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Spatio-specific regulation of endocrine-responsive gene transcription by periovulatory endocrine profiles in the bovine reproductive tract

Estela R. Araújo[A], *Mariana Sponchiado*[A], *Guilherme Pugliesi*[A], *Veerle Van Hoeck*[A], *Fernando S. Mesquita*[B], *Claudia M. B. Membrive*[C] *and Mario Binelli*[A,D]

[A]Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo, Avenida Duque de Caxias Norte, 225, Pirassununga, SP, 13635-900, Brazil.
[B]School of Veterinary Medicine, Federal University of Pampa, Rodovia BR 472, 592, Uruguaiana, RS, 97508-000, Brazil.
[C]College of Animal Science, University of São Paulo State 'Júlio de Mesquita Filho', DracenaRodovia Comandante João Ribeiro de Barros, km 651, Dracena, SP, 17900-000, Brazil.
[D]Corresponding author. Email: binelli@usp.br

Abstract. In cattle, pro-oestrous oestradiol and dioestrous progesterone concentrations modulate endometrial gene expression and fertility. The aim was to compare the effects of different periovulatory endocrine profiles on the expression of progesterone receptor (*PGR*), oestrogen receptor 2 (*ESR2*), oxytocin receptor (*OXTR*), member C4 of aldo–keto reductase family 1 (*AKR1C4*), lipoprotein lipase (*LPL*), solute carrier family 2, member 1 (*SLC2A1*) and serpin peptidase inhibitor, clade A member 14 (*SERPINA14*): (1) between uterine horns ipsi- and contralateral to the corpus luteum (CL), (2) between regions of the ipsilateral horn and (3) in the vagina. Endometrium and vagina tissue samples were collected from cows that ovulated a larger (large follicle-large CL, LF-LCL; $n = 6$) or smaller follicle (small follicle-small CL, SF-SCL; $n = 6$) 7 days after oestrus. Cows in the LF-LCL group had a greater abundance of transcripts encoding *ESR2*, *AKR1C4*, *LPL*, *SLC2A1* and *SERPINA14*, but a reduced expression of *PGR* and *OXTR* in the endometrium versus the SF-SCL group ($P < 0.05$). Expression of *PGR* and *OXTR* was greater in the contralateral compared with the ipsilateral horn ($P < 0.05$). Regardless of group, the anterior region of the ipsilateral horn had increased expression of *PGR*, *ESR2*, *LPL*, *SLC2A1* and *SERPINA14* ($P < 0.05$). Different periovulatory endocrine profiles, i.e. LF-LCL or SF-SCL, did not influence gene expression in the vagina and had no interaction with inter- or intra-uterine horn gene expression. In conclusion, inter- and intra-uterine horn variations in gene expression indicate that the expression of specific genes in the bovine reproductive tract is location dependent. However, spatial distribution of transcripts was not influenced by distinct periovulatory sex-steroid environments.

Additional keywords: oestradiol, progesterone, transcript abundance, uterine horn, vagina.

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Introduction

Embryonic mortality (Diskin and Morris 2008) during the first weeks after ovulation is a major cause of economic losses in beef cattle commercial operations (Ayres *et al.* 2008; Sales *et al.* 2011). Losses are associated with preimplantation embryo mortality that reaches 30 to 40% of inseminated cows (Diskin and Sreenan 1980). Between Day 4 and 5 after oestrus the embryo leaves the oviduct and enters the anterior region of the uterine horn relative to the ovary containing the corpus luteum (CL; ipsilateral horn), where it continues its further development supported by uterine secretions until implantation (Spencer and Bazer 2003). Volume and composition of uterine secretions are regulated by endometrial function, specifically, by the continuum of changes in the endometrial transcriptome and associated proteome that occur before implantation.

Indeed, up to the time of maternal recognition of pregnancy during late dioestrus, progressive changes in the endometrial transcriptome occur independently of the presence of the conceptus (Bauersachs *et al.* 2005; Mitko *et al.* 2008; Clemente *et al.* 2009; Forde *et al.* 2011). This indicates that the uterine

tissues play a key role in providing an optimal environment for the early developing embryo. It may be proposed that the uterine environment is modulated by steroid hormones to reach a maternal receptive state, as supported by the positive association between the preovulatory follicle size and subsequent circulating progesterone (P4) concentrations during the early luteal phase and the greater probability of embryo survival in beef cows (Baruselli *et al.* 2012). In this context, coordinated actions of oestradiol (E2) and P4 during pro-oestrus and dioestrus, respectively, stimulate uterine function towards embryo receptivity (Lopes *et al.* 2007; Bridges *et al.* 2012). Furthermore, there are positive associations between periovulatory concentrations of E2 and P4, conceptus development and elongation (Garrett *et al.* 1988; Satterfield *et al.* 2006; Carter *et al.* 2008) and overall fertility (Demetrio *et al.* 2007; Meneghetti *et al.* 2009; Peres *et al.* 2009; Baruselli *et al.* 2012).

Changes in the periovulatory endocrine milieu that stimulate embryo development, i.e. pro-oestrus–oestrus E2 and metaoestrus–early dioestrus P4, affect endometrial gene expression. For example, Forde *et al.* (2009) increased P4 concentrations during the early luteal phase through insertion of a progestin-releasing device. They reported changes in expression of several genes in the endometrium 5, 7, 13 or 16 days after insemination and such changes were associated with increased elongation of conceptuses recovered on Day 16. More recently, Mesquita *et al.* (2014) manipulated the growth of the preovulatory follicle (POF) to generate two groups of cows that presented distinctly different pro-oestrus concentrations of E2 and early dioestrus concentrations of P4. These different periovulatory endocrine environments significantly affected the expression of genes associated with endometrial function, such as steroid signalling (progesterone receptor, PGR; oestrogen receptor 2, ESR2), lipid metabolism (lipoprotein lipase, LPL) and eicosanoid synthesis (member C4 of aldo–keto reductase family 1, AKR1C4). More importantly, cows treated to ovulate larger POFs presented a 20% increase in pregnancy in a large AI fertility trial (G. Pugliesi and M. Binelli, unpubl. data).

An important technical aspect of most reports on uterine gene expression is that either tissue from a single region within the uterine horn (i.e. medial region; Forde *et al.* 2009) or a pool of tissues from different regions (Mesquita *et al.* 2014) was used for treatment comparisons. However, regional gradients of gene expression levels, previously reported in the reproductive tract of cattle (Bauersachs *et al.* 2005), pigs (Buhi and Alvarez 2003) and sheep (Meikle *et al.* 1997, 2001), might be highly relevant for receptivity and pregnancy success. For example, early embryo transfer work determined that when two embryos were transferred bilaterally, the greatest chances of twinning were achieved when at least one embryo was transferred to the tip of the horn relative to the CL (Newcomb *et al.* 1980). In contrast, the lowest embryo survival was noted when embryos were transferred to the base of each horn. It is tempting to speculate that regional differences in receptivity within the uterus are due to molecular microenvironments specific for each region. Specific microenvironments could be associated with different regional abundance of transcripts. Furthermore, differences in P4 concentrations between different regions of the uterine horn relative to the CL and between horns were reported earlier (Pope *et al.* 1982). This provides mechanistic support for a regional control of expression of P4-stimulated genes.

In the present study we hypothesised that endocrine changes associated with growth, ovulation of follicles of different sizes and subsequent changes in dioestrous P4 concentrations modulate the spatial expression patterns of specific steroid-responsive transcripts in the uterine and vaginal tissue of beef cows at a specific moment during the early luteal phase (Day 7 after induction of ovulation). Therefore, we aimed to compare periovulatory endocrine influences on the expression levels of *PGR*, *ESR2*, oxytocin receptor (*OXTR*), solute carrier family 2, member 1 (*SLC2A1*), *AKR1C4*, *LPL* and serpin peptidase inhibitor, clade A member 14 (*SERPINA14*) between the ipsi- and contralateral uterine horns, between different regions within the ipsilateral uterine horn and in the vagina.

Materials and methods

Animal model and reproductive management

Animal procedures were approved by the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo (Protocol number 6732280414). Forty-two non-lactating, multiparous Nelore (*Bos indicus*) cows containing no gross reproductive anomalies were kept in grazing conditions, supplemented with sugarcane and corn silage, concentrate and minerals and water *ad libitum*. As previously described by Mesquita *et al.* (2014), follicular growth was pharmacologically manipulated to generate two groups of animals, presenting a large (LF-LCL) or small (SF-SCL) preovulatory follicle and subsequent CL. Briefly, all cows were pre-synchronised by two intramuscular (i.m.) treatments of prostaglandin F2α (PGF2α) analogue (0.5 mg of sodium clopostrenol, Sincrocio; Ouro Fino, Cravinhos, Brazil) 14 days apart. Cows that did not present oestrus after the second PGF2α treatment were excluded. To modulate the growth of the preovulatory follicle (POF), on Day −10 animals received a single i.m. treatment of PGF2α (LF-LCL) or not (SF-SCL) along with oestradiol benzoate (2 mg; Sincrodiol, Ouro Fino). On the same day, all animals received an intravaginal P4-releasing device (1 g, Sincrogest; Ouro Fino) that was removed 42–60 h or 30–36 h before the gonadotrophin-releasing hormone (GnRH) treatment in the LF-LCL and SF-SCL groups, respectively. Ovulation was induced with buserelin acetate (GnRH, 10 μg i.m., Sincroforte; Ouro Fino) on Day 0. Expectation was that POF growth would be reduced in the presence of greater circulating P4 concentrations in cows from the SF-SCL group because of P4 from both exogenous (device) and endogenous (CL) sources. All animals received an i.m. PGF2α treatment at P4 device removal and a second treatment 6 h later. Blood plasma samples were obtained for measurement of E2 concentrations from Day −2 to Day 0 and for P4 measurements from Day 0 to Day 7. Blood was collected by jugular venipuncture in heparinised tubes (BD, São Paulo, Brazil) and stored on ice. Plasma was obtained by centrifugation at 4°C, 1500*g* for 30 min and stored at −20°C.

Transrectal ultrasound examinations of the ovaries were performed on Days −10 and −6, daily from Day −2 to Day 0, every 12 h on Day 1 and Day 2 and daily from Day 3 to Day 7 to

assess follicle growth, ovulation and CL development and vascularisation. The examinations were performed using a duplex B-mode (grey-scale) and pulsed-wave colour Doppler ultrasound instrument (MyLab30; Esaote Healthcare, São Paulo, Brazil) equipped with a multi-frequency linear transducer. From the 42 animals that started the synchronisation protocols, six animals per group were selected because they corresponded to the treatments as planned.

Collection of reproductive tract samples

Following slaughter on Day 7, the reproductive tract was removed, transported to the laboratory on ice within 10 min and the uterus and the vagina were dissected. Each horn was opened from the antimesometrial side and intercaruncular endometrium dissected from the anterior, middle and posterior regions. Endometrium samples from each region of the uterine horn relative to the ovary containing the CL, from pooled regions of the contralateral horn and from vaginal tissue collected in the vaginal fornix were stored individually at −80°C. Furthermore, endometrial fragments from the anterior and middle regions of the ipsilateral horn relative to the CL were collected from cows in the LF-LCL and SF-SCL groups, fixed in 4% buffered formalin for 24 h and embedded in paraffin for microscopy.

The CL was excised, measured and weighed. Volume (V) of the CL was calculated by applying measurements of height, width and length in the ellipsoid volume formula: $V = 4/3\pi \times r_1 \times r_2 \times r_3$, where π is the mathematical constant, r_1 is height radius, r_2 is width radius and r_3 is length radius.

Sample processing

Radioimmunoassay for measuring P4 and E2 concentrations

Plasma P4 concentrations were assayed with a solid-phase radioimmunoassay (RIA) kit containing antibody-coated tubes and ^{125}I-labelled P4 (Coat-A-Count Progesterone; Diagnostic Products Corporation, Los Angeles, CA, USA) as reported previously (Garbarino *et al.* 2004). Plasma E2 concentrations were assayed using a commercial RIA kit (Double Antibody Oestradiol; Diagnostic Products Corporation) as reported (Siddiqui *et al.* 2009). The intra-assay CV and sensitivity for P4 and E2, respectively, were 6.2% and 0.07 ng mL^{-1} and 4.0% and 0.06 pg mL^{-1}.

Extraction of RNA and synthesis of cDNA

RNA extraction was performed using an RNeasy mini columns kit (Qiagen Laboratories, São Paulo, Brazil), as per the manufacturer's instructions. To maximise lysis and total RNA isolation, ~30 mg of endometrial and vaginal tissue was submerged in liquid nitrogen and macerated using a stainless steel apparatus. The macerate was mixed with buffer RLT from the kit and the tissue suspension was passed at least 10 times through a 21-guage needle and centrifuged at 12 000*g* for 1 min at 4°C for removal of debris, before supernatant loading and processing in RNeasy columns. The samples were treated with DNAse I (Life Technologies, São Paulo, Brazil) for 15 min at room temperature in 80-μL reactions during the RNA extraction protocol. Columns were eluted with 40 or 60 μL of RNase free water for endometrium and vagina, respectively. Concentration and purity of total RNA in extracts was estimated by a spectrophotometer (NanoDrop; Thermo Scientific, Waltham, MA, USA) by the absorbance at 260 nm and the 260/280 nm and 260/230 nm ratios, respectively.

The RNA extracted samples were submitted to cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), as per the manufacturer's instructions. Samples were incubated at 25°C for 10 min, followed by incubation at 37°C for 2 h and reverse-transcriptase inactivation at 85°C for 5 min and storage at −20°C.

Quantitative polymerase chain reaction (PCR)

We selected seven genes participating in different pathways relevant to endometrium receptivity (Table 1). Gene expression of *PGR*, *ESR2* and *OXTR* are upregulated by E2 and downregulated by P4 (Okumu *et al.* 2010; Bishop 2013). *SLC2A1*, *AKR1C4*, *LPL* and *SERPINA14* are genes related to histotroph composition and conceptus maintenance (Ulbrich *et al.* 2009*a*, 2009*b*; Forde *et al.* 2010). Peptidylprolyl isomerase A (*PPIA*), cyclophilin A, actin-β (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein L15 (*RPL15*) were used as reference genes for normalisation of expression. Primer sequences were obtained from the literature or were designed based on Gen-Bank Ref Seq mRNA sequences using the National Center for Biotechnology Information tool, Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) and PrimerQuest (http://www.idtdna.com/primerquest). Equivalent specificity searches were done using the software Basic Local Alignment Search Tool (BLAST; Altschul *et al.* 1990; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Real-time reverse transcription polymerase chain reactions were carried out in 96-well plates in a Step One Plus apparatus (Life Technologies). The 20-μL reactions contained power SYBR green reagent (10 μL), cDNA samples (diluted 1 : 40) or diethylpyrocarbonate (DEPC) water in the negative control (4 μL) and a mixture of specific forward and reverse primers (6 μL). The thermal cycling parameters were set at 95°C for 10 min then 40 cycles of 15 s at 95°C and 1 min at 60°C. For each gene the concentration of primers in the reaction mixture was tested (150, 300, 600 or 900 nM) and selected according to efficiency of amplification, lack of amplification in the negative control and melting (dissociation) curve analysis. Transcript abundance was determined using the relative standard curve method using serial dilutions of a cDNA pool (1 : 40 to 1 : 320). Primers were considered for further analysis when efficiency of amplification of the standard curve was between 85 and 110% and the coefficient of determination of the regression equation was >0.97. There was no linear amplification of the AKR1C4 gene in the vagina and this gene was not further analysed in this tissue. Cycle quantification (Cq) values were obtained from LinReg PCR software (http://www.hartfaalcentrum.nl/index.php?main=files&sub=LinRegPCR). The expression of each target gene was calculated following normalisation using GeNorm software (http://medgen.ugent.be/~jvdesomp/genorm/; Vandesompele *et al.* 2002). Initially four reference genes were used (*PPIA*, *ACTB*, *GAPDH* and *RPL15*). After selection performed by the software, *PPIA* and *ACTB* were chosen.

Table 1. Symbols of genes, accession numbers, primer pair sequences, amplicon size and literature references used to generate primers

Target gene	GenBank number	Sense and anti-sense primer sequences	Size of amplicon (bp)	References
PGR	NM_001205356.1	5′-GCCGCAGGTCTACCAGCCCTA-3′ 3′-GTTATGCTGTCCTTCCATTGCCCTT-5′	199	Primer-Blast
ESR2	NM_174051.3	5′-TCACGTCAGGCACGCCAGTAAC-3′ 3′-CACCAGGTTGCGCTCAGACCC-5′	155	Primer-Blast
OXTR	NM_174134.2	5′-AAGATGACCTTCATCGTCGTG-3′ 3′-CGTGAAGAGCATGTAGATCCAG-5′	177	Krishnaswamy *et al.* 2009
AKR1C4	NM_181027.2	5′- TGAGCTATTACTGGGTGTTGGTCACCC-3′ 3′-ACAGCCCTAGAAGCAAGATGTTCTGGT-5′	106	Primer-Blast
SERPINA14	NM_174797.3	5′-ATATCATCTTCTCCCCCATGG-3′ 3′-GTGCACATCCAACAGTTTGG-5′	126	Ulbrich *et al.* 2009*a*
LPL	NM_001075120	5′-CAGGTCGAAGTATCGGAATCCA-3′ 3′-GAAAGTGCCTCCGTTAGGGTAAA-5′	68	Forde *et al.* 2010
SLC2A1	NM_174602.2	5′-ATCATCTTCACCGTGCTCCTGGTT-3′ 3′-TGTCACTTTGACTTGCTCCTCCCT-5′	127	PrimerQuest
GAPDH	NM_001034034.2	5′-GCCATCAATGACCCCTTCAT-3′ 3′-TGCCGTGGGTGGAATCA-5′	70	Bettegowda *et al.* 2006
ACTB	NM_173979.3	5′-GGATGAGGCTCAGAGCAAGAGA-3′ 3′-TCGTCCCAGTTGGTGACGAT-5′	78	Bettegowda *et al.* 2006
RPL15	NM_001077866.1	5′-TGGAGAGTATTGCGCCTTCTC-3′ 3′-CACAAGTTCCACCACACTATTGG-5′	65	Bettegowda *et al.* 2006
PPIA	NM_178320.2	5′-GCCATGGAGCGCTTTGG-3′ 3′-CCACAGTCAGCAATGGTGATCT-5′	66	Bettegowda *et al.* 2006

The geometric mean of the expression of these two genes was used for normalisation of the expression of the target genes. Identity of amplified PCR fragments was verified by sequencing and the resulting sequences were assessed by Chomas software (http://technelysium.com.au/) and tested for specificity using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Immunohistochemistry

Paraffin-embedded endometrium samples of the anterior and middle regions of the ipsilateral horn were cut into 4-μm sections and mounted on an adhesive slide (StarFrost; Knittel Glass, Braunschweig, Germany). Sections were deparaffinised in xylene and rehydrated in a series of increasing dilutions of ethanol. Sections were incubated for 5 min in 10 nM citrate buffer (pH 6) at room temperature and then incubated slides were processed for four cycles of 5 min each at 750 W in a microwave oven for antigen retrieval. The container was refilled with distilled water as needed to replace evaporation of the solution and avoid section drying. Slides were cooled at room temperature for 20 min and washed three times for 5 min in phosphate-buffered saline (PBS) containing 0.3% Triton (PBS–Triton; pH 7.4). Endogenous peroxidase activity was blocked by incubation of the sections in 1.5% hydrogen peroxide in methanol for 30 min at room temperature then sections were washed in PBS–Triton (3 × 5 min). To block nonspecific binding, protein block was carried out using 2% non-fat dry milk for 15 min at room temperature. Tissue sections were incubated in a humid chamber with a mouse monoclonal anti-progesterone receptor (PGR) primary antibody (Clone PR10A9, 1 : 75; Beckman Coulter, Villepinte, France) overnight at 4°C. Negative-control reactions contained normal mouse IgG instead of primary antibody. Tissue sections were then washed (3 × 5 min) in PBS–Triton. The Biotinylated Link Universal Solution (Dako, Glostrup, Denmark) was used as secondary antibody and the incubation was conducted for 15 min at room temperature in a humid chamber. Next, slides were washed in PBS–Triton buffer (3 × 5 min), incubated with a streptavidin–horseradish peroxidase complex (streptavidin–HRP; Dako) for 15 min in a humid chamber and washed again in PBS–Triton. Sections were then incubated in diaminobenzidine (DAB; Dako) solution for 5 min and washed in distilled water (3 × 5 min). Finally, sections were counterstained with haematoxylin, washed in running water for 10 min, dehydrated in a series of increasing concentrations of ethanol, cleared in xylene and mounted on coverslips. The slides were photographed using light microscopy and staining intensity was subjectively scored in each of the following tissue compartments: luminal epithelium, stromal cells and glandular epithelium, by six independent evaluators. Intensity scores ranged from zero (i.e. absence of staining) to three (i.e. very strong staining).

Statistical analyses

All data were tested for normality of distribution of residues using Shapiro–Wilk's test and homogeneity of variances using Welch's method and transformed by natural or inverse logarithms if necessary. Discrete dependent variables were analysed by one-way ANOVA for the effect of group using the PROC MIXED procedure (SAS Software, Version 9.2; SAS Institute, Cary, NC, USA) with animal-within-group as a random variable. For gene expression, data was analysed by split-plot ANOVA. For measures repeated in space using PROC MIXED procedure (SAS software) two distinct models were used. The first model estimated the effects of group, side and their interaction, whereas the second model estimated the effects of group,

Table 2. Follicle, CL and P4 measurements of cows ovulating within the first 48 h after GnRH
Values are expressed as mean ± s.e.m. Day 0 is the day of GnRH treatment to induce ovulation

Parameter	Group		*P* value
	SF-SCL ($n = 6$)	LF-LCL ($n = 6$)	
Follicle diameter (mm)			
D-2	6.92 ± 0.47	10.88 ± 0.76	0.002
D0	10.11 ± 0.41	13.35 ± 0.48	0.0004
Pre-ovulatory follicle	10.33 ± 0.33	13.88 ± 0.47	<0.0001
Plasma E2 concentrations (pg mL^{-1})[A]			
Day −1	0.36 ± 0.23	2.30 ± 0.57	0.04
Day 0	0.93 ± 0.16	2.96 ± 0.36	0.006
CL			
Weight (g)[B]	1.64 ± 0.23	2.88 ± 0.27	0.005
Volume (cm^3)	1.72 ± 0.31	2.31 ± 0.24	0.17
Plasma P4 concentrations (ng mL^{-1})			
Day 6	1.27 ± 0.25	3.52 ± 0.48	0.002
Day 3 to Day 6[C]	2.71 ± 0.59	7.83 ± 1.16	0.002
Difference between Day 6 and Day 3[D]	1.03 ± 0.19	3.03 ± 0.40	0.001
Interval (days)			
Day 0 to day of P4 >1 ng mL^{-1} [E]	6.17 ± 0.31	4.33 ± 0.33	0.003

[A]Oestradiol concentrations were assayed in a subset of animals ($n = 3$–5 per group).
[B]Measured post mortem on Day 7.
[C]Sum of P4 concentration of Day 3 to Day 6.
[D]Subtraction of P4 concentration on Day 3 from P4 concentration on Day 6.
[E]Number of days between Day 0 and the day that P4 was >1 ng mL^{-1}.

region and their interaction using a compound symmetry (CS) covariance matrix structure with animal-within-group as a random variable. For target gene abundance in vaginal tissue, only the effect of group was considered. A probability of $P \leq 0.05$ indicated significant effects and a probability of $0.05 < P \leq 0.1$ indicated that significance was approached. Data are presented as the arithmetic mean ± s.e.m.

Results

Animal model

The animal model succeeded to generate two groups with larger or smaller follicle and subsequent CL and, consequently, greater or smaller plasma concentrations of E2 and P4 (Table 2). Briefly, follicle diameter on Day −2 and Day 0 and POF diameter were greater ($P \leq 0.05$) in the LF-LCL group than in the SF-SCL group. Concentrations of E2 were higher ($P \leq 0.05$) on Day −1 and Day 0 in the LF-LCL group ($n = 3$) compared with the SF-SCL group ($n = 5$). Although weight of CL on Day 7 was greater ($P \leq 0.05$) in the LF-LCL than in the SF-SCL group, CL volume did not differ ($P > 0.1$) between groups. Plasma P4 concentrations were also greater ($P \leq 0.05$) for several P4 endpoints during early dioestrus in the LF-LCL group compared with the SF-SCL group.

Endometrial transcript abundance

Effect of side of the uterine horn relative to the to CL

Abundance of transcripts for *ESR2*, *AKR1C4*, *SLC2A1*, *LPL* and *SERPINA14* did not differ ($P > 0.1$) between the ipsilateral and contralateral horns to the CL. However, abundance of transcripts for *PGR* and *OXTR* was greater ($P \leq 0.05$) in the side contralateral to the CL (Fig. 1). For the proposed model to modulate the periovulatory endocrine profile, an effect of group (SF-SCL or LF-LCL) was detected for the expression of *ESR2*, *AKR1C4*, *LPL*, *SLC2A1* and *OXTR* genes. Abundance of transcripts for *ESR2*, *AKR1C4*, *LPL* and *SLC2A1* was greater ($P \leq 0.05$) and for *SERPINA14* tended to be greater ($P \leq 0.1$) in the LF-LCL group than in SF-SCL group. In contrast, the transcript abundance was lesser ($P \leq 0.05$) or tended to be lesser ($P \leq 0.1$) in the LF-LCL group than in the SF-SCL group for *OXTR* and *PGR*, respectively. An interaction of group-by-side was observed only for the *OXTR* gene. Interpretation of the interaction revealed that while abundance of *OXTR* was increased in the contralateral side for the SF-SCL group, it was similar between sides in the LF-LCL group.

Effect of region of the uterine horn relative to the CL

An effect ($P < 0.05$) or an approached effect ($P < 0.1$) of region (anterior, medial or posterior) of the horn relative to the CL was detected for all transcripts studied, except for *OXTR* (Fig. 2). Generally, expression was greatest in the anterior region and lowest in the posterior region of the uterine horn. There was no significant group-by-region interaction. An effect of group (SF-SCL or LF-LCL) was also detected for all target genes studied and followed the same abundance direction observed in the above-mentioned side-by-group analysis. Therefore, transcript abundance was greater ($P \leq 0.05$) for *ESR2*, *AKR1C4*, *SLC2A1*, *LPL* and *SERPINA14*

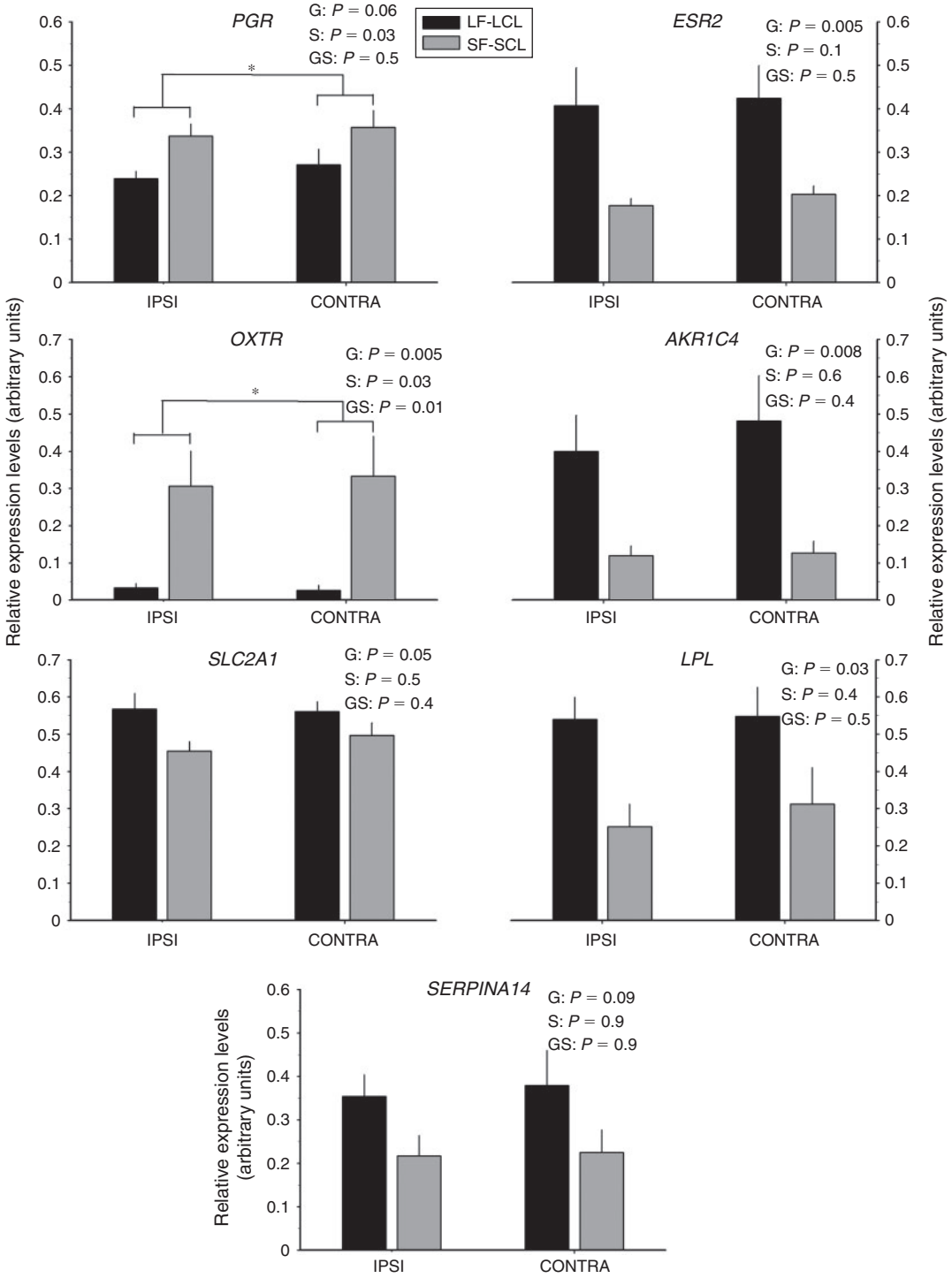

Fig. 1. Mean ± s.e.m. transcript abundance of target gene expression normalised to peptidylprolyl isomerase A (*PPIA*) and β-actin (*ACTB*) in the uterine horns ipsi (IPSI)- and contralateral (CONTRA) to the CL from beef cows synchronised to ovulate a large (LF-LCL) or small follicle (SF-SCL). Effect of group (G), side (S) and interaction of group-by-side (GS) are indicated. IPSI, horn ipsilateral to the ovary bearing the CL; CONTRA, horn contralateral to the ovary bearing the CL. Genes: progesterone receptor (*PGR*), oestrogen receptor 2 (*ESR2*), oxytocin receptor (*OXTR*), aldo–keto reductase family 1, member C4 (*AKR1C4*), solute carrier family 2, member 1 (*SLC2A1*), lipoprotein lipase (*LPL*) and serpin peptidase inhibitor, clade A member 14 (*SERPINA14*, formerly known as *UTMP*).

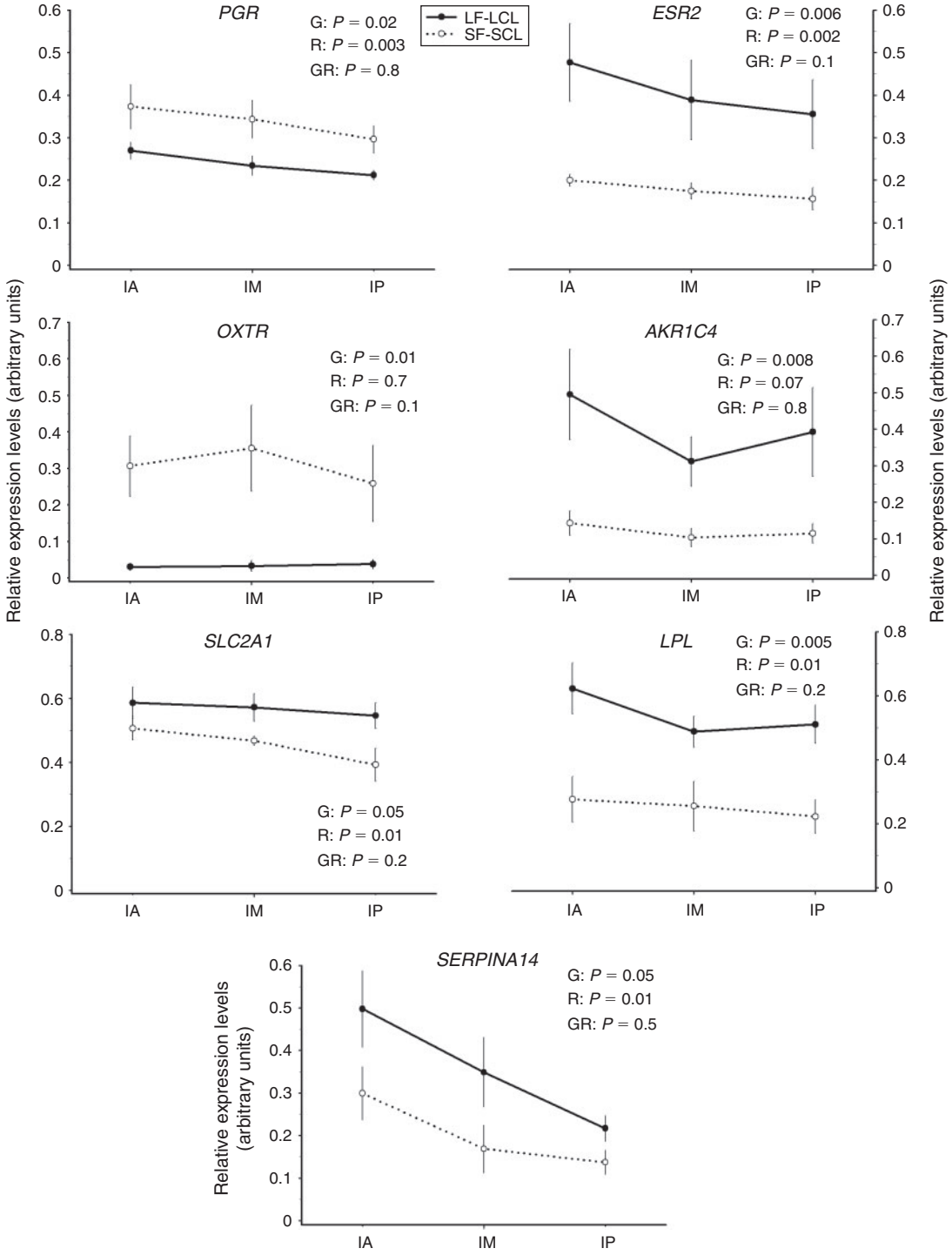

Fig. 2. Mean ± s.e.m. transcript abundance of target gene expression normalised to peptidylprolyl isomerase A (*PPIA*) and β-actin (*ACTB*) in the regions of the horn ipsilateral to the CL from beef cows synchronised to ovulate a large (LF-LCL) or small follicle (SF-SCL). Effect of group (G), region (R) and interaction of group-by-region (GR) are indicated. IA, ipsilateral anterior; IM, ipsilateral medial and IP, ipsilateral posterior regions. Genes: progesterone receptor (*PGR*), oestrogen receptor 2 (*ESR2*), oxytocin receptor (*OXTR*), solute carrier family 2, member 1 (*SLC2A1*), aldo–keto reductase family 1, member C4 (*AKR1C4*), lipoprotein lipase (*LPL*), serpin peptidase inhibitor, clade A member 14 (*SERPINA14*, formerly known as *UTMP*).

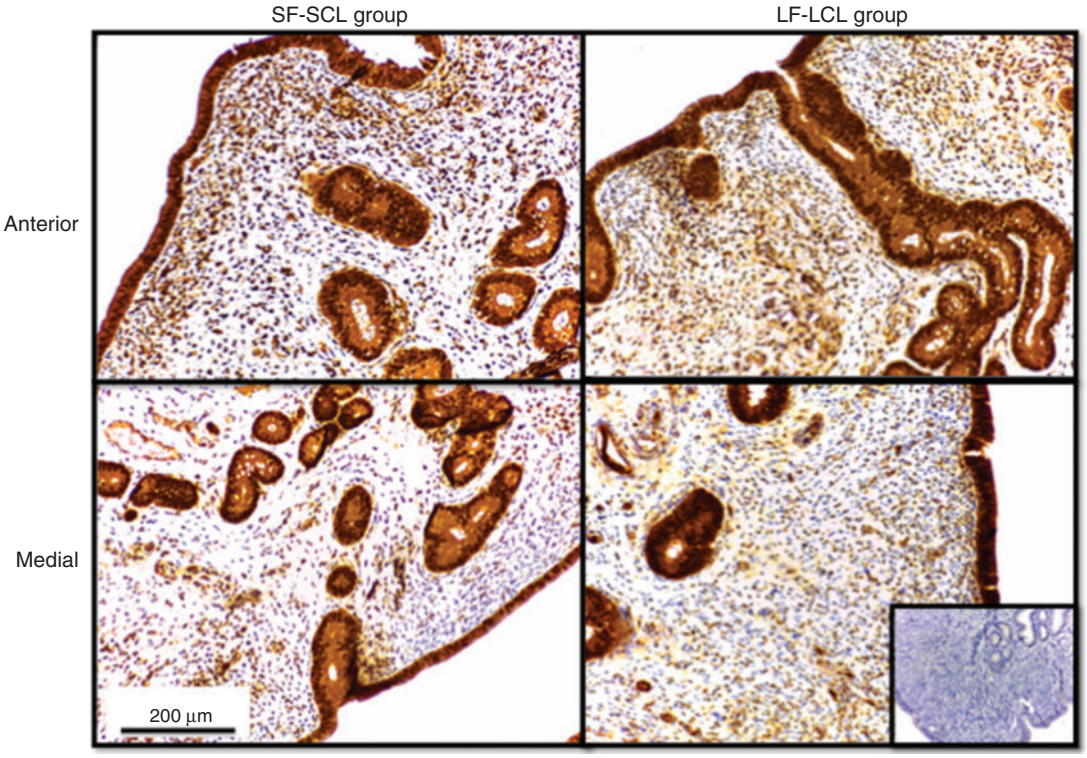

Fig. 3. Localisation of PGR in the bovine uterus by immunohistochemistry. Representative images of PGR immunohistochemical localisation in the anterior and medial regions of LF–LCL and SF–SCL groups at Day 7 of the oestrous cycle. Original magnification 200×. Scale bar = 200 μm. Inset: negative control.

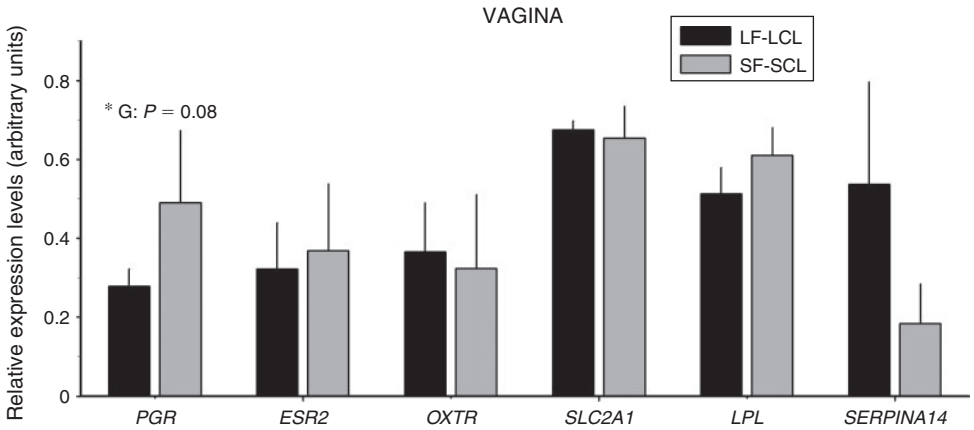

Fig. 4. Mean ± s.e.m. transcript abundance of target gene expression normalised to peptidylprolyl isomerase A (*PPIA*) and β-actin (*ACTB*) in the vagina of beef cows synchronised to ovulate a large (LF-LCL) or small follicle (SF-SCL). An approached effect ($P < 0.01$) of group is indicated by an asterisk between group bars within each gene. Genes: progesterone receptor (*PGR*), oestrogen receptor 2 (*ESR2*), oxytocin receptor (*OXTR*), solute carrier family 2, member 1 (*SLC2A1*), lipoprotein lipase (*LPL*), serpin peptidase inhibitor, clade A member 14 (*SERPINA14*, formerly known as *UTMP*).

and lesser ($P \leq 0.05$) for *PGR* and *OXTR* in the LF-LCL group than in the SF-SCL group. Regarding intensity of immunostaining for PGR, average staining intensity was similar between groups and regions (Fig. 3).

Vaginal transcript abundance

There was no difference ($P > 0.01$) between groups in transcript abundance for *ESR2*, *OXTR*, *LPL*, *SLC2A1* and *SERPINA14* genes in the vagina (Fig. 4). However, the transcript abundance

for *PGR* approached being greater ($P \leq 0.1$) in the SF-SCL group compared with the LF-LCL group (Fig. 4).

Discussion

Needed increases in reproductive efficiency depend on in-depth understanding of endocrine, cellular and molecular mechanisms regulating fertility. Focussing on the maternal reproductive tract, the endometrium is a highly dynamic tissue that has the capacity to undergo transcriptional and physiological changes coordinated by ovarian hormones. Circulating E2 and P4 concentrations orchestrate uterine events in a spatio–temporal manner. In the present study, we have for the first time evaluated whether specific periovulatory endocrine profiles can regionally influence gene transcription patterns in the different sections of the bovine uterus and in the vagina at a specific moment during early dioestrus (Day 7). Therefore, the gene expression patterns of genes encoding endocrine receptors, substrate provision pathways and receptivity markers were compared in the endometrium, between the uterine horns ipsi- and contralateral to the CL and in the vagina. In summary, we found that the transcript abundance of *ESR2*, *AKR1C4*, *LPL*, *SLC2A1* and *SERPINA14* was greater, and the abundance of *PGR* and *OXTR* was lower, in the endometrium of cows from the LF-LCL group (higher preovulatory E2 and postovulatory P4) when compared with the SF-SCL group. The expression patterns of the specific genes displayed inter- and intra-uterine horn variations at Day 7, which indicates that the transcriptome signature of the bovine female reproductive tract is modulated regionally. However, the absence of group-by-side and group-by-region interactions on the abundance of transcripts indicates that regional effects were not influenced by the endocrine changes associated with the treatment groups. This finding did not support our initial hypothesis. Finally, no effect of group was observed on gene transcript abundance in the vagina.

Abundance of transcripts for *PGR* and *OXTR* was downregulated in the endometrial tissue of cows with larger POF and CL, and thus higher circulating pro-oestrous E2 and early dioestrous P4 levels, compared with the cows with smaller POF and CL. In line with this finding, elevated concentrations of P4 during early dioestrus are known to downregulate P4 receptor (Okumu *et al.* 2010; Forde *et al.* 2011). Moreover, downregulated mRNA and protein expression of PGR in the luminal and glandular epithelium have been proposed as a prerequisite for proper embryo implantation (Spencer *et al.* 2008; Bazer *et al.* 2010). However, no difference in PGR staining intensity in the endometrium was observed between anterior and medial regions or between the LF-LCL and SF-SCL groups. Similarly, the expression of *OXTR* is known to be downregulated during dioestrus; its transcript abundance is maximal around oestrus and decreases progressively during early to mid-dioestrus (Wijayagunawardane *et al.* 1998; Spencer and Bazer 2004; Spencer *et al.* 2004). The downregulation of *OXTR* expression may be triggered by increased P4 levels via non-genomic actions (Bishop 2013). Interestingly, we observed a higher *ESR2* expression in cows with greater circulating P4 at Day 7, which contrasts with the previous report of Okumu *et al.* (2010), who documented an inhibiting effect of P4 on *ESR2* gene expression at mid-dioestrus.

Bazer and Slayden (2008) suggested that the dioestrous inhibition of endocrine receptors might trigger the expression of genes encoding secretory proteins and transporter molecules that selectively transfer substrates into the uterine lumen in order to produce histotroph. In the present study, the favourable periovulatory endocrine profiles (higher E2 and P4) indeed resulted in an increased expression of the genes related to histotroph composition and conceptus maintenance (*AKR1C4*, *SLC2A1*, *LPL*, *SERPINA14*). The *AKR1C4* gene encodes an enzyme related to PGF2α synthesis and P4 clearance (Gauvreau *et al.* 2010; Seo *et al.* 2011). Therefore, the greater expression of *AKR1C4* in the LF-LCL group could be associated with the beneficial role of uterine PGF2α during early pregnancy, i.e. stimulating vascularisation of the endometrium to support blastocyst growth and embryo implantation (Charpigny *et al.* 1997; Reese *et al.* 1999; Ulbrich *et al.* 2009*b*). LPL and SLC2A1 may contribute to the composition of the histotroph, as LPL is involved in delivering triacylglycerol (TAG) to target tissues (Mead *et al.* 2002) and SLC2A1 is responsible for the facilitated transport of glucose across the plasma membrane by diffusion gradient (Wood and Trayhurn 2003). Actions of the latter molecules are important for energy source provision towards the embryo during early development in the uterus (Ferguson and Leese 2006). Interestingly, P4 sequentially enhances LPL expression on Day 7 and decreases it when the embryo starts to elongate (Day 13; Forde *et al.* 2010, 2011). Regarding SERPINA14, it is one of the most abundant proteins present in the pregnant ruminants uterus (Segerson and Bazer 1989). Its main functions are immune modulation and transport of proteins such as immunoglobulins (Ulbrich *et al.* 2009*a*; Padua and Hansen 2010; Ledgard *et al.* 2011). A previous study (Bauersachs *et al.* 2005) reported that the expression of SERPINA14 is increased at late dioestrus compared with early dioestrus, indicating that the main function of SERPINA14 could be much later during pregnancy. However, the present results indicated a difference in SERPINA14 transcript abundance at Day 7 between the different periovulatory profiles, which may suggest favourable earlier modulation of SERPINA14 expression by higher preovulatory oestradiol and postovulatory P4 on embryo survival.

In the present study, the maternal tissue samples were collected around early dioestrus (Day 7 after GnRH treatment to induce ovulation), which coincides with the presence of the embryo in the ipsilateral horn, i.e. located on the side of the ovary containing the CL. The embryo remains confined to that region until elongation, which starts 12 to 15 days after oestrus (Peippo *et al.* 2011). This implies that, during the first 10 days in the uterus, the embryo is free-floating in the endometrial tissue secretion (histotroph) at the anterior region of the ipsilateral horn, in which the first maternal–embryonic interactions occur. Around this timing, the expression of PGR and OXTR was less in the uterine horn relative to the CL compared with the contralateral horn, whereas expression of the remaining five genes was similar between the two horns. It is proposed that local P4 concentrations were different between horns as observed by Pope *et al.* (1982) and this affected horn expression of PGR and OXTR. It is possible that the differential expression between horns may be needed to provide an optimal

environment for the developing embryo in the lumen of the anterior region of the ipsilateral horn (Wallenhorst and Holtz 1999). This can be reinforced by the fact that embryos transferred to the ipsilateral horn have greater chance of survival than embryos transferred to the contralateral horn (Tervit *et al.* 1977; Christie *et al.* 1979).

When focusing on the regional transcript abundance patterns within the ipsilateral uterine horn, it is important to consider the importance of the specific regions, with particular focus on the anterior part, in providing the embryonic nutrition for its first 2 weeks of development (Bazer *et al.* 2012). The abundance of transcripts coding for PGR, ESR2, LPL, SERPINA14 and SLC2A1 was upregulated in the anterior region of the ipsilateral uterine horn. Greater expression of PGR and ESR2 in the anterior part was not expected, as greater P4 concentrations reported (Pope *et al.* 1982) in this portion may downregulate these receptors. However, similar results were previously reported by Meikle *et al.* (1997) in sheep treated with P4 or E2. In that study, higher levels of PGR and ESR2 were observed in the anterior section of the myometrium, endometrium and caruncles than in the medial.

The analysis of vaginal gene expression was performed in an attempt to determine possible similarities between modulation of gene expression by the periovulatory endocrine environments in the uterus and in the vagina. Due to its easy access and to avoid invasive approaches such as endometrial biopsies, vaginal biopsies might provide hands-on information in order to forecast maternal receptivity without disturbing maternal–embryonic interactions (Bonnett *et al.* 1993; LeBlanc *et al.* 2002; Pugliesi *et al.* 2014). Although a trend was observed for an upregulated abundance of PGR in the vagina of SF-SCL cows compared with the LF-LCL cows, there was no effect of our endocrine model on the expression of any of the other five genes evaluated in vaginal tissue. The PGR gene expression in our study is in line with findings reported by Sağsöz *et al.* (2011), who reported that E2 and P4 receptors in the bovine cervix and vagina were higher during the follicular phase than the luteal phase. Such endocrine responsiveness of the vaginal transcriptome profile has been demonstrated previously in rats by Ohta *et al.* (1993), who showed that PGR levels were increased by E2 administration, but not by P4. From the results presented here, we concluded that periovulatory endocrine milieu-induced changes in the expression of genes in the endometrium are not similar to changes in the vagina.

In conclusion, the present study showed that the expression pattern of specific genes displays uterine inter- and intra-horn variations in response to periovulatory endocrine modifications, which indicates that the transcriptome signature of the bovine female uterus is endocrine- and location-dependent at Day 7 after oestrus, but that of the vagina is not. This implies that the model used in the present study might provide a useful tool to modulate and fine-tune, within physiological limits, the uterine 'receptive' environment. However, to which extent the regional changes observed in this study are critical for further embryo development and survival and, ultimately, pregnancy success, remains to be determined. Mechanisms that regulate regional expression of transcripts have not been elucidated, but may include vascular specialisations to deliver different sex-steroid concentrations to particular regions of the reproductive tract and specific intrinsic regional programming of expression across the uterine horn.

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