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PHOTOPERIOD ALTERS THE CHOROID PLEXUS RESPONSE TO LPS-INDUCED ACUTE INFLAMMATION IN EWES*

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Abstract

This study determined the influence of photoperiod on the expression of toll-like receptor 2 and 4 (*TLR2* and *TLR4*), interleukin 1β (*IL1B*), IL-1 receptor type I (*IL1R1*) and II (*IL1R2*), interleukin 6 (*IL6*), the IL-6 receptor (*IL6R*) and signal transducer (*IL6ST*), tumor necrosis factor α (*TNF*), and TNFα receptor type I (*TNFRSF1A*) and II (*TNFRSF1B*) in the choroid plexus (ChP) of ewes with lipopolysaccharide (LPS)-induced acute inflammation. Under short days (SD, n = 12, anestrus) and long days (LD, n = 12, synchronized follicular phase), ewes were treated with saline or LPS. Compared to LD conditions, the ewes under SD were characterized by a greater (P<0.05) area under the curve (AUC) of cortisol in the LPS-treated group and by a lower (P<0.05) AUC of prolactin in the saline-treated group. Under both photoperiods, LPS increased (P<0.05) the expression of all examined genes except for *TNFRSF1B* (only under SD), *TNF* and *TNFRSF1A* (no stimulation), and *IL6R* (decreased (P<0.05) under SD). The LPS-induced increases in *TLR2*, *TLR4*, *IL1B* and its receptors, *IL6* and *TNFRSF1B* were higher (P<0.05) under SD than LD. *TLR4* was positively correlated with *IL1B* and *IL6* in both saline- ($r^2 = 0.64$, P<0.01 and $r^2 = 0.52$, P<0.01) and LPS-treated ($r^2 = 0.81$, P<0.0001 and $r^2 = 0.51$, P<0.001) ewes. *IL1B* ($r^2 = 0.56$, P<0.01 and $r^2 = 0.77$, P<0.0001) and *IL6* ($r^2 = 0.77$, P<0.005 and $r^2 = 0.35$, P<0.05) were positively correlated with *TLR2* in saline- and LPS-treated ewes, respectively. This indicates that in ewes, the ChP response to acute systemic inflammation is dependent upon the photoperiod with stronger effects being observed under SD. Our results also suggest that gonadal hormones altering TLR4 signaling events are involved in the photoperiodic modulation of the ChP response to LPS. Further experiments are required to explain the mechanism involved in this phenomenon.

Key words: innate immunity, toll-like receptors, pro-inflammatory cytokines, ruminants

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At higher latitudes, seasonal changes in day length (photoperiod) provide the primary environmental cue that synchronizes animal physiology to environmental living conditions. As day length changes over the course of the year, longer- and shorter-duration melatonin signals are generated during the short days (SD) of winter and the long days (LD) of summer, respectively. Melatonin acting directly on the pituitary gland mediates the effects of photoperiod on the secretion of prolactin (PRL), which is higher during LD than SD (Lincoln and Clark, 1994). The broad spectrum of seasonal physiological adaptations includes fattening and weight loss, molting and pelage growth and reproduction.

The choroid plexus (ChP) of the brain ventricles plays an important role in the communication between the periphery and the brain; it receives and responds to blood-borne signals as well as signals coming from the brain via the cerebrospinal fluid (CSF) (Skipor and Thiery, 2008). The blood-CSF barrier (BCSFB), located in the epithelial cells of the ChP, allows the access of molecules to the CSF and then to the brain to be regulated. In ewes, greater access of sex steroids to the CSF under LD than SD was postulated to participate in the mechanism regulating the seasonal shift in responsiveness to steroids (Thiery and Malpaux, 2003; Thiery et al., 2003, 2006), which is important for the seasonal inhibition of reproductive activity during LD (Weems et al., 2015). Additionally, brain access to leptin, a hormone that responds to photoperiod-driven changes in food intake and adiposity in sheep, is higher under LD than SD (Marie et al., 2001; Adam et al., 2006). The ChP is the main source of CSF, whose turnover rate in ewes is modulated by photoperiod and is approximately two times higher under SD conditions (Thiery et al., 2009). In turn, melatonin modulates the expression of proteins responsible for water transport in the ChP (Szczepkowska et al., 2018, 2019). In ewes, photoperiod-dependent changes have been identified in the CSF proteome composition (Teixeira-Gomes et al., 2015) as well as the concentration of thyroid hormones in CSF (Skipor et al., 2010, 2012).

The ChP is also indicated to be an important link between systemic inflammation and cerebral innate immune responses and is actively involved in the control of immune cell and pathogen entry into the brain (Engelhardt et al., 2001; Kunis et al., 2013; Schwerk et al., 2015). The ChP is not a homogeneous structure; it contains endothelial and epithelial cells as well as macrophages and dendritic cells residing in the stromal matrix (Meeker et al., 2012). At least the epithelial and immune cells express specialized receptors of the innate immune system belonging to the Toll-like receptor (TLR) family and therefore have the potential to respond to lipopolysaccharide (LPS), a TLR4 ligand (Akira et al., 2006). Numerous TLR receptors (TLR1-7 and TLR9-10) have been detected at the transcript level in the ovine ChP (Skipor et al., 2015). We demonstrated that LPS-induced immune stress stimulates the mRNA expression of *TLR2*, *TLR4* and *CD14* (cluster of differentiation 14) in the ovine ChP (Kowalewska et al., 2017 b). The activation of the TLR signaling pathway leads to the synthesis of pro-inflammatory cytokines, type I interferon and other mediators that induce innate immune responses (Takeda et al., 2003). Indeed, the upregulation of the expression of pro-inflammatory cytokine and corresponding receptor genes was observed in the ovine ChP under LPS-challenged conditions (Kowalewska et al., 2017 a; Herman et al., 2018). In ewes, the local synthesis of interleukin (IL)-1β in the

ChP is an important source of IL-1β in the CSF at the onset of inflammation (Skipor et al., 2017). Centrally acting pro-inflammatory cytokines have been linked with the inhibition of gonadotropin-releasing hormone gene expression in the preoptic area (POA) in ewes during LPS-induced systemic inflammation in both anestrus during LD and the breeding season during SD (Herman and Tomaszewska-Zaremba, 2010; Herman et al., 2010, 2017). However, there are no data comparing the ChP response to acute systemic inflammation during these two seasons in ewes. Considering that in most vertebrates, innate immunity, which is the first line of defense activated immediately after infection, varies significantly throughout the year (Nelson, 2004), we hypothesized that photoperiod may also affect the ChP response to systemic acute inflammation. Therefore, the aim of the present study was to determine the influence of photoperiodic conditions under natural SD and LD on the mRNA expression of *TLR4* and *TLR2,* interleukin 1β (*IL1B*) and its type I (*IL1R1*) and type II (*IL1R2*) receptors, tumor necrosis factor α (*TNF*) and its type I (*TNFRSF1A*) and type II (*TNFRSF1B*) receptors, and interleukin 6 (*IL6*) and its receptor (*IL6R*) and signal-transducing component (*IL6RST*) in the ChP in ewes with or without LPS-induced acute systemic inflammation.

Material and methods

Animals and experimental design

The studies were performed on frozen samples of the ChP obtained from adult female blackface sheep (n = 24, 2-year-old, body condition score = 3) consistent with the studies of Krawczyńska et al. (2019 a). The ewes were maintained under the natural (latitude 52°N, 21°E) light conditions of SD (n = 12, December, day:night 8:16) and LD (n = 12, June, day:night 16:8) photoperiods and were fed a consistent diet of commercial concentrates with hay and water available *ad libitum* according to the recommendations of the National Research Institute of Animal Production (Strzetelski et al., 2014). All animal procedures were approved by the 3rd Local Ethical Commission of the Warsaw University of Life Sciences – SGGW (Warsaw, Poland, authorization no. 56/2013). To avoid variability connected with the phase of the estrous cycle during SD, the ewes were synchronized via the Chronogest® CR (Merck Animal Health, Boxmeer, The Netherlands) method using an intravaginal sponge impregnated with 20 mg of cronolone (flugestone acetate), a synthetic progesterone-like hormone. After sponge removal 14 days after intravaginal insertion, the ewes were intramuscularly injected with 500 IU of pregnant mare's serum gonadotropin (PMSG, Merck Animal Health, Boxmeer, The Netherlands). The experimental procedure began 24 h after PMSG injection, so the ewes were in the follicular phase of the estrous cycle. For LD ewes, synchronization was not required, as the animals were in seasonal anestrus. In both SD and LD experiments, the animals were randomly divided into 2 groups: one group was treated intravenously with LPS (LPS group, n = 6, *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO, USA)) at a dose of 400 ng/kg of body mass, and the other was treated intravenously with

physiological saline (control group, n = 6). During the experiment, the animals were kept in individual pens, and the stress of social isolation was limited by visual contact with other members of the flock. Body temperature was measured before and every 30 min after LPS administration. Ewes were euthanized 3 h after LPS/saline administration, the brain was removed, and the ChP was dissected from the third and lateral ventricles, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis.

Blood collection and hormone concentration measurement

Blood samples were collected every 15 min through a jugular vein catheter (starting 1 h before and continuing for 3 h after LPS/saline administration) for cortisol (controlling the LPS response), prolactin (PRL, controlling the photoperiod response), progesterone (P4) and estradiol (E2) measurement. After centrifugation, the plasma was stored at −20°C until hormone concentrations were analyzed. Plasma PRL and cortisol concentrations were determined at the Radioisotope Laboratory of the Kielanowski Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences in Jabłonna (Poland). The sensitivity of the assay for PRL was 2 ng/ml, and the intra- and inter-assay coefficients of variation were 9 and 12%, respectively. For cortisol measurements, the sensitivity of the assay was 0.95 ng/ml, and the intra- and inter-assay coefficients of variation were 10 and 12%, respectively. The plasma levels of P4 and E2 were measured using RIA assay kits from DSL Beckman Coulter (Brea, CA, USA; cat. no. IM1188 and DSL4800, respectively) according to the manufacturer's instructions. Samples were quantified in duplicate in a single assay with intra-assay coefficients of variations of 8.15% for P4 and 8.9% for E2 and sensitivity on the order of 0.03 ng/ml and 2.2 pg/ml for P4 and E2, respectively.

Gene expression analysis

Total RNA from the ChP was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. The initial homogenization of the frozen ChP was carried out in Lysing Matrix D (MP Biomedicals, Irvine, CA, USA) filled with lysing buffer from the NucleoSpin RNA II Kit (RA1) using a FastPrep24 instrument (MP Biomedicals, Irvine, CA, USA) at an oscillation speed of 6.5 m/s for 30 s. A step for genomic DNA digestion was included in the isolation procedure. The purity and concentration of the isolated RNA were quantified spectrophotometrically using a NanoDrop 1000 instrument (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was confirmed by electrophoresis in a 1.2% agarose gel stained with ethidium bromide. The DyNAmo cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA) and 1 μg of total RNA were used to synthetize cDNA according to the manufacturer's protocol. The resulting cDNA was stored at −20°C until further analysis. Gene expression in the ChP was determined via real-time PCR. Specific primer pairs for different genes, presented in Table 1, were used according to the literature or were designed using Primer-BLAST (National Center for Biotechnology Information, Bethesda, MD, USA) and were synthesized by Genomed (Warsaw, Poland). Real-time PCR was performed using a Viia7 instrument (Applied Biosystems by Life

Technologies, Waltham, MA, USA). Three housekeeping genes were examined: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTB*) and histone deacetylase 1 (*HDAC1*). For real-time PCR, each reaction (10 µl) contained 3 µl of diluted cDNA (1:10), the forward and reverse primers at 0.2 µM each and 5 µl of the DyNAmo SYBR Green qPCR kit with ROX reagent (Thermo Fisher Scientific, Waltham, MA, USA). The following protocol was used: 95°C for 10 min for the hot-start modified Tbr DNA polymerase, followed by 40 cycles of 95°C for 15 s (denaturation), 60°C for 30 s (primer annealing) and 72°C for 30 s (extension). A final cycle was performed to evaluate the specificity of amplification via a melting curve until continuous fluorescence measurement. The results were analyzed using Real-Time PCR Miner (available online: http://ewindup.info/miner/) based on the algorithm developed by Zhao and Fernald (2005).

Table 1. Sequences of oligonucleotide primers used for real-time PCR

Gene	(Forward/Reverse) Sequence 5'→3'	Amplicon size (bp)/ annealing temp. (°C)	References
TLR2	F: GGC TGT AAT CAG CGT GTT CA R: GAT CTC GTT GTC GGA CAG GT	159/55	Chang et al. (2009)
TLR4	F: TGG ATT TAT CCA GAT GCG AAA R: GGC CAC CAG CTT CTG TAA AC	152/55	Chang et al. (2009)
IL1B	F: CAG CCG TGC AGT CAG TAA AA R: GAA GCT CAT GCA GAA CAC CA	137/59	Herman et al. (2018)
IL1R1	F: GGG AAG GGT CCA CCT GTA AC R: ACA ATG CTT TCC CCA ACG TA	124/59	Herman et al. (2018)
IL1R2	F: CGC CAG GCA TAC TCA GAA A R: GAG AAC GTG GCA GCT TCT TT	162/59	Herman et al. (2018)
TNF	F: CAA ATA ACA AGC CGG TAG CC R: AGA TGA GGT AAA GCC CGT CA	153/59	Herman et al. (2018)
TNFRSF1A	F: AGG TGC CGG GAT GAA ATG TT R: CAG AGG CTG CAG TTC AGA CA	137/59	Herman et al. (2018)
TNFRSF1B	F: ACC TTC TTC CTC CTC CCA AA R: AGA AGC AGA CCC AAT GCT GT	122/59	Herman et al. (2018)
IL6	F: GTT CAA TCA GGC GAT TTG CT R: CCT GCG ATC TTT TCC TTC AG	165/59	Herman et al. (2018)
IL6R	F: TCA GCG ACTC CGG AAA CTA T R: CCG AGG ACT CCA CTC ACA AT	149/59	Herman et al. (2018)
IL6ST	F: GGC TTG CCT CCT GAA AAA CC R: ACT TCT CTG TTG CCC ACT CAG	139/59	Kowalewska et al. (2017 b)
GAPDH*	F: TGA CCC CTT CAT TGA CCT TC R: GAT CTC GCT CCT GGA AGA TG	143/60	Herman et al. (2018)
ACTB*	F: CTT CCT TCC TGG GCA TGG R: GGG CAG TGA TCT CTT TCT GC	168/60	Herman et al. (2018)
HDAC1*	F: CTG GGG ACC TAC GGG ATA TT R: GAC ATG ACC GGC TTG AAA AT	115/60	Herman et al. (2018)

TLR – toll-like receptors 2 and 4; IL1B – interleukin 1-beta; IL1R1 – interleukin 1 receptor, type I; IL1R2 – interleukin 1 receptor, type II; TNF – tumor necrosis factor; TNFRSF1A – tumor necrosis factor receptor superfamily member 1A; TNFRSF1B – tumor necrosis factor receptor superfamily member 1B; IL6 – interleukin 6; IL6R – interleukin 6 receptor; IL6ST –glycoprotein 130; *reference genes: GAPDH – glyceraldehyde-3-phosphate dehydrogenase; ACTB – beta actin; HDAC1 – histone deacetylase 1.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM 8 (GraphPad Software, San Diego, CA, USA). After the verification of the assumption of normality (Shapiro-Wilk's test), the raw data were subjected to the appropriate test. Body temperature, cortisol and PRL concentrations were subjected to repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc test. The areas under the time × concentration curve (AUC) for cortisol and PRL and the mean P4 and E2 plasma concentrations were analyzed using two-way ANOVA with multiple comparisons of Tukey's post hoc test. Real-time PCR results are presented as the relative gene expression of all examined genes vs. the mean of three reference genes. Real-time PCR data were subjected to two-way ANOVA followed by a post hoc Tukey's multiple comparison test. The relationship between variables was analyzed using Pearson's correlation coefficient. All data are presented as the mean ± standard error of the mean (SEM). Statistical significance was established at $P<0.05$.

Results

Effect of photoperiod and LPS on body temperature and hormone concentrations

The mean body temperature of LPS-treated ewes increased ($P<0.05$) from 39.8 ± 0.3°C or 40.0 ± 0.2°C 1 h before LPS administration to 41.4 ± 0.2°C or 40.8 ± 0.2°C 3 h after LPS administration during LD and SD, respectively. In saline-treated ewes, body temperature was 39.3 ± 0.1°C (LD) or 39.6 ± 0.3°C (SD) 1 h before saline administration and did not change during the experiment.

The mean concentration of cortisol in the blood plasma of LPS-treated ewes increased ($P<0.05$) from 12.3 ± 5.9 to 52.6 ± 5.6 ng/ml and from 23.9 ± 6.0 to 82.3 ± 11.2 ng/ml in both the LPS/LD and LPS/SD groups, respectively. The mean cortisol concentration before saline infusion was 7.4 ± 2.2 ng/ml in the C/LD group and 27.4 ± 9.4 ng/ml in the C/SD group and did not change during the entire period of the experiment (Figure 1 A). The AUC of the plasma cortisol profiles was higher ($P<0.05$) in the LPS/SD group than in the LPS/LD group (Figure 1 A). The mean blood plasma concentration of PRL in control ewes ranged from 38.0 ± 4 to 62.7 ± 7.7 ng/ml and from 9.5 ± 0.5 to 15.9 ± 3.7 ng/ml in the C/LD and C/SD groups, respectively, while in LPS-treated ewes, it ranged from 69.3 ± 10.8 to 194.4 ± 61 ng/ml and from 12.0 ± 2.9 to 141.2 ± 67.5 ng/ml in the LPS/LD and LPS/SD groups, respectively (Figure 1 B). The AUC of the plasma PRL profile was higher ($P<0.05$) in the C/LD than in the C/SD group (Figure 1 B).

In ewes, the mean concentrations of P4 in blood plasma were 0.6 ± 0.30 and 0.6 ± 0.1 ng/ml in the C/LD and LPS/LD groups, respectively, and 2.0 ± 0.90 and 1.4 ± 0.30 ng/ml in the C/SD and LPS/SD groups, respectively. In saline-treated ewes, the P4 concentration was higher ($P<0.05$) during SD than LD (Figure 2 A). As indicated in Figure 2 B, there were no differences in the mean plasma concentrations of E2 between the C/LD and C/SD groups (3.7 ± 0.37 vs. 5.0 ± 0.99 pg/ml) or the LPS/LD and LPS/SD groups (2.6 ± 0.49 vs. 3.5 ± 0.56 pg/ml).

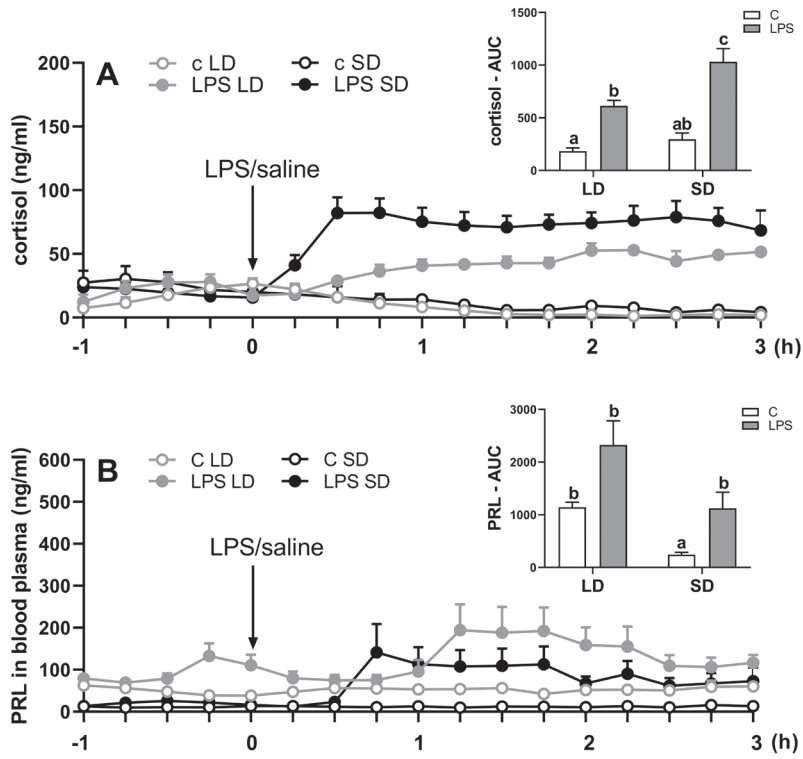


Figure 1. Mean (±SEM) concentrations of cortisol (A) and prolactin – PRL (B) in blood plasma in saline- and LPS-treated ewes during short-day (SD) and long-day (LD) photoperiods. The areas under the time × concentration curve (AUC) for each hormone are presented in inserts. Different lowercase letters indicate significant differences at P<0.05

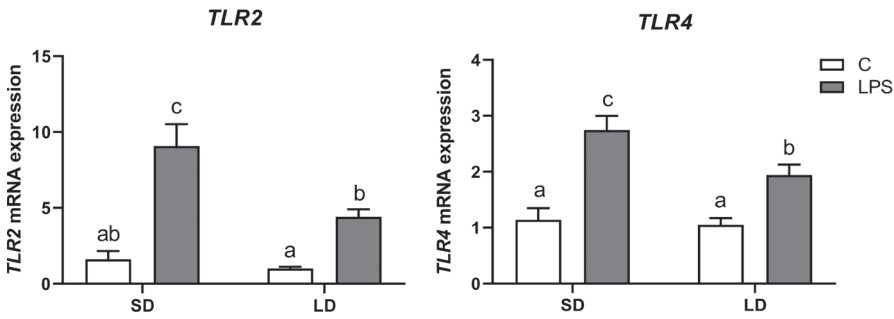


Figure 2. Overall mean (±SEM) concentrations of progesterone – P4 (A) and 17β-estradiol – E2 (B) in the blood plasma of ewes during long-day (LD) and short-day (SD) photoperiods. Different lowercase letters indicate significant differences at P<0.05

Effect of photoperiod on basal and LPS-induced gene expression of TLRs, and pro-inflammatory cytokines and their receptors

The mRNA expression of *TLR2* and *TLR4* in LPS-treated ewes was higher (P<0.05) than that in saline-treated ewes in both the SD and LD photoperiods. Moreover, the LPS-induced upregulation of TLRs expression was higher (P<0.05) under SD than LD (Figure 3). There were no photoperiod-driven changes in *TLR2* and *TLR4* expression in saline-treated ewes.

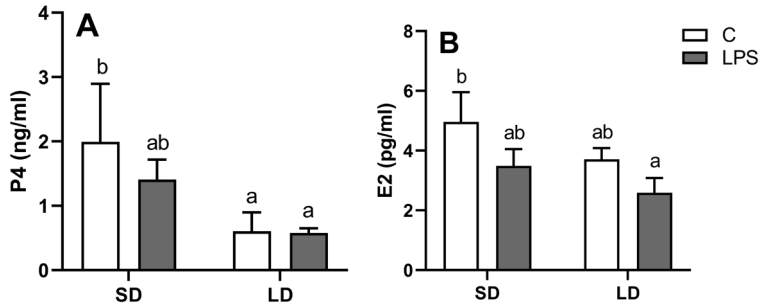


Figure 3. Mean (±SEM) relative *TLR2* (A) and *TLR4* (B) mRNA expression in the ovine choroid plexus, during short-day (SD) and long-day (LD) photoperiods. Different lowercase letters indicate significant differences at P<0.05

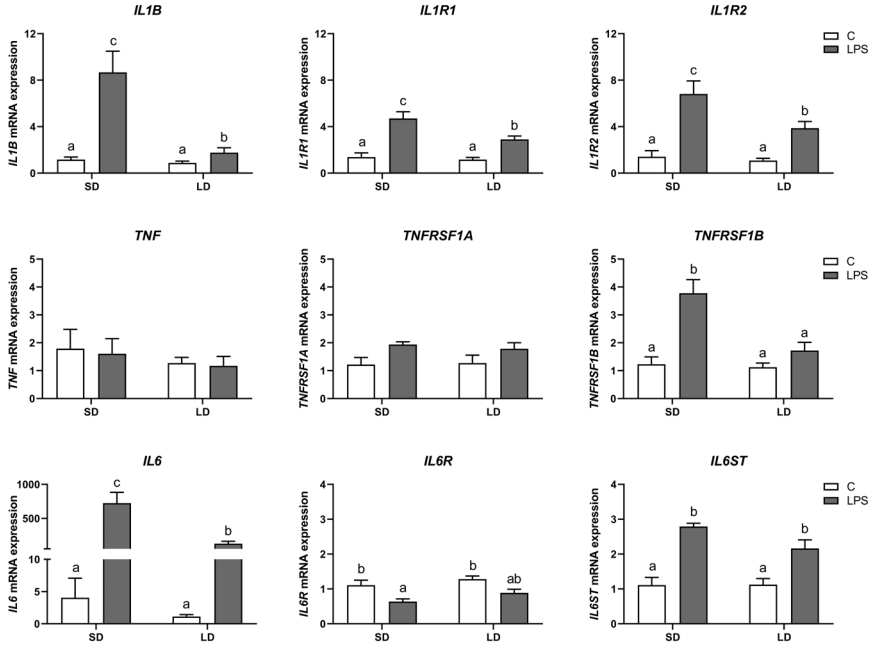


Figure 4. Mean (±SEM) relative *IL1B* (A), *IL1R1* (B), *IL1R2* (C), *IL6* (D) and *IL6ST* (E) mRNA expression in the ovine choroid plexus, during short-day (SD) and long-day (LD) photoperiods. Different lowercase letters indicate significant differences at P<0.05

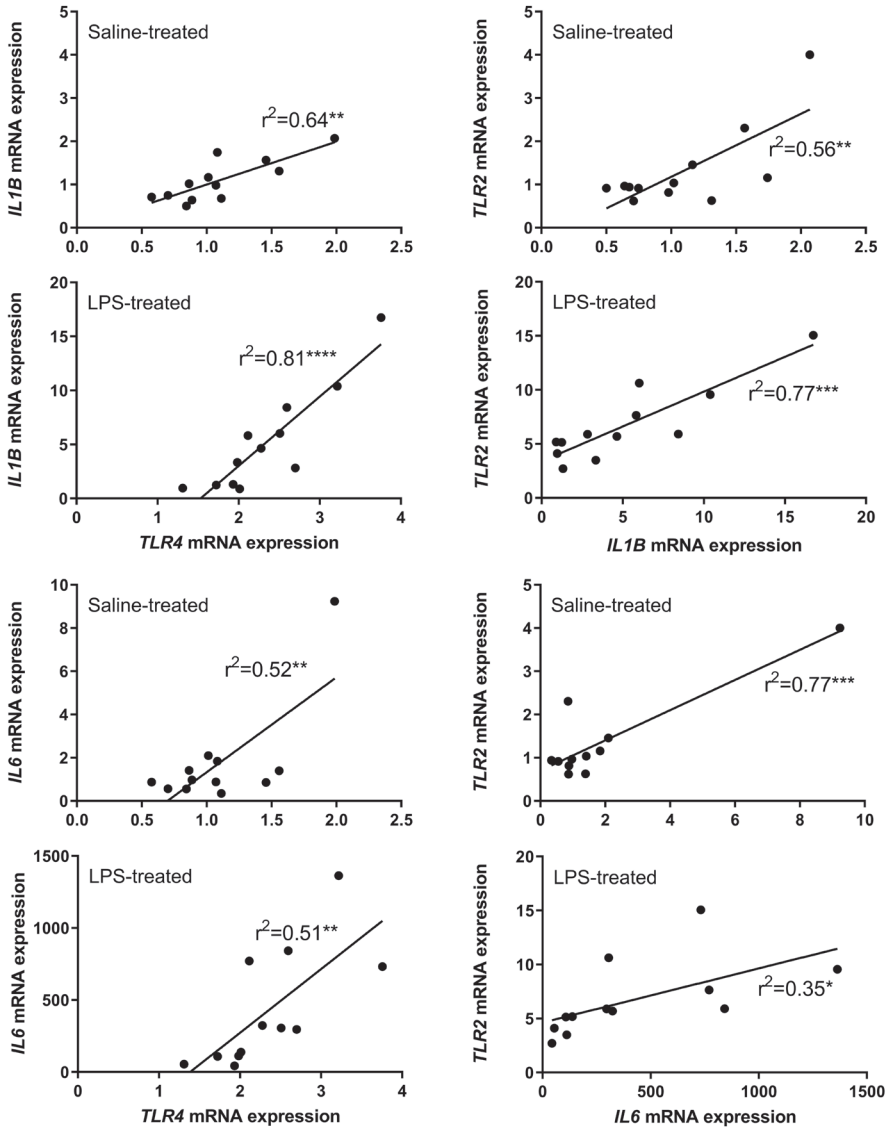


Figure 5. Correlations between the relative mRNA expression of *TLR4* and selected pro-inflammatory cytokines (left panel) and between the relative mRNA expression of *IL1B* and *IL6* and that of *TLR2* (right panel) in the ovine choroid plexus of saline- and LPS-treated ewes from pulled short-day (SD) and long-day (LD) photoperiods. Pearson rank order analysis with significance at P<0.001 (****), P<0.005 (***), P<0.001 (**) and P<0.05 (*)

In both LD and SD photoperiods LPS treatment induced ($P<0.05$) the gene expression of *IL1B* and its receptors (*IL1R1* and *IL1R2*), *IL6* and its signal transducing component (*IL6ST*) and *TNFRSF1B* and decreased that of *IL6R* but had no effect on *TNF* or *TNFRST1A* expression (Figure 4). The LPS-induced upregulation of both interleukins and their receptors was higher ($P<0.05$) under the SD photoperiod than the LD photoperiod. Photoperiod-driven changes in the gene expression of pro-inflammatory cytokines and their receptors were not observed in saline-treated ewes.

Additionally, there was a relationship between the relative expression of *TLR4* and that of *IL1B* and *IL6* in saline-treated ($r^2 = 0.64$, $P<0.01$ and $r^2 = 052$, $P<0.01$, Figure 5) and LPS-treated ewes ($r^2 = 0.81$, $P<0.0001$ and $r^2 = 0.51$, $P<0.01$), respectively. There were also correlations between relative *IL1B* and *IL6* expression and *TLR2* expression in saline-treated ($r^2 = 0.56$, $P<0.01$ and in $r^2 = 0.77$, $P<0.0001$, Figure 5) and LPS-treated ewes ($r^2 = 0.77$, $P<0.005$ and $r^2 = 0.35$, $P<0.05$), respectively.

Discussion

Seasonal immunological plasticity is common in many vertebrates and is trait- and species-specific (Stevenson and Prendergast, 2015). In this paper, we present the first study describing photoperiod-driven differences in the ChP response to acute LPS-induced systemic inflammation in ewes. To the best of our knowledge, similar studies have never been performed in other species. We observed a stronger LPS effect on *TLR2*, *TLR4*, *IL1B*, *IL1R1*, *IL1R2*, *IL6* and *TNFRSF1B* expression under SD than LD. In the same ewes, a stronger response to LPS stimulation during SD than LD was also observed in the anterior pituitary (Wójcik et al., 2020). Moreover, in these ewes, the sensitivity of the perivascular adipose tissue and aorta to the action of leptin on the gene expression of pro-inflammatory cytokines and their receptors was photoperiod driven, with a stronger effect being observed in the SD photoperiod (Krawczyńska et al., 2019 a, b). It should be emphasized that photoperiod-driven differences were not observed in the ChP under basal conditions (no LPS stimulation), which was confirmed by our previous finding that the profile of TLR gene expression in the pineal gland was generally independent of photoperiodic conditions and/or the circulating levels of melatonin (Bochenek et al., 2015). In turn, in the anterior pituitary, only the expression of *IL1B* and *IL1R2* was similar between LD and SD, while the expression of other cytokines and their receptors was higher under SD than LD (Wójcik et al., 2020).

TLR4 is the only member of the TLR4 subfamily and binds LPS and its endogenous ligands involved in the inflammatory response, even in the absence of infection, while TLR2 belongs to the TLR1 subfamily and because of heterodimerization with other subfamily members (TLR1, TLR6 and TLR10), it binds to a variety of microbial components including lipoproteins/lipopeptides, peptidoglycans and lipoteichoic acid, zymosan, glycolipids and atypical LPS (Takeda and Akira, 2005). Considering that LPS binding to its receptor activates the TLR signaling pathway,

leading to an increase in pro-inflammatory cytokine expression, one might expect a correlation between the expression of *TLR4* and that of *IL1B* and *IL6* in the ovine ChP. Indeed, we observed a positive correlation, indicating that *TLR4* is directly responsible for the stronger ChP response to LPS-induced systemic inflammation observed in ewes during the SD photoperiod. In turn, the higher *IL1B* expression and plasma cortisol concentration observed in LPS-treated ewes during the SD photoperiod might be responsible for the higher *TLR2* expression under SD than LD, since the synergistic action of glucocorticoids and pro-inflammatory cytokines in the induction of *TLR2* expression has been demonstrated in multiple cell types (Hermoso et al., 2004; Homma et al., 2004; Sakai et al., 2004).

The mechanism by which the photoperiod modulates the ChP response to acute systemic inflammation is not known. Generally, photoperiodic changes in immunity are dependent on the duration of pineal melatonin secretion (Stevenson and Prendergast, 2015). Melatonin has been suggested to act as an immune buffer with a stimulatory effect under basal or immunosuppressive conditions or an inhibitory effect in the presence of exacerbated immune responses, such as acute inflammation (Carrillo-Vico et al., 2013). The ChP is under the influence of very high concentrations of melatonin, which can act on its receptors, MT1 and MT2 (Cogé et al., 2009). In ewes, the mean diurnal and nocturnal concentrations of melatonin reach levels of 71 ± 8 and 1497 ± 216 pg/ml in the CSF and 8 ± 2 and 117 ± 15 pg/ml in jugular blood, respectively (Skinner and Malpaux, 1999). Accordingly, an attenuated ChP response to inflammation during SD would be expected. In our study, we observed an opposite effect. Therefore, we can assume that other factors mask the effect of melatonin. Studies in Siberian hamsters, a key model species for the study of immunological photoperiodism under laboratory conditions, demonstrated that the photoperiodic regulation of immune function may be both dependent on and independent of gonadal hormones (Bilbo and Nelson, 2001; Prendergast et al., 2008). Sheep are SD breeders, which means that they are in the nonbreeding season when the gonads become relatively inactive during LD and exhibit normal estrous cycles during SD. Our experiment was performed on ewes in the anestrous season and in the follicular phase of the breeding season, which was confirmed by the presence of preovulatory follicles. The mean plasma E2 concentrations did not differ significantly between the C/SD and C/LD groups (5.0 ± 0.99 vs. 3.7 ± 0.37 pg/ml) or between the LPS/SD and LPS/LD groups (3.5 ± 0.56 vs. 2.6 ± 0.49 pg/ml). The P4 concentration was higher in SD ewes than in LD ewes (2.0 ± 0.9 and 0.6 ± 0.3 ng/ml in saline-treated ewes and 1.4 ± 0.3 and 0.6 ± 0.1 ng/ml in LPS-treated ewes, respectively), but this difference was significant only in saline-treated ewes. All the plasma concentrations of sex hormones were in the range reported by Bartlewski et al. (1999, 2000) for ewes during the anestrous and breeding seasons. Despite the lack of significant differences in E2 concentrations at the time of the experiment, we can assume that ewes during the breeding season are under the influence of higher E2 concentrations than those in seasonal anestrus. Sex hormone receptors such as the P4 receptor, α and β estrogen receptor (ERα and ERβ, respectively), and androgen receptor are expressed in the ChP (Santos et al., 2017). Unfortunately, there is a lack of data in the literature regarding the effects of photoperiod or reproductive activity on the expression of these

receptors in the ChP. Studies by Calippe et al. (2008, 2010) demonstrated that action of circulating blood E2 via ERα in murine resident peritoneal macrophages *in vivo* enhances the ability of the cells to produce pro-inflammatory cytokines upon TLR4 activation. According to their study, exposure to E2 alters signaling events but does not influence TLR4 surface expression. Macrophages are residual in the ChP; therefore, the above results may at least partly explain why a photoperiod-driven effect on pro-inflammatory cytokine gene expression in the ovine ChP was observed only under LPS-challenged conditions. Similarly, Navara et al. (2007) observed no differences in the mRNA expression of *TLR2* and *TLR4* between SD and LD in Siberian hamster thioglycollate-elicited peritoneal macrophages that were not stimulated with LPS. In the same study, macrophages collected during LD were found to be more sensitive to LPS treatment than those collected during SD, as indicated by higher *TNF* expression. Additionally, hypothalamic *IL1B* and *TNF* gene expression was attenuated in SD hamsters compared to LD hamsters (Pyter et al., 2005). Unlike sheep, hamsters are LD breeders and are reproductively active during the LD of spring and summer (Weems et al., 2015).

The seasonal rhythm of PRL secretion, in which higher plasma PRL concentrations occur during LD than during SD, plays a key role in the adaptation of sheep to the external environment. Indeed, this pattern of PRL release was observed in our sheep, which indicated that the animals were under the expected influence of LD and SD photoperiod. PRL affects the innate immune system by stimulating the secretion of pro-inflammatory and regulatory cytokines as well as other regulatory factors in different cell types (Boutet et al., 2007; Lopez-Meza et al., 2010). In ewes, PRL secretion by the anterior pituitary cells increases following LPS administration (Tomaszewska-Zaremba et al., 2018), which was manifested in our study by the higher plasma PRL concentration in LPS- than saline-treated ewes during the SD photoperiod. The ChP has been found to contain the highest level of PRL receptors present anywhere in the brain (Pi and Grattan, 1998). As demonstrated by Auchtung et al. (2003) decreased circulating PRL levels in the SD photoperiod parallel increased mRNA expression of both the short and long forms of PRL receptors in the bovine liver, mammary gland and peripheral blood lymphocytes. In ewes, the mRNA expression of PRL receptors in the ChP and the paraventricular nucleus showed the same level during the lactation and anestrous periods, while in the POA and supraoptic nucleus, it was higher during lactation than anestrus despite the higher plasma PRL concentration in lactating ewes (Hasiec et al., 2016). Unfortunately, there are no available data on the impact of PRL on PRL receptors in the ChP in nonlactating ewes, which is very important to this discussion.

In conclusion, we demonstrated photoperiod-driven differences in the ChP response to LPS-induced acute systemic inflammation in ewes for the first time, which were manifested in higher gene expression levels of TLRs, pro-inflammatory cytokines and their receptors in the SD than the LD photoperiod. We also provide new data demonstrating a correlation between *TLR4* and *TLR2* expression and *IL1B* and *IL6* expression, which indicates that the photoperiodic modulation of the ChP response to LPS-induced immune stress occurs through the regulation of *TLR4* expression. Our results also suggest that gonadal hormones, altering TLR4 signaling

events, are involved in the photoperiodic modulation of the ChP response to acute systemic inflammation. However, further experiments are required to explain the mechanism involved in this phenomenon.

Author contributions

JS drafted and interpreted the data from the experiment and prepared the manuscript, AK designed and performed the animal experiment and performed article revision, APH performed the animal experiment, AS and MK performed the laboratory analysis, MD performed the statistical analysis. All authors read and approved the final manuscript.

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