metabolism (including glucose balance and insulin resistance), and energy balance. (Lamia *et al*., 2008; Rudic *et al*., 2004; Shimba *et al*., 2005).

In sheep, the oscillating pattern of *Clock* expression is similar to that observed in other mammals (Kennaway *et al*., 1981). In this study, we found that *Clock* and *BMAL1* were expressed in all the reproductive tissues, which reflects the expression patterns of these genes in humans and mice. This result supports the suggestion that *Clock* and *BMAL1* influence the reproductive activity of rams directly by regulating the expression of related clock genes in the hypothalamic-pituitary-gonadal axis, as well as indirectly influencing ram breeding activities by regulating energy metabolism in other tissues (Pugazhendhi *et al*., 2019). In addition, the expression patterns of *BMAL1* and *Clock* in the hypothalamus and pituitary (which have key roles in circadian rhythm) are similar to those of ewes (Lincoln *et al*., 2002), which implies similar roles of these genes in the two sexes.

Many studies have reported the joint effects of *Clock* and *BMAL1* on reproduction (Kondratov *et al*., 2006; Tamayo *et al*., 2015; Zhang *et al*., 2016). Briefly, both genes contain the transcription factor PAS-HLH domain (Kondratov, *et al*., 2003), which contributes to the formation of Clock: BMAL1 dimers from the gene products. The dimer form activates transcription and translation of PER and CRY (Jung, *et al*., 2003; Kurbatova *et al*., 2012; Li *et al*., 2008), and regulates the frequency of the gonadotropin-releasing hormone pulse generator (Chappell *et al*., 2003), which in turn affects rhythmic biological activities such as seasonal estrous. In the present study, we found that the expression levels of *Clock* and *BMAL1* in the brain, cerebellum, hypothalamus, pituitary and epididymis were higher in STH than in SNT rams (although this difference was not significant for *Clock* expression in the brain and cerebellum; $p>0.05$). Our findings are in agreement with reported expression patterns of *Clock* and *BMAL1* in male black-line hamsters (Liu, 2016), indicating that these genes have similar regulatory functions in rams and other male seasonal estrous animals.

It seems plausible that the long period of estrous in rams may be related to the high expression levels of *Clock* and *BMAL1*. However, although expression of *BMAL1* in the testis was significantly higher in STH rams compared with SNT rams, there was no significant difference in the expression levels of *Clock* between STH and SNT testis tissues. One possible explanation is that, in testis, *Clock* and *BMAL1* do not have primary roles that affect ram reproduction. Our findings are in agreement with Morse *et al*. who found that testis did not exhibit rhythmicity of clock gene expression (Morse *et al*., 2003). Further studies are needed to investigate the relationship between *Clock*/*BMAL1* and ram reproduction in more depth.

Period and cryptochromes

Besides *Clock* and *BMAL1*, the molecular mechanism of seasonal estrous involves the transcriptional activation of *Per* and *Cry* by the BMAL1: Clock dimer (Hirayama *et al*., 2003; Jang *et al*., 2015; Spoelstra *et al*., 2014). Cryptochrome is the only negative feedback loop involved in the regulation of circadian rhythm, and this has a strong effect on biological rhythms. In mammals, there are two variants, *Cry1* and *Cry2*, which play opposite roles in the feedback loop (Duong *et al*., 2011). *Cry1* knock-out mice exhibit a short-period circadian rhythm at behavioral and tissue/cell levels, whereas *Cry2* knock-out mice exhibit a completely opposite phenotype (van der Horst *et al*., 1999). The reason behind these opposing phenotypes is still unclear. In mammals, the period gene family has three members, *Per1*, *Per2,* and *Per3* (Tei *et al*., 1997). Mutations or deletions in *Per1* or *Per2* have been shown to cause changes in circadian rhythms, but no evidence of the effect of *Per3* on circadian rhythms has been reported so far (Bae *et al*., 2001). Therefore, *Per3* is considered to be unnecessary for circadian rhythm, and so *Cry1*, *Cry2*, *Per1* and *Per2* were selected for investigation of the seasonal estrous of rams in this study.

In the first study of period genes in mice (Tei *et al*., 1997), it was reported that *Per*/*Cry* genes are expressed in mouse heart, brain and testis. Further studies revealed that equivalent genes are expressed in many tissues in human (Hawkins *et al*., 2008). Our results demonstrate that *Per1/2* and *Cry1/2* are expressed in all eight of the selected tissues of STH and SNT rams, and are highly expressed in the brain, cerebellum, and hypothalamic-pituitary-gonadal axis. This implies a role of Per/Cry genes in ram reproduction.

Studies have shown that over-expression of *Cry* in mammalian cells can directly inhibit the transcriptional activity of the BMAL1: Clock dimer, whereas over-expression of *Per* has little effect on this process (Akashi *et al*., 2014; Leloup and Goldbeter, 2003). Therefore, we compared the expression levels of *Per1, Per2, Cry1*, *Cry2*, *BMAL1* and *Clock* in different tissues, which revealed significantly higher expression of *BMAL1* and *Clock* in the pituitary tissue of STH rams compared with SNT rams. This was also the case for *Cry1* and *Cry2*. Considering the core function of the pituitary gland in circadian rhythm, we speculate that *Cry1* and *Cry2* may play opposite roles in the circadian rhythm of rams, similar to their reported functions in other mammals. Surprisingly, we found that *Per1* and *Per2* have similar expression patterns as *Clock*, *BMAL1*, and *Cry1* in the pituitary tissue, which indicated that PER may be associated with the ram reproduction to some degree. Further research is necessary to confirm this idea.

It is worth noting that the stability of CRY is largely dependent on the presence of PER, they can form a complex to stabilize each other and regulate circadian rhythm (Zhou *et al*., 2018). We found the expression levels of *Cry1*, *Per1,* and *Per2* were significantly higher in the pituitary tissue of SNT rams compared with STH rams, whereas, *Cry2* showed the opposite pattern. Given the expression of the four genes in the pituitary gland in ewes (Lincoln *et al*., 2002), we speculate that their regulatory mechanism is similar in rams, namely, PER and CRY bind to form a complex that plays a role in circadian rhythm in the pituitary. However, these four genes did not have similar expression patterns in other tissues, indicating that CRY and PER can work either by forming a dimer or as monomeric proteins.

CONCLUSION

This study describes the expression pattern of six canonical clock genes in year-round estrous (STH) and seasonal estrous (SNT) rams. All six genes were expressed in the eight selected tissues, with high expression levels seen in the brain, cerebellum, hypothalamus, testis and epididymis. Our results suggest that circadian rhythmicity may regulate the estrous mode of rams via clock gene transcription/translation feedback/feedforward loops. However, the specific mechanism remains to be further explored. This is the first study to investigate the tissue-specific expression patterns of the six canonical clock genes in rams, and it provides a foundation for elucidating the molecular mechanisms of the estrous mode in rams.

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Statement of conflicts of Interest

All authors declare no conflicts of interest.

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