



## The Toll-like receptors mRNA expression profile in the pineal gland of sheep during long and short days

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**ABSTRACT.** Interaction between the immune system and pineal gland seems to be bidirectional. The feedback effect of an inflammatory response on the pineal gland is poorly understood, however. This study was performed to analyse the pattern of mRNA expression of receptors belonging to the Toll-like receptors (TLR) family in the pineal gland of ewes under different photoperiod conditions. TLRs are involved in the initiation of the innate immune response because they recognize different pathogen-associated molecular patterns (PAMPs). The experiment was carried out on adult Blackface ewes during long-day (LD, 18:6, June; n = 6) and short-day (SD, 6:18, December; n = 6) periods. The animals were euthanized 5 h after sunset (at 21:00 during SD and 2:00 during LD). The mean concentration of circulating melatonin in the last hour of the experiment was higher ( $P < 0.05$ ) during the SD ( $270 \pm 15 \text{ pg} \cdot \text{ml}^{-1}$ ) compared with the LD period ( $108 \pm 5 \text{ pg} \cdot \text{ml}^{-1}$ ). The expression of genes encoding each TLR was differentiated. *TLR3* had the highest mRNA expression, followed by TLRs 1, 2, 5, 7, 9, 8, 4, 10 and 6. Gene expression also varied for each TLR and was influenced by the photoperiod and/or melatonin. The gene whose expression varied the most was *TLR10* (coefficient of variation, 42%) during the LD period, but during the SD period, the expression of *TLR6* varied most (coefficient of variation, 42%). The study showed that the cells of the pineal gland express transcripts for all ten members of the TLR family, therefore, the functioning of this gland may be affected by number of PAMPs recognized by these receptors.

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### Introduction

In the vertebrate, the pineal gland is a neuroendocrine organ converting environmental photoperiodic information into a biochemical messenger – melatonin. This molecule regulates the activity of numerous target tissues after its release into the bloodstream and cerebrospinal fluid (Skinner and Malpoux, 1999). The pineal gland has evolved from the roof of the forebrain in the main part of the epithalamus, and is formed by three types of cells:

pinealocytes, glia cells and neurons. In non-mammalian vertebrates, the pinealocytes are photoreceptors and react directly to light, while in mammals, they are not directly sensitive to light (Markus and Ferreira, 2011). A phylogenetically conserved feature is increased melatonin synthesis during darkness. The magnitude and duration of the nocturnal increase in melatonin synthesis is dependent on the length of the dark phase of the photoperiodic cycle. Melatonin synthesis acts as a 'clock' and 'calendar' for

other biological activities. It occurs in all vertebrate species, regardless of their diurnal or nocturnal locomotor activity, and it is thus a message of darkness and not of the rest or sleep period (Skwarlo-Sonta et al., 2003). It is noteworthy that melatonin secretion from the pinealocytes is controlled by the suprachiasmatic nucleus (SCN) in the anterior hypothalamus, which receives light/dark signals *via* the retinohypothalamic pathway (Moore, 1997). The rhythm generated by the SCN is then transmitted *via* the paraventricular nucleus (PVN), hindbrain, spinal cord, superior cervical ganglion (SCG) to pineal noradrenergic (NA) receptors (Arendt, 1998). In mammals, spontaneous bioelectrical and metabolic activity of the SCN neurons is highest during the day (or during the light phase of the circadian cycle). In the same phase, the impulsion of sympathetic neurons connecting the superior cervical ganglion with the pineal gland is negligible. At night, when the activity of the SCN neurons is decreased, there is an increase in the electrophysiological activity of sympathetic postganglionic fibres resulting in the release of NA from the nerve terminals (Gupta et al., 2005). Acting *via*  $\beta$ 1- and  $\alpha$ 1-adrenergic membrane receptors, NA induces the synthesis of melatonin in the pinealocytes (Schomerus and Korf, 2005).

It is well established that among its numerous roles, melatonin acts as an immunomodulator, regulating the development, differentiation and function of lymphoid tissues. Moreover, diurnal and seasonal changes in immune function are thought to directly reflect changes in pineal melatonin production, which suggests an important role of circulating melatonin in the development and maintenance of immune function (Skwarlo-Sonta et al., 2003). The influence of melatonin on inflammatory and immune processes is ambiguous because it may act as both an activator and inhibitor of these responses (Carrillo-Vico et al., 2013). In the early phase of inflammation, melatonin enhances the inflammatory response, activating the production of inflammatory mediators such as phospholipase A2 (PLA2), 5-lipoxygenase (LOX), interleukin (IL)-1 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Radogna et al., 2010). On the other hand, melatonin contributes to the attenuation of chronic inflammation, down-regulating the production of PLA2, LOX, IL-1 and TNF- $\alpha$ . Melatonin also induces the survival pathway in leukocytes and, due to its antioxidant properties, reduces oxidative stress in inflamed tissues (Radogna et al., 2010).

The interaction between the immune system and pineal gland seems to be bidirectional. The feedback effect of the inflammatory response on pineal gland neuroendocrine functions is poorly understood,

however. Cytokines and other immune factors may possibly regulate pineal function indirectly, acting at the level of the SCN. It was found that inflammatory mediators such as cytokines, prostaglandins and histamine penetrate this region of the brain during an immune/inflammatory challenge (Fabris, 1994) and a study carried out on rodents showed high densities of cytokine receptors in the hypothalamus (Vitkovic et al., 2000). Even so, immune mediators and antigens may also directly affect the secretory activity of the pineal gland because it is a part of the brain lacking the blood-brain barrier and all molecules present in the blood can easily access this tissue. A few studies have shown that the secretory activity of pinealocytes could be modified by antigenic stimulation (Markowska et al., 2000), histamine (Zawilska et al., 1997), cytokines (Mucha et al., 1994) and prostaglandins (Voisin et al., 1993). Nonetheless, the spectrum of molecules affecting the pineal gland activity may be much broader.

In the present study we analysed the pattern of mRNA expression of receptors belonging to the Toll-like receptor (TLR) family. Mammalian TLRs comprise a large family consisting of at least 13 members, all of which have been identified in mice (Takeda and Akira, 2005), but only 10 members of this family have been found in sheep (Chang et al., 2009). TLRs play an important role in the initiation of the innate immune response because they are responsible for the recognition of different pathogen-associated molecular patterns (PAMPs). Recognition of microbial components by TLRs initiates signal transduction pathways that trigger gene expression. These gene products control innate immune responses and further instruct development of antigen-specific acquired immunity (Takeda and Akira, 2005). Determining the expression of TLR mRNAs in the pineal gland will shed new light on pineal-immune interaction.

This study was designed to verify the hypothesis that mRNAs encoding all of the receptors belonging to the TLR family identified in sheep to date are expressed in the pineal gland of sheep and that the transcription of these receptors may be influenced by the photoperiod and/or circulating melatonin.

## Material and methods

### Animals and experimental design

This study was performed on two groups of adult, two-year-old Blackface ewes during long-day (LD) (16:8, June;  $n = 6$ ) and short-day (SD) (8:16, December;  $n = 6$ ) periods. The animals were maintained indoors in individual pens and were exposed

to the natural daylight present at 52°N latitude and 21°E longitude. The ewes were in good condition, i.e. their body condition was estimated to be 3 on a five-point scale (Russel, 1991). The animals were acclimated to the experimental conditions for one month. The ewes were always within visual contact with other members of the flock to prevent isolation stress. The animals were fed a constant diet of commercial concentrates with hay and water available *ad libitum*, according to the recommendations proposed by the National Research Institute of Animal Production for adult ewes (IZ PIB-INRA, 2009). The experimental procedures were performed during the night, in the darkness under red light. One hour prior to euthanasia, four blood samples (8 ml) were collected in 15-min intervals through a catheter implanted into the jugular vein the day prior to the experiment. After centrifugation in heparinized tubes, plasma was stored at -20°C until assayed. The animals were euthanized 5 h after sunset at 21:00 during SD and 2:00 during LD. The brain was immediately removed from the skulls, and the pineal gland was dissected and immediately frozen in liquid nitrogen and stored at -80°C until assay.

All procedures were performed with the consent of the Local Ethics Committee for Animal Experimentation of the Warsaw University of Life Sciences – SGGW.

### Assays

**Melatonin.** Melatonin was assayed in the experimental media according to the method of Fraser et al. (1983) modified in the laboratory of the Kielanowski Institute of Animal Physiology and Nutrition, using ovine anti-melatonin serum (AB/S/01, Stockgrand Ltd., Surrey, UK). Synthetic melatonin (Sigma-Aldrich Co., St. Louis, MO, USA) was used as a standard and [O-methyl-3H]-melatonin (Amersham PLC, Amersham, UK) as a tracer. The sensitivity of the assay was  $16.8 \pm 8.0 \text{ pg} \cdot \text{ml}^{-1}$  and the intra- and inter-assay coefficients of variation were 10.5% and 13.2%, respectively.

**Isolation of mRNA from the pineal gland.** Total RNA from the pineal fragments was isolated using the NucleoSpin® RNA/Protein Kit (MACHEREY-NAGEL GmbH & Co., Düren, Germany). All steps of the isolation were performed according to the manufacturer's protocol. The purity and concentration of the isolated RNA was quantified spectrophotometrically. RNA integrity was confirmed by electrophoresis using 1% agarose gel stained with ethidium bromide.

**Real-Time PCR.** To synthesize cDNA, the Maxima™ First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA) and 2 µg of total RNA were used. Real-Time RT-PCR was performed using the HOT FIREPol EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and HPLC-grade oligonucleotide primers (Genomed, Warszawa, Poland). Specific primer pairs for all of the analysed genes were used according to the literature (Table 1). One reaction mixture (total volume: 20 µl) contained: 4 µl of PCR Master Mix (5×), 14 µl of RNase-free water, 1 µl of primers (0.5 µl each primer, working concentration 0.25 µM) and 1 µl of the cDNA template. The reactions were run on a Rotor-Gene 6000 instrument (Qiagen, Düsseldorf, Germany). The following protocol was used: 95°C for 15 min and 30 cycles of 95°C for 10 s for denaturation, the temperature of annealing was dependent on the primers used for reaction (Table 1), but in all cases the hold time of this step was 20 s, and 72°C for 10 s for extension. A final melting curve analysis was performed to confirm the specificity of the amplification.

Relative gene expression was calculated using the comparative quantification option (Rasmussen, 2001) of Rotor Gene 6000 software 1.7. (Qiagen, Düsseldorf, Germany). Three housekeeping genes were examined: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*),  $\beta$ -actin (*ACTB*) and histone deacetylase 1 (*HDAC1*). For normalization of the expression of TLR genes, the mean expression of these three housekeeping genes was used. The results are presented in arbitrary units as the ratio of the target gene expression to the mean expression of the housekeeping genes.

### Statistical analysis

The concentration of melatonin is presented as the mean  $\pm$  SEM. Real-time RT-PCR results are presented as the relative gene expression (mean  $\pm$  SEM) of the TLR genes vs the mean expression of reference genes (*GAPDH*, *ACTB* and *HDAC1*). All analyses were performed using GraphPad PRISM 6 (La Jolla, CA, USA), including calculations of the coefficient of variation for the expression values of each TLR gene, t-test for analysis of melatonin concentration during SD and LD, and, after log transformation, two-way analysis of variance (ANOVA) with multiple comparison Tukey's post-hoc test for analysis of expression of TLR genes in the pineal gland harvested during LD and SD.

**Table 1.** Sequences of oligonucleotide primers used for Real-Time PCR assay

GenBank Acc. No.	Gene	Amplicon size (bp)	Temperature of primers annealing	Forward/reverse	Sequence 5'→3'	Reference
AY957612	<i>TLR1</i>	208	58°C	forward reverse	CCCACAGGAAAGAAATTCCA GGAGGATCGTGATGAAGGAA	Menzies and Ingham, 2006
AM981300	<i>TLR2</i>	159	58°C	forward reverse	GGTGTAATCAGCGTGTTCA GATCTCGTTGTCGGACAGGT	Chang et al., 2009
AM981301	<i>TLR3</i>	150	60°C	forward reverse	TCAGCTCCAAGTGGAGAACC CACCCAGGAGAGAAGTCTTTGA	Chang et al., 2009
AY957615	<i>TLR4</i>	117	58°C	forward reverse	GGTTCCCAGAACTGCAAGTG GGATAGGGTTTCCCGTCAGT	Herman et al., 2013
AM981303	<i>TLR5</i>	152	58°C	forward reverse	CGACAACCTCCAAGTTCTCAA TTTCCCAGGAATTTGAATG	Chang et al., 2009
AM981304	<i>TLR6</i>	213	58°C	forward reverse	TTTGTCTCAGGAACCAAGC TCATATTCAAAGAATTCCAGCTA	Chang et al., 2009
AM981305	<i>TLR7</i>	153	60°C	forward reverse	CTGGACCATCTGGTGGAGAT GCTGGTTTCCATCCAGGTAA	Chang et al., 2009
AY957619	<i>TLR8</i>	150	58°C	forward reverse	TCCACATCCCAGACTTTCTACGA GGTCCAATCCCTTTCTCTA	Menzies and Ingham, 2006
AM981307	<i>TLR9</i>	175	60°C	forward reverse	CCCTGGAGAAGCTGGACAT GACAGGTCCACGAAGAGCAG	Chang et al., 2009
AM981308	<i>TLR10</i>	149	58°C	forward reverse	TGCCAAGACATCCATTCTGT TGCCAAGACATCCATTCTGT	Chang et al., 2009
NM_001034034	<i>GAPDH</i>	134	58°C	forward reverse	AGAAGGCTGGGGCTCACT GGCATTGCTGACAATCTTGA	Herman et al., 2015
BC108088.1	<i>HDAC1</i>	115	58°C	forward reverse	CTGGGGACCTACGGGATATT GACATGACCGGCTTGAAAAT	Herman et al., 2015
U39357	<i>ACTB</i>	168	58°C	forward reverse	CTTCTTCTCTGGGCATGG GGGCAGTGATCTCTTTCTGC	Herman et al., 2015

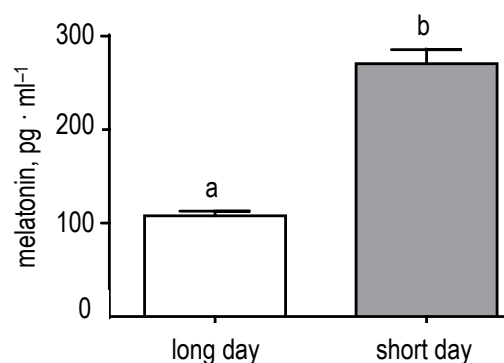
*TLR* – Toll-like receptor, *GAPDH* – glyceraldehyde-3-phosphate dehydrogenase, *HDAC1* – histone deacetylase 1, *ACTB* – beta-actin

## Results

The mean concentration of circulating melatonin in the last hour of the experiment (at 21:00 during SD and 2:00 during LD) was higher ( $P < 0.05$ ) during the SD ( $270 \pm 15 \text{ pg} \cdot \text{ml}^{-1}$ ) compared with the LD period ( $108 \pm 5 \text{ pg} \cdot \text{ml}^{-1}$ ) (Figure 1).

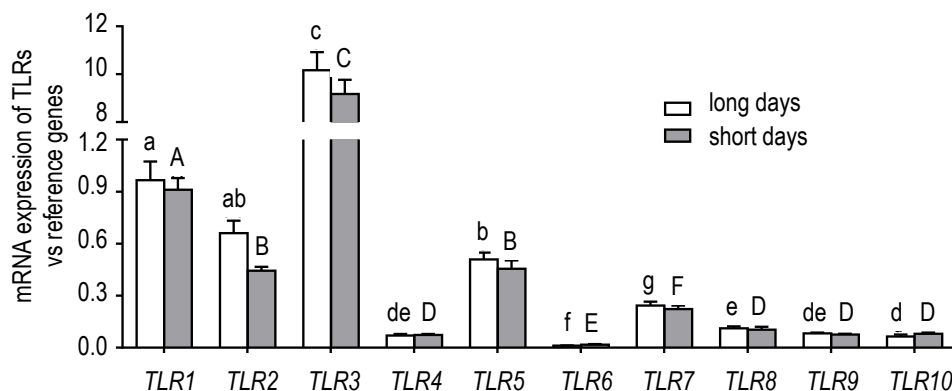
Transcripts for all TLR genes were found in the pineal gland. The level of gene expression for each particular TLR gene differed, however (Figure 2). *TLR3* had the highest mRNA expression, followed by TLRs 1, 2, 5, 7, 9, 8, 4, 10 and 6. Moreover, the expression of each TLR gene also varied and was influenced by the photoperiod and/or circulating melatonin. The gene characterized by the highest variation in the degree of expression was *TLR10* (coefficient of variation, 42%) during the LD period, but during the SD period, *TLR6* expression varied most (coefficient of variation, 42%) (Table 2). On the other hand, the gene showing the lowest variation in the degree of expression was *TLR9* (coefficient of variation, 12%)

during the LD period, but during the SD period the lowest variation was found for *TLR2* (coefficient of variation, 14%). Photoperiodic conditions did not influence TLR gene expression.



**Figure 1.** Mean ( $\pm$  SEM) concentration of melatonin in blood plasma collected at the last hour of the experiment during long day (LD, 2:00, white bar) and short day (SD, 21:00, grey bar); a,b – bars with different letters differ significantly at  $P < 0.05$





**Figure 2.** TLRs relative mRNA expression vs reference genes, during long day (LD, white bars) and short day (SD, grey bars) in the pineal gland of adult ewes. Data are presented as a mean  $\pm$  SEM. Bars with different letters (a–f for LD and A–F for SD) differ significantly at  $P \leq 0.05$ ; TLR – Toll-like receptor

**Table 2.** The coefficient of variations (%) for Toll-like receptors (TLRs) mRNA expression in the pineal gland collected during long and short days

Period	TLR1 <sup>1</sup>	TLR2	TLR3	TLR4	TLR5	TLR6	TLR7	TLR8	TLR9	TLR10
Long days	28	27	18	28	19	26	23	24	13	42
Short days	18	12	16	19	26	42	23	38	15	23

<sup>1</sup> see Table 1

## Discussion

The results of our study demonstrate that cells located in the pineal gland express mRNA encoding all 10 members of the TLR family. These receptors are able to recognize a wide range of exogenous and endogenous ligands. TLR2 recognizes a variety of microbial components such as lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from gram-positive bacteria, liparabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from *Trypanosoma cruzi*, a phenol-soluble modulins from *Staphylococcus epidermidis*, zymosan from fungi, glycolipids from *Treponema maltophilum* and lipopolysaccharides (LPS) found in *Leptospira interrogans* and *Porphyromonas gingivalis* (Takeda et al., 2003; Takeda and Akira, 2005). TLR2 recognizes a wide range of microbial products through functional cooperation with several proteins that are either structurally related or unrelated. TLR2 forms heterophilic dimers with other TLRs such as TLR1 and TLR6, both of which are structurally related to TLR2. These receptors functionally associate with TLR2 and discriminate between diacyl or triacyl lipopeptides, respectively (Alexopoulou et al., 2002; Takeuchi et al., 2002). TLR2 has also been shown to functionally collaborate with distinct types of receptors such as dectin-1, a lectin family receptor for the fungal cell wall component  $\beta$ -glucan (Takeda and Akira, 2005). TLR3, whose mRNA is predominantly expressed in the pineal gland, is involved in the detection

of double-stranded RNA and viruses (Alexopoulou et al., 2001). TLR4 is an essential receptor for LPS recognition, but is also involved in the detection of taxol and some endogenous ligands such as heat shock proteins, extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulphate and fibrinogen (Takeda and Akira, 2005). TLR5 recognizes flagellin, thus enabling detection of flagellated bacterial pathogens (Hayashi et al., 2001). TLR7 and TLR8 detect single-stranded RNA from viruses such as human immunodeficiency virus, vesicular stomatitis virus and influenza virus (Takeda and Akira, 2005). TLR9 has been shown to recognize various bacterial and viral components, including unmethylated CpG-DNA (Takeda and Akira, 2005). The ligand of TLR10 is still unidentified, however, it was demonstrated that TLR10 homodimerizes but also heterodimerizes with TLRs 1 and 2 (Hasan et al., 2005). The pineal gland is the circumventricular organ, which is characterized by fenestrated capillaries allowing for the movement of molecules in the paracellular space (Joly et al., 2007), therefore the activity of the pineal cells may be affected by a number of circulating factors. The fact that the expression of genes encoding all 10 members of the TLR family was demonstrated in our study suggests that their ligands may cross the fenestrated capillaries and influence the secretory activity of the pineal gland. This strongly supports the thesis about involvement of the pineal tissue in immune-endocrine interactions. It is worth mentioning that just recently we presented the profile of TLRs in the ovine

choroid plexus (Skipor et al., 2015) in which the capillaries are fenestrated, but also covered by epithelial cells with tight junctions forming what is known as the blood-cerebrospinal fluid barrier (Skipor and Thiery, 2008). Transcripts for all ten TLRs except *TLR8* were present in the choroid plexus, but only *TLR3*, *TLR7*, *TLR9* and *TLR10* presented relatively invariant expression levels (coefficient of variation, 7% to 17%). There was high variation in the degree of expression of the *TLR5* and *TLR1* genes (61% and 46%, respectively) as well as moderate variation in the expression of the *TLR4* (34%), *TLR2* (27%) and *TLR6* (26%) genes (Skipor et al., 2015). Our study shows that the profile of TLR mRNA expression in the pineal gland was generally independent of the photoperiodic conditions and/or the circulating level of melatonin. However, it was found that only *TLR3*, *TLR7* and *TLR9* were characterized by similar inter-individual variability of mRNA expression in the pineal gland, regardless of different photoperiodic conditions. Our study does not allow drawing conclusions about which factors influence the interindividual variation in the mRNAs encoding other TLRs in the SD and LD photoperiods. Although it was suggested that the expression of TLRs may be modulated by circulating glucocorticoids (Shuto et al., 2002), no correlation between cortisol concentration and mRNA expression of any of the examined TLRs was found in our previous study performed on sheep (Skipor et al., 2015).

Based on our results, it is not possible to infer about the type of pineal cells expressing TLRs, however, their existence may have a profound influence on the secretory activity of the whole gland. It could be assumed that these receptors are mainly expressed on the glial cells. It was previously described that rat pineal astrocytes and microglia react to bacterial endotoxins such as LPS and lipoteichoic acid thanks to the existence of membrane receptors for LPS, such as *TLR4* and cluster of differentiation 14 (CD14) (da Silveira Cruz-Machado et al., 2010). However, more recent studies showed the expression of *TLR4* in isolated pinealocytes themselves and suggested that LPS may reduce melatonin secretion by both acting directly on pinealocytes and indirectly through activated glial cells (da Silveira Cruz-Machado et al., 2012). Our results suggest that the spectrum of molecules affecting the function of pineal cells could be much wider. Nevertheless, studies analysing the possible influence of diverse ligands of other TLRs on the secretory activity of the pineal gland have yet to be carried out. The dominant expression of mRNA for *TLR3* in the pineal gland deserves special attention

as this suggests that pineal tissue is particularly sensitive to viral-derived PAMPs. Notwithstanding, the role of *TLR3* in the pineal gland may be more complex. Recent studies carried out on mice (Pan et al., 2014) showed that astrocytic *TLR3* signaling plays an important role in suppression of the post-ischaemic inflammatory response and thereby protects against ischaemic damage. Therefore, the expression of *TLR3* in the pineal gland may be involved in the protection of this tissue against strong inflammatory stimulation. Worth mentioning is that the involvement of TLRs in the modulation of melatonin secretion may result from the induction of inflammatory mediator synthesis in the cells of the pineal gland. It has been reported that ligation of *TLR2*, *TLR3* and *TLR4* by these receptors' agonists resulted in activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), c-Jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases (MAP), leading to the expression of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 and IL-6. *TLR3* and *TLR4* are known to induce the synthesis of type I interferon (IFN- $\alpha$  and IFN- $\beta$ ) in response to viral and bacterial antigens (Schaefer et al., 2004). These locally synthesized inflammatory mediators may, in turn, affect melatonin secretion by acting directly on pinealocytes or indirectly on pineal glial cells. It has been suggested that the effect of cytokines such as IFN- $\gamma$  and IL-1 $\beta$  on pineal melatonin synthesis may be mediated by microglia, whereas TNF- $\alpha$  may exert its biological effects directly on pinealocytes (Tsai et al., 2001).

Although our findings indicate that the mRNA of all 10 members of the TLR family is expressed in the pineal gland of ewes, it should be stressed that further studies are necessary to examine the protein profiles of the TLRs. Mishra et al. (2006) demonstrated that not all TLRs for which mRNA was found were expressed as proteins. They suggested that the expression of some TLRs may be regulated at the posttranscriptional level. However, a more recent study suggests that the variability found in TLR protein expression may result from the use of antibodies lacking high specificity (Mallard, 2012).

## Conclusions

Our study shows that the cells of the sheep pineal gland express transcript for all ten members of the Toll-like receptors family, which suggests that the functioning of this gland may be affected by a number of pathogen-associated molecular patterns recognized by these receptors.

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