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TRANSCRIPT ABUNDANCES OF THE PROLACTIN RECEPTOR, THE LEPTIN RECEPTOR AND THEIR MAJOR SUPPRESSOR IN THE SHEEP MAMMARY GLAND DURING PREGNANCY AND LACTATION*

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Abstract

This study aimed to expand the knowledge of the interactions between prolactin (PRL) and leptin in the ovine mammary gland during pregnancy and lactation; we examined the mRNA expression of prolactin receptor (PRLR), the long form of the leptin receptor (LRb) and suppressor of cytokine signaling (SOCS)-3 in mammary gland biopsies collected on days 60, 90 and 120 of pregnancy and on days 30, 60 and 90 of lactation (n = 6 for each time point), along with the plasma PRL and leptin concentrations. The PRL concentrations were stable throughout pregnancy and increased during lactation. The plasma leptin concentrations were comparable among nonpregnant, early-pregnant, late-pregnant and lactating ewes, but this metric peaked during mid-pregnancy. Expression of *PRLR* and *SOCS-3* in the mammary gland fluctuated during the transition from pregnancy to lactation, and differences in *LRb* expression occurred during the late stages of lactation. The *LRb* transcript abundance was approximately 31 times higher in ewes on day 60 of lactation than in early-lactating ewes. Expression of *SOCS-3* mRNA in biopsies gradually decreased over the course of pregnancy and reached a minimum value during late pregnancy. After lambing, the transcript level of *SOCS-3* increased and peaked on day 60 of lactation. During pregnancy, the plasma PRL concentration positively correlated with the abundances of PRLR ($r = 0.971$, $P<0.01$) and SOCS-3 ($r = 0.818$, $P<0.05$). Positive correlations were also observed between the transcript abundances of SOCS-3 and LRb ($r = 0.854$, $P<0.05$). The variations observed in the plasma PRL and leptin concentrations and the changes in expression of key leptin and PRL signal transduction pathway components, such as PRLR, LRb and SOCS-3, indicate that the efficacies of both hormone actions are modulated in a multilevel manner throughout pregnancy and lactation. These interactions may regulate the ability of the mammary gland to respond to current energy requirements and challenges, thus affecting milk yield and lactation duration.

Key words: pregnancy, lactation, mammary gland, prolactin, leptin

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Lactation significantly increases the energy requirements of females, and these demands may be fulfilled by increased appetite and metabolic efficiency or by the use of lipid reserves and other nutrients. Mammary gland development and subsequent lactation are controlled by numerous factors. Among them, prolactin (PRL) and leptin appear to exhibit a specific interaction as a result of involvement in the modulation of metabolic processes and similar signaling pathways through which these molecules exert their biological activities (Bole-Feysot et al., 1998; Banks et al., 2000; Szczesna et al., 2011). Lactation- and pregnancy-related fluctuations in their concentrations have been observed (Szczesna et al., 2018). Furthermore, both hormones are involved in the phenomena of pregnancy-induced hyperphagia and gestational energy storage in adipose tissue and affect adipose tissue metabolism, including fatty acid mobilization during lactation (Vernon et al., 1981, 2002). Thus, PRL-leptin interactions may be a functional link between mammary glands and adipose tissue, the existence of which is essential for the initiation and maintenance of milk production. Prolactin not only promotes mammary epithelial cell proliferation but also affects milk yield and lactation parameters; stimulates the synthesis of milk components, such as proteins, lactose or lipids; and plays an important role in galactopoiesis (Lacasse et al., 2016). The role of leptin in the regulation of lactation is not widely known, but the presence of leptin receptor (LR) in mammary tissue (Laud et al., 1999) suggests that it directly affects the physiology of the udder. The action of leptin in this tissue includes endocrine, paracrine and autocrine modulation of mammary epithelial cell growth, differentiation or apoptosis (Chilliard et al., 2001) and other regulatory functions, such as secretory activity or casein expression (Baratta et al., 2003). In sheep, leptin can also indirectly affect mammary gland physiology by modulating the secretion of other hormones, such as thyroid hormones (Klocek-Gorka et al., 2010) or growth hormone (Szczesna et al., 2018), which are galactopoietic and help to establish the metabolic priority of the mammary gland during lactation.

In target tissues, such as the mammary gland, leptin and PRL bind to the long forms of the leptin receptor (LRb) and prolactin receptor (PRLR), respectively, and activate similar signaling cascades, including the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Banks et al., 2000; Bole-Feysot et al., 1998). The effectiveness of PRL and leptin signal transduction may be modified by the abundance of PRLR and LRb, respectively, and by the activity of common intracellular factors, such as suppressors of cytokine signaling (SOCSs), especially SOCS-3, which inhibits leptin- and PRL-induced signaling (Tam et al., 2001; Sutherland et al., 2007 a). It has been shown that SOCS proteins regulate the development (proliferation and differentiation) and physiology (lactogenesis) of the mammary gland during pregnancy and lactation (Lindeman et al., 2001).

The objective of the present study was to investigate fluctuations in the mRNA expression of *LRb* and *PRLR* in the ovine mammary gland during pregnancy and lactation and to quantify the transcription of the *SOCS-3* gene, which encodes important negative regulators of the LRb and PRLR signaling pathways. Concomitant changes in the concentrations of leptin and PRL in the blood were also measured. The variable expression of these proteins may contribute to mammary gland physiology and pathology and may thus affect milk yield and duration of lactation.

Material and methods

All of the animal-related procedures used in these studies were approved by the First Local Ethical Committee on Animal Testing in Krakow (No. 83/2013).

Animals

The studies were performed at the Experimental Station of the Department of Animal Nutrition and Biotechnology, and Fisheries at the University of Agriculture in Krakow (19°57'E, 50°04'N). The experiments were carried out on six (n = 6) fourth-parity 4- to 5-year-old adult female Polish Longwool sheep with body conditions that rated 3 on a five-point scale (Russel et al., 1969); these sheep weighed 54.3 ± 5.3 kg (before mating), 66.3 ± 5.1 kg (on day 120 of gestation) and 57.7 ± 4.6 kg (on day 90 of lactation). Maintenance and feeding followed standard procedures used for pregnant and lactating ewes, including group housing and group feeding. The ewes were fed twice daily at 07:00 and 16:00 with a diet formulated to fully cover their nutrient requirements (Polish National Research Institute of Animal Production recommendations for maintenance, 1997) according to their physiological status and in a uniform manner to eliminate the impact of nutrition on mammary gland physiology (Molik et al., 2007). Before pregnancy until day 120 of pregnancy, the ewes were fed 1.4 kg silage/sheep/day (per 1 kg: dry matter, 382 g; crude protein, 58 g; net energy, 1.95 MJ) and hay *ad libitum* (per 1 kg: dry matter, 882 g; crude protein, 185 g; net energy, 3.24 MJ), with half of the silage fed in the morning and half in the afternoon. From day 121 of gestation until day 90 of lactation, the ewes were fed 1.5 kg of pelleted compound feed (per 1 kg: crude protein, 220 g and net energy, 7.5 MJ; ingredients: cereal grains, rapeseed, dried legume plants, dried beet pulp, and corn flour) and hay *ad libitum* (the same used in earlier stages of the study), with half of the pelleted concentrate fed in the morning and half in the afternoon. The hay originated from the first cut (before heading), whereas the silage was prepared from the second cut (before heading) from the same meadow. Water and mineral licks were available *ad libitum*. Sheep were housed under natural photoperiodic and thermoperiodic conditions.

Procedures and treatments

In the reproductive season, the estrous cycles of the ewes were synchronized using a 14-day treatment of intravaginal progestagen-impregnated sponges (40 mg fluorogestone acetate, FGA; Chronogest, Intervet International, Boxmeer, The Netherlands). On the day of sponge removal, ewes were intramuscularly treated with a single dose of 500 IU of pregnant mare serogonadotropin (PMSG, Biowet, Drwalew, Poland). Estrus detection was performed twice daily (at 08:00 and 20:00) with an adult ram equipped with an apron. Estrus was defined as the acceptance of mounting. After estrus detection, the ewes were individually presented to the male and naturally mated. All ewes were mated 36 ± 12 h after PMSG injection at the same time of the year (November). Pregnancy was verified 40 days after mating using an ultrasonographic scanner (Aloka SSD 500 Micrus, Equine Therapy System,

Inc., Greenwood Village, CO, USA). All experimental ewes were in their 4th consecutive lactation, gave birth to twins and were suckling two lambs.

Serial blood samples were collected from 6 ewes before mating and at different stages of pregnancy (on days 30, 60, 90, and 120) and lactation (on days 30, 60 and 90). Mammary gland biopsies were performed on the same 6 ewes during pregnancy (on days 60, 90, and 120) and during subsequent lactation (on days 30, 60 and 90). On the morning of each day indicated above, the sheep were fitted with jugular catheters. Blood samples (5 ml) were collected at 15-min intervals for 1 h, starting at 9:00. The blood samples were dispensed into tubes containing 150 µl of a heparin solution (10 000 IU/ml; heparinum natricum, Polfa, Warsaw, Poland). The plasma was separated by centrifugation at 3 000 × g at 4°C for 10 min and stored at –20°C for subsequent measurements of PRL and leptin concentrations. Immediately after blood sampling, mammary gland biopsies were performed after subcutaneous anesthesia (2% lignocainum hydrochloricum, Polfa, Warsaw, Poland) on a 2 × 2-cm area on the side surface of the mammary gland. Xylazine (0.5 mg/kg, Sedazin, Biowet, Pulawy, Poland) was intramuscularly administered 10 minutes before the biopsy to minimize the stress induced by puncture. Sample collection was performed in triplicate, from one-half of the udder (2–7 cm inside, depending on the stage of mammary gland development), using a semiautomatic spring loaded biopsy system with adjustable penetration depth (Velox2 14 G × 160 mm, Medax, Poggio Rusco, Italy). The presence of glandular tissue in the samples was checked using a light microscope. Proper explants were snap frozen in liquid nitrogen and then transferred and stored at –80°C until use for subsequent analyses.

Assessment of hormone concentrations

Plasma PRL concentrations were assayed by radioimmunoassay (RIA) using a double-antibody method with anti-ovine-PRL and anti-rabbit-γ-globulin antisera, as described by Wolińska et al. (1977). The standard was iodinated according to the chloramine T method and then purified on a Sephadex G-100 (Sigma-Aldrich, St. Louis, MO, USA) column. The range of the calibration curve was 0.3–400 ng/ml, and the working dilution of the anti-ovine PRL antiserum was 1:20 000. The assay sensitivity was 2.0 ng/ml, and the intra-assay and interassay coefficients of variation were 9.1% and 12.0%, respectively.

The plasma leptin concentrations were determined using a highly specific ovine leptin RIA with the double-antibody method, with a specific, high-affinity rabbit antibody generated against recombinant ovine leptin, anti-rabbit γ-globulin antisera and a recombinant ovine leptin standard, as described by Delavaud et al. (2000). The intra-assay and interassay coefficients of variation in the leptin assay were 3.2% and 11.0%, respectively, and the assay sensitivity was 0.3 ng/ml.

Molecular analysis

Real-time PCR was used to measure *LRb* and both forms of *PRLR* and *SOCS-3* mRNA expression. Samples were homogenized using a rotor-stator tissue homogenizer (Omni TH, Omni International, Inc., Kennesaw, GA, USA) equipped with single-use tips (Soft Tissue Omni Tip Plastic Homogenizing Probes, Omni Inter-

national, Inc., Kennesaw, GA, USA). Total RNA was obtained using TRIzol Reagent (Ambion, Inc., Austin, TX, USA) according to the manufacturer's instructions. A total of 1 μg of RNA was then reverse transcribed into cDNA using Quantiscript Reverse Transcriptase and RT Primer Mix (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany) by incubating the samples at 42°C for 15 min; the reaction was then terminated by an incubation at 94°C for 3 min. Genomic DNA was eliminated by adding gDNA Wipeout Buffer (QuantiTect Reverse Transcription Kit; as above) and incubating the samples at 42°C for 2 min. Each cDNA was amplified using TaqMan Gene Expression Master Mix (Life Technologies, Foster City, CA, USA); specific primers corresponding to the target genes and reference gene, each at a final concentration of 900 nM (Sequence Detection Primers, Life Technologies, Foster City, CA, USA); specific probes corresponding to the target genes and reference gene, each at a final concentration of 250 nM (TaqMan MGB Probes; Life Technologies, Foster City, CA, USA); and an Applied Biosystems 7300 Real-Time PCR System. The primers and probes were designed using Primer Express v. 2.0 software (Applied Biosystems; Foster City, CA, USA). The sequences of the primers and probes, amplicon sizes and sequence accession numbers are listed in Table 1.

Table 1. Sequences of the oligonucleotides used as primers and probes to analyze the mRNA expression of cyclophilin (CPH; reference gene), the long form of the leptin receptor (LRb; target gene), the prolactin receptor (PRLR; target gene) and suppressor of cytokine signaling-3 (SOCS-3; target gene) in sheep

Gene	Primer sequence (5'–3')	Probe sequence (5'–3')	Amplicon size	GenBank accession number
CPH	CGGCTCCCAGTTCTTCATCA ACTACGTGCTTCCCATCCAAA	FAM-CGTTCCGACTC-CGC-MGB	64 bp	D14074
LRb	CGACGAGGGTGGCATATTTAA CAGACATAACCTGTGAGGATG-GAA	FAM-CAGGAGACAGC-CCTC-MGB	63 bp	U62124.1
PRL	CAGGTACGTACAGGGAA-GCATTC GAGTGCTTTTCATTCTGC-TACTTTTTC	FAM-TTCGCCAGC-CTTC-MGB	66 bp	NM_001009204.1
SOCS-3	CCTCAAGACCTTCAGCTCCAA CTTGCGCACTGCGTTCAC	FAM-AGCGAGTAC-CAGCTGG-MGB	68 bp	NM_174466

Amplification was performed under the following conditions: 1) initial incubation at 50°C for 2 min, 2) polymerase activation at 95°C for 10 min, and 3) 45 cycles of denaturation (95°C for 15 s) and annealing/elongation (60°C for 60 s). Data were collected and recorded using the Applied Biosystem 7300 Real-Time PCR System SDS software, and the results were expressed as a function of the threshold cycle. Using diluted samples, the amplification efficacies for the target genes and the reference gene were found to be identical.

Statistical analysis

Hormone data were analyzed with a series of one-way analyses of variance using SigmaPlot statistical software (version 11.0; Systat Software, Inc., Richmond, CA, USA) for repeated measures. The values determined for all samples at the indicated stages of gestation or lactation were averaged to calculate the means used for comparisons among groups. The statistical models included the main effects of the gestational/lactation stage. After determining a significant F-value, the means were contrasted using all pairwise multiple comparison procedures (Tukey's test). A P value of <0.05 was considered statistically significant. All data are expressed as the mean ± SEM.

The expression levels were calculated using relative quantification (RQ) analysis. The amplification plot consisted of the plot of fluorescence versus the PCR cycle number. The threshold cycle value (Ct) was the fractional PCR cycle number at which the fluorescence signal reached the detection threshold. Therefore, the input cDNA copy number and Ct were inversely related. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and the Ct values were converted to fold-change RQ values. The RQ values from each gene were used to compare target gene expression across all groups. The mean mRNA expression level for each target gene in each sample was normalized to the expression of a reference gene (cyclophilin) and was expressed relative to the calibrator. There were no variations ($P>0.05$) in the Ct values of cyclophilin among the treatment groups. We used the mean ΔCt value for samples collected from ewes at the earliest stage of pregnancy in which the expression of the target gene was confirmed as a calibrator to compare the changes in gene expression levels among the biopsies isolated during other stages of gestation and lactation. Following the determination of a significant F-value, differences in the means were compared using all pairwise multiple comparison procedures (Tukey's test) using SigmaPlot statistical software (version 11.0, Systat Software, Inc., Richmond, CA, USA). All differences with P values <0.05 were considered statistically significant.

Pearson's correlation analysis using SigmaPlot statistical software (version 11.0, Systat Software, Inc., Richmond, CA, USA) was performed to examine the relationships among the means of plasma PRL concentrations, plasma leptin concentrations, *PRLR* transcript abundance, *LRb* transcript abundance and *SOCS-3* transcript abundance. A P value of <0.05 was considered to indicate statistical significance.

Results

Assessment of hormone concentrations

The profile of the plasma PRL concentrations during pregnancy and lactation is shown in Figure 1. There were no differences ($P>0.05$) in plasma PRL concentrations throughout pregnancy. Regardless of the day, the concentrations of PRL during lactation increased approximately 30-fold ($P<0.01$) compared to those during all stages of gestation and before breeding (N-P).

The profile of the plasma leptin concentrations during pregnancy and lactation is shown in Figure 2. The leptin concentrations of early-pregnant (on day 30 of gestation) and late-pregnant (on day 120 of gestation) ewes were similar to those of nonpregnant individuals (P>0.05); however, during mid-pregnancy (on day 60 of gestation), the leptin concentration reached the highest level (P<0.05) among all time points. During lactation, leptin concentrations were maintained at stable levels, similar (P>0.05) to those detected before pregnancy.

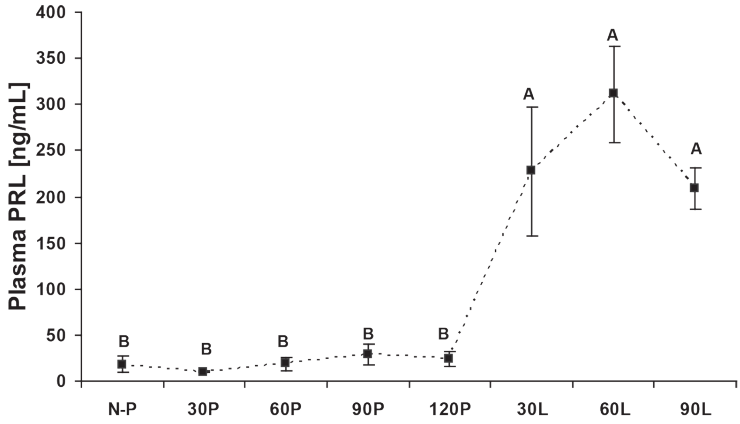

Figure 1. Prolactin (PRL) concentrations (mean ± SEM) in ewes before pregnancy (N-P) on days 30 (30P), 60 (60P), 90 (90P), and 120 (120P) of pregnancy and on days 30 (30L), 60 (60L), and 90 (90L) of lactation. Means without the same letter (A, B) differ significantly (P<0.01)

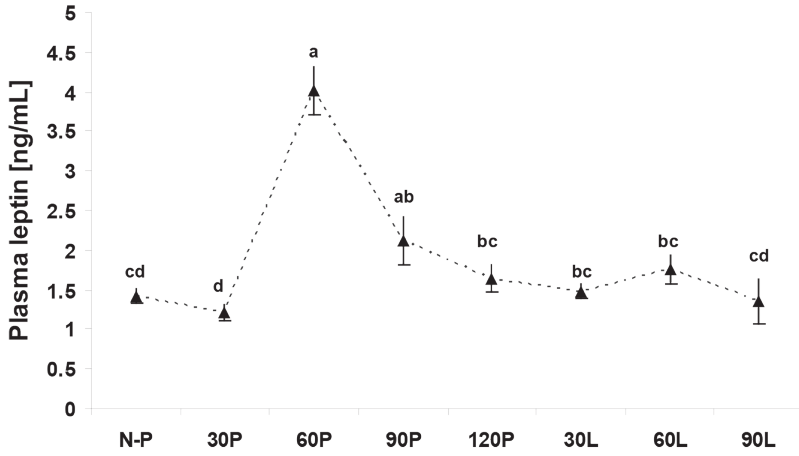

Figure 2. Leptin concentrations (mean ± SEM) in ewes before pregnancy (N-P) on days 30 (30P), 60 (60P), 90 (90P), and 120 (120P) of pregnancy and on days 30 (30L), 60 (60L), 90 (90L) of lactation. Means without the same letter (a, b) differ significantly (P<0.05)

Expression of *PRLR*

The fluctuations in the expression profile of *PRLR* are shown in Figure 3. At mid-pregnancy (day 60 of gestation), no *PRLR* transcripts were detected in the mammary gland. On the following days of measurement (90 and 120 days of gestation), the abundance of *PRLR* transcripts was lower (P<0.01) than that measured during lactation. There were no significant differences in the *PRLR* mRNA levels among mammary gland samples collected at different stages of lactation (P>0.05).

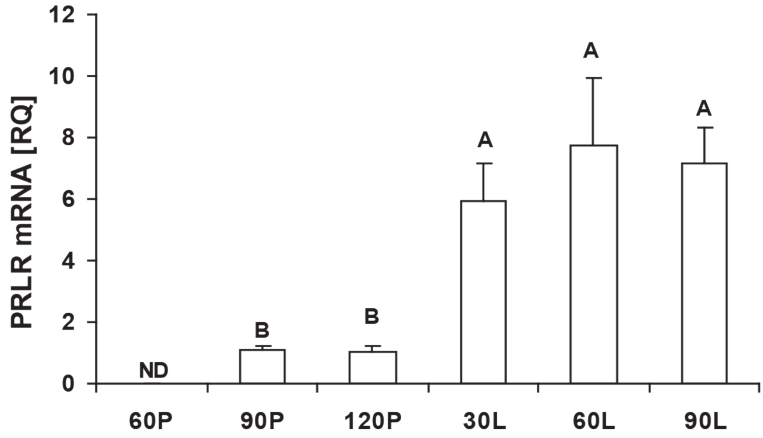

Figure 3. Relative mRNA expression (mean± SEM) of prolactin receptor (PRLR) in mammary gland biopsies collected on days 60 (60P), 90 (90P), and 120 (120P) of pregnancy and on days 30 (30L), 60 (60L), and 90 (90L) of lactation. The mRNA expression of PRLR is reported in arbitrary units (RQ) relative to the expression of cyclophilin mRNA. The mean value calculated for samples collected from ewes on day 90 of pregnancy was used as a calibrator. Samples in which the expression of the target gene was undetectable are denoted with ND. Means without the same letter (A, B) differ significantly (P<0.01)

Expression of *LRb*

Transcripts of *LRb* in the mammary gland were quantified beginning in the third month of pregnancy (Figure 4). On day 60 of gestation, *LRb* expression was undetectable. The abundances of *LRb* mRNA were similar during late pregnancy (on days 90 and 120 of gestation) and early lactation (on day 30). The expression of *LRb* transcripts increased (P<0.01) in the late stages of lactation compared to the expression in the early stages of lactation and on day 90 of pregnancy in ewes. Expression of *LRb* was approximately 31-fold higher on day 60 of lactation than during early lactation and was over 13.6-fold greater on day 60 of lactation than the highest level of transcription determined during pregnancy (in the mammary glands of late-pregnant ewes).

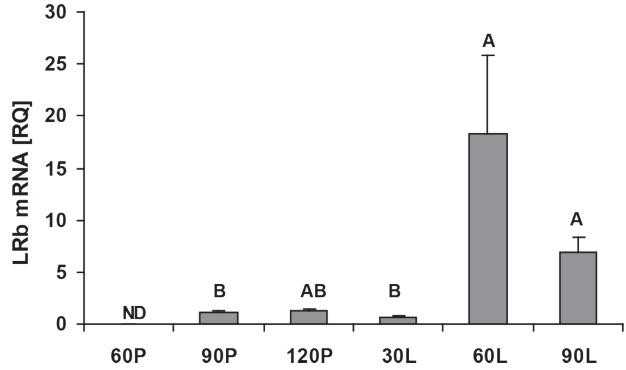

Figure 4. Relative mRNA expression (mean± SEM) of the long form of leptin receptor (LRb) in mammary gland biopsies collected on days 60 (60P), 90 (90P), and 120 (120P) of pregnancy and on days 30 (30L), 60 (60L), and 90 (90L) of lactation. The mRNA expression of LRb is reported in arbitrary units (RQ) relative to the mRNA expression of cyclophilin. The mean value calculated for samples collected from ewes on day 90 of pregnancy was used as a calibrator. Samples in which expression of the target gene was undetectable are denoted with ND. Means without the same letter (A, B) differ significantly (P<0.01)

Expression of *SOCS-3*

The presence of *SOCS-3* transcripts in the mammary gland was confirmed at all the studied stages of pregnancy and lactation (Figure 5); however, the level of *SOCS-3* expression varied depending on the time. The mRNA expression of *SOCS-3* gradually decreased over the course of pregnancy and reached a minimum value in late-pregnant ewes. The level of *SOCS-3* transcripts after lambing increased (P<0.01) compared to the level on day 120 of pregnancy.

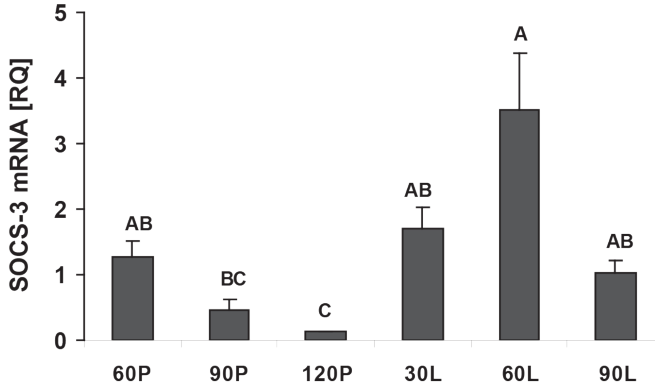

Figure 5. Relative mRNA expression (mean± SEM) of suppressor of cytokine signaling-3 (*SOCS-3*) in mammary gland biopsies collected on days 60 (60P), 90 (90P), and 120 (120P) of pregnancy and on days 30 (30L), 60 (60L), and 90 (90L) of lactation. The expression of *SOCS-3* mRNA is reported in arbitrary units (RQ) relative to the expression of cyclophilin mRNA. The mean value calculated for samples collected from ewes on day 60 of pregnancy was used as a calibrator. Means without the same letter (A, B, C) differ significantly (P<0.01)

Correlation analysis

During pregnancy, the plasma PRL concentration positively correlated with the *PRLR* abundance ($r = 0.971$, $P<0.01$; Figure 6) and *SOCS-3* ($r = 0.818$, $P<0.05$; Figure 7). Positive correlations were also observed between the transcript abundances of *SOCS-3* and *LRb* ($r = 0.854$, $P<0.05$; Figure 8).

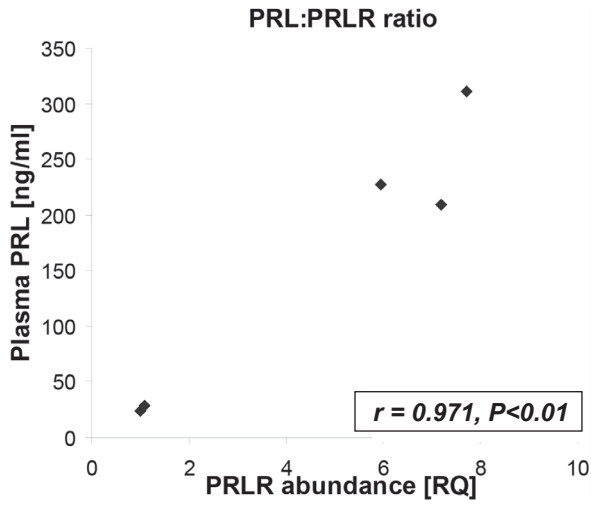

Figure 6. Ratio of plasma prolactin (PRL) concentration to prolactin receptor (*PRLR*) mRNA abundances in the ovine mammary gland across the different stages of pregnancy and lactation

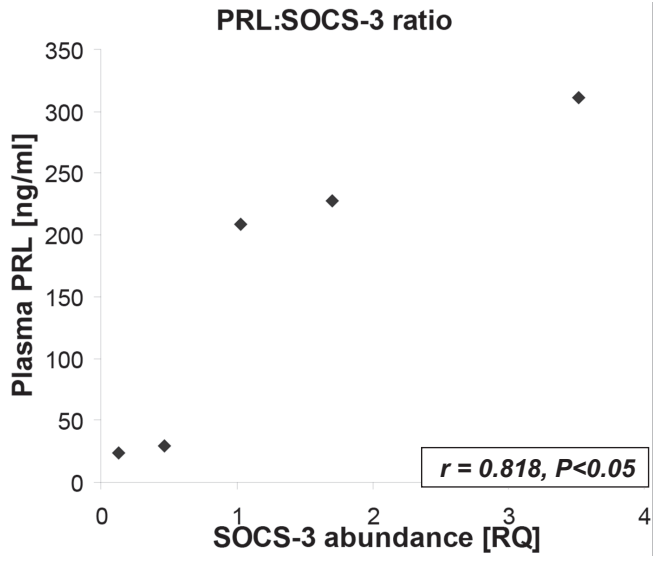

Figure 7. Ratio of plasma prolactin (PRL) concentration to suppressor of cytokine signaling-3 (*SOCS-3*) mRNA abundances in the ovine mammary gland across the different stages of pregnancy and lactation

r = 0.818, P<0.05

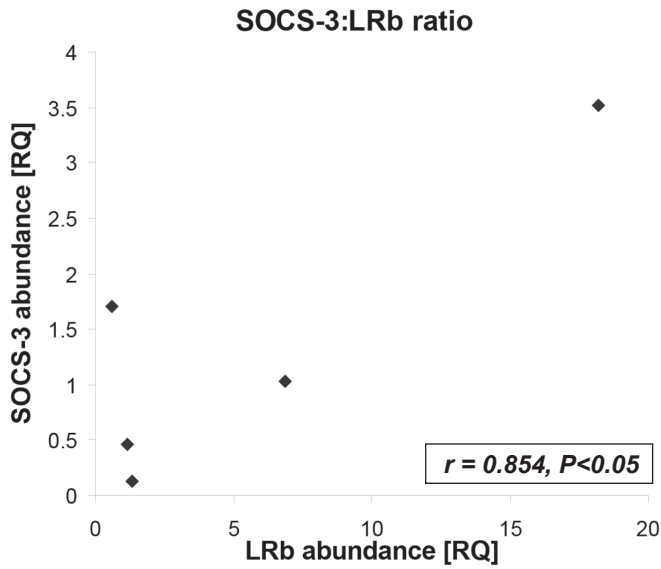

Figure 8. Ratio of suppressor of cytokine signaling-3 (*SOCS-3*) and long form of leptin receptor (*LRb*) mRNA abundances in the ovine mammary gland across the different stages of pregnancy and lactation

Discussion

In the present experiments, we demonstrated that the expression of *PRLR* and *SOCS-3* in the ovine mammary gland fluctuates between pregnancy and lactation. Additionally, although the impact of the transition from pregnancy to lactation on *LRb* expression in the mammary gland was not highly evident, an increase in the expression of this gene occurred at other stages of lactation. In our study, we observed a strong positive correlation between circulating PRL and *SOCS-3* expression, as well as between the transcript abundances of *SOCS-3* and *LRb*, suggesting that SOCS-3 may be an important factor in the PRL-leptin relationship in the mammary gland.

The results of previous studies indicated that the abundances of both PRLR mRNA and protein increased in the mammary gland during gestation in sheep (Cassy et al., 1998) and pigs (Trott et al., 2009); however, in mice, expression of *PRLR* decreased during pregnancy (Hovey et al., 2001). In the present study, we found that the abundance of *PRLR* mRNA in the ovine mammary gland during pregnancy was upregulated toward parturition, but the highest levels of *PRLR* mRNA were observed in lactating ewes. These findings are convergent with evidence from rabbits (Djiane et al., 1977) and rats (Jahn et al., 1991) but differ from the results of previous experiments on sheep, in which increased expression of *PRLR* was observed in late pregnancy compared with in the early stages of pregnancy and subsequent lactation (Cassy et al., 1998). However, the significance of the decrease in the *PRLR* mRNA levels observed between late pregnancy and lactation by Cassy et al. (1998) was

dependent on the analytical methods used (northern blot, slot-blot hybridization or ribonuclease protection assays) and was probably related to a dilution of specific *PRLR* mRNA by rRNA and milk protein transcripts, the levels of which increased dramatically at parturition. The results obtained in our study using a highly sensitive real-time PCR procedure may provide new insight into the current understanding of the pattern of *PRLR* expression in ovine mammary glands. Data from our experiments complement the results obtained earlier by Colitti and Pulina (2010), who used the same method and observed that *PRLR* expression in the udders of ewes just before delivery is higher than that during lactation; however, Colitti and Pulina did not examine *PRLR* expression at earlier stages of pregnancy. The influence of breed (dairy vs. meat/wool breed) or photoperiod (spring/summer vs. autumn/winter) on the discrepancies of the results of different experiments cannot be excluded, as both of these factors may affect *PRLR* expression. Auchtung et al. (2005) indicated that cows exposed to short-day conditions had greater *PRLR* expression than cows exposed to long-day conditions. In sheep, extended melatonin treatment caused a decrease in the abundance of PRLR in the udder (Misztal et al., 2018). In the present study, an increase in *PRLR* transcripts in the mammary gland coincided with a rise in plasma PRL concentrations during lactation, suggesting that for Polish Longwool ewes mated in November, the mammary gland requires strong PRL stimulation enhanced by not only hyperprolactinemia but also PRLR overexpression.

Pregnancy- and lactation-linked fluctuations in *LR* expression were also observed. In the experiments reported herein, we determined upregulation of *LRb* mRNA expression in the second half of pregnancy compared with that in the early stages of gestation. The abundance of *LRb* transcripts was maintained at a similar level during early lactation and then increased by over 15-fold during the late stages of lactation. The increased expression of *LRb* was maintained until at least day 90 of lactation. Studies conducted by Laud et al. (1999) on ovine mammary glands indicated that LRs are localized within alveolar epithelial cells during both pregnancy and lactation. These researchers also suggested that the highest expression of *LR* among different stages of pregnancy and lactation occurred during mid-pregnancy (Laud et al., 1999). This evidence deviates from our results; however, the previous study used a ribonuclease protection assay (Laud et al., 1999), which is less sensitive than the real-time PCR method used herein, and the previous study did not analyze the expression of *LR* after day 48 of lactation, when we found the largest increase in the mRNA expression of *LRb*. Using ribonuclease protection assays, Laud et al. (1999) were unable to detect *LRb* transcripts, although they did confirm its presence in the ovine mammary gland using RT-PCR. Regardless of these discrepancies, both studies showed that the expression of these receptors in the mammary gland varies depending on the physiological state of the ewe. The increased expression of *LRb* observed by Laud (1999) on day 70 of pregnancy coincides with the initiation of mammary epithelial cell proliferation, which occurs in this stage of ovine gestation. The rise observed in our study beginning on day 60 of lactation may be linked to the beginning of involution processes.

The observations mentioned above suggest the contribution of leptin to mammary tissue remodeling that occurs from gestation throughout lactation to the dry

period, although the exact role of leptin in the development of the mammary gland and lactation is still unclear. Mounzih et al. (1998) demonstrated that pregnancy and delivery of offspring may be induced in leptin-deficient mice but that lactation does not occur in these mice. A decrease in the concentration of leptin with accompanying hyperprolactinemia may be responsible for lactational hyperphagia and obtaining the energy resources necessary for milk production. Many of leptin's actions in the mammary gland are linked to interactions with prolactin. In the presence of PRL, leptin stimulates fatty acid synthesis and milk protein expression in bovine mammary glands (Feuermann et al., 2004), while leptin antagonists eliminate the PRL-induced stimulation of casein expression in bovine mammary gland explants (Feuermann et al., 2006). Prolactin increases leptin and *LR* expression in explants of bovine mammary glands during lactation by 2.2-fold and 25-fold, respectively, without affecting this process in calves (Feuermann et al., 2004). The association between circulating PRL and leptin mRNA in the mammary gland has also been confirmed in pregnant sows (Palin et al., 2004). These associations and interactions between leptin and PRL signaling and mammary function indicate that leptin plays important roles in mammary development and lactation.

The evidence mentioned above indicates the existence of PRL-leptin interactions within the mammary gland of lactating females, but the exact mechanism of this relationship remains unknown. An essential factor linking both hormones may be the SOCS-3 protein, which acts in a negative feedback loop as an inhibitor of PRL and leptin intracellular signaling pathways to attenuate different biological responses in the mammary gland. A SOCS-3 deficiency is linked to accelerated mammary epithelial apoptosis and stimulation of tissue remodeling (Sutherland et al., 2007 b). Wall and coworkers (2005) demonstrated that the mRNA expression of *SOCS-3* in the mammary glands of dairy cattle decreases in response to frequent milking. In rats, the lack of milk flow associated with the filling of the gland induces *SOCS-3* expression and prevents milk overproduction by suppressing the stimulatory effect of PRL (Tam et al., 2001). Moreover, *in vitro* experiments indicate that SOCS-3 directly inhibits the PRL-induced expression of milk proteins by interacting with PRLR (Dif et al., 2001), and Le Provost et al. (2005) proved that PRL enhances *SOCS-3* expression in murine mammary tissue. The present results indicate that in the ovine mammary gland, the abundance of *SOCS-3* transcripts positively correlates with the plasma PRL concentration. Some evidence suggests that SOCS-3 may be involved in mediating photoperiodic effects on mammary gland function in ewes (Szczesna et al., 2015) and cows (Wall et al., 2005). We also found that the pattern of *SOCS-3* mRNA expression in the ovine mammary gland varied depending on the physiological state of the ewe. In the second half of pregnancy, the levels of *SOCS-3* transcripts decreased gradually to reach a nadir in late-pregnant ewes in a manner similar to the pattern observed in mice (Tonko-Geymayer et al., 2002) but different from the profile observed in dairy cattle (Wall et al., 2005). Our current results are also consistent with those obtained by Le Provost et al. (2005), who demonstrated that in murine mammary glands, the expression of *SOCS-3* occurs at low levels throughout the entire prepubertal period and during pregnancy but immediately increases on the first day of lactation and remains active throughout the involution of the gland. In the

current study, expression of *SOCS-3* in the ovine mammary gland was significantly upregulated during lactation compared with that in the prepartum stages. We also found that the abundance of *SOCS-3* mRNA positively correlated with the level of *LRb* transcripts. All of these findings confirm the physiological significance of this suppressor during remodeling of the mammary gland and indicate its potential engagement in leptin-PRL crosstalk within the udder. Nonetheless, the discrepancies observed in the results obtained in different dairy species indicate the necessity of conducting in-depth research in this field. Further investigation of leptin-PRL relationship may provide new insight for a better understanding of the mechanisms that link nutritional status, body adiposity and mammary gland productivity.

In summary, it can be concluded that the transcript abundances of *PRLR*, *LR* and *SOCS-3*, which affect leptin and PRL signaling, are important for mammary gland performance and thus, may be taken into account as factors that affect the milk yield and lactation duration. However, the protein abundances of PRLR, LR and SOCS-3, which may differ from their transcript levels, should be studied to confirm these assumptions.

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Declaration of interest

None.

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