**CSIRO** PUBLISHING

*Reproduction, Fertility and Development*, 2016, **28**, 1642–1651
http://dx.doi.org/10.1071/RD14334

# Expression of leukaemia inhibitory factor at the conceptus–maternal interface during preimplantation development and in the endometrium during the oestrous cycle in the mare

*M. de Ruijter-Villani*[A,C], *C. Deelen*[A] *and T. A. E. Stout*[A,B]

[A]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 112, 3584 CM Utrecht, The Netherlands.
[B]Section of Reproduction, Faculty of Veterinary Science, University of Pretoria, Private Bag XO4, Onderstepoort, 0110, South Africa.
[C]Corresponding author. Email: m.villani@uu.nl

**Abstract.** Leukaemia inhibitory factor (LIF) plays a critical role in blastocyst development and implantation in several species. The present study investigated mRNA and protein expression for LIF, as well as the low-affinity LIF receptor (LIFR) and interleukin-6 signal transducer (IL6ST), in equine endometrium, trophoblast and histotroph during early pregnancy and in the endometrium during the oestrous cycle. Endometrial *LIF* mRNA expression was upregulated after Day 21 of pregnancy, whereas LIF immunoreactivity increased in the endometrium on Day 28. Expression of *LIF* mRNA in the yolk sac membrane increased from Day 21 of pregnancy, whereas LIF immunoreactivity increased from Day 28 in the trophoblast. *LIFR* and *IL6ST* mRNA was expressed in the endometrium during both the oestrous cycle and early pregnancy and, although LIFR and IL6ST protein were localised to the glandular epithelium during the cycle and first 14 days of pregnancy, from Day 21 they were located in the luminal epithelium. Trophoblast expression of LIFR and IL6ST increased as pregnancy proceeded. In conclusion, LIF expression increased at the conceptus–maternal interface during capsule attenuation. Because contemporaneous upregulation of both LIFR and IL6ST was also observed in the trophoblast, we propose that LIF plays an important role in the development of endometrial receptivity for trophoblast growth, apposition and adhesion in mares.

**Additional keywords:** early pregnancy, equine, histotroph.

Received 9 September 2014, accepted 17 March 2015, published online 17 April 2015

## Introduction

Early pregnancy loss is a common source of economic loss within the horse breeding industry. Indeed, recent surveys indicate that 15%–20% of pregnancies detected at Day 15 fail to survive to term (Ball 1988; Carnevale *et al.* 2000; Morris and Allen 2002; Allen *et al.* 2007) and that most of these losses occur between Days 15 and 42 of gestation (Morris and Allen 2002; Allen *et al.* 2007), that is, during the preimplantation period. The equine preimplantation period is characterised by several critical developmental processes, such as formation of the embryo proper, initial organogenesis and dissolution of the acellular blastocyst capsule (Days 20–22 of gestation) to finally allow direct contact between the trophoblast and the endometrium, and the formation of a stable attachment between the conceptus and the endometrium (Allen 2001). For most of this early intrauterine period, the conceptus is entirely dependent on progesterone produced by the primary corpus luteum (CL) for its survival. In particular, progesterone stimulates the endometrial glands to proliferate and produce the protein-rich histotroph ('uterine milk'; Kenney 1978; Clarke and Sutherland 1990). Histotrophic nutrition (i.e. the provision of nutrients through secretions produced by the uterine glands) represents the primary form of nutrition for the equine conceptus before the establishment of the definitive placenta between Days 40 and 45 (Kenney 1978; Clarke and Sutherland 1990). The exact composition of equine histotroph is not known; however, studies in other species indicate that uterine secretions contain not only nutrients, such as amino acids, glucose, fructose and vitamins, but also other substances, including mitogens, cytokines, lymphokines, enzymes, hormones, growth factors, proteases and protease inhibitors, that actively stimulate conceptus growth and development (Bazer *et al.* 2011). Both the transport and the *de novo* synthesis of histotroph components necessitate a fine-tuning of the transcription and translation of specific genes in the luminal and glandular endometrial epithelium (Filant and Spencer 2014). Although the expression of many of these genes

in the uterine epithelium is primarily progesterone dependent, available evidence suggests that progesterone stimulation alone is not sufficient to fully explain the regulation of endometrial expression of most of these genes, and that factors from the conceptus (e.g. oestrogens, cytokines, interferons and prostaglandins) are equally important in enhancing or tailoring their expression (Spencer *et al.* 2007; Bazer *et al.* 2010).

A better understanding of the endocrine and molecular mechanisms by which uterine secretions enhance growth, development and survival of the equine conceptus may improve methods for detecting and treating mares prone to early embryonic loss.

Leukaemia inhibitory factor (LIF) is a pleiotropic cytokine of the interleukin (IL)-6 family that signals via a heterodimeric receptor complex composed of the specific low-affinity LIF receptor (LIFR) associated with the common signalling component gp130 (also known as IL-6 signal transducer (IL6ST)); this receptor acts primarily through the Janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction pathway (Heinrich *et al.* 1998). The biological functions of LIF include stimulation of cell proliferation, differentiation and survival (Hilton 1992). In particular, LIF plays a critical role in murine blastocyst development and implantation, as demonstrated by the failure of wild-type embryos to survive in the uterus of LIF-knockout dams unless the latter receive LIF supplementation (Stewart *et al.* 1992). LIF has also been implicated in the establishment of uterine receptivity to implantation in women, primates, rodents, pigs, cattle and sheep (Anegon *et al.* 1994; Auernhammer and Melmed 2000; Kimber 2005). Moreover LIF has been shown to enhance adhesion of both endometrial epithelial cells and trophoblast cells to extracellular matrix via phosphorylation of STAT3 *in vitro* (Tapia *et al.* 2008; Marwood *et al.* 2009). LIF and its receptor have been detected in the endometrium and conceptus membranes of various species during the peri-implantation period (Charnock-Jones *et al.* 1994; Vogiagis *et al.* 1997; Modrić *et al.* 2000), but there are currently no reports of LIF expression during equine pregnancy. The aim of the present study was to examine the expression of LIF and the subunits of its receptor at the conceptus–maternal interface during the preimplantation period in the pregnant mare. In addition, to distinguish between the effects of pregnancy and progesterone alone, the expression of LIF and its receptor subunits was also investigated during the oestrous cycle.

## Materials and methods

### Animals

All animal procedures were approved by Utrecht University's Animal Experimentation Committee (permission no. 2007.III.02.036). A group of 18 fertile Warmblood mares aged 5–15 years was used in the present study. Mares were monitored during the oestrous cycle by transrectal palpation and ultrasonographic examination of the reproductive tract. During early oestrus, mares were examined three times a week and, once the dominant follicle exceeded 35 mm, the frequency of examination was increased to daily. When a pregnancy was required, oestrous mares with a follicle ≥35 mm in diameter were inseminated with a minimum of $500 \times 10^6$ progressively motile spermatozoa from a single stallion of proven fertility. Daily monitoring was then continued until ovulation was detected by the disappearance of the preovulatory follicle and replacement by a CL or corpus haemorrhagicum. Insemination was repeated at 48-h intervals until ovulation. The day on which ovulation was first detected was recorded as Day 0 of the cycle. An 'oestrous mare' was defined as a mare showing obvious signs of oestrus together with an ovarian follicle ≥35 mm in diameter and uterine oedema. Pregnancy status was diagnosed before tissue collection by transrectal ultrasonographic detection of the conceptus vesicle during Days 14–28; on Day 7 after ovulation, pregnancy was confirmed when an embryo was recovered by standard non-surgical uterine lavage (Stout 2006). None of the mares was used more than once within a single experimental group and, after recovery of an endometrial biopsy, mares were allowed a minimum of two cycles rest before being re-used.

### Histotroph

Aspiration of the histotroph was performed using a strobed-light video endoscope and a polytetrafluoroethylene (PTFE) cannula connected to a 20-mL syringe. Histotroph collection ($n = 4$ mares per group) was performed in cycling mares on Days 14 and 21 after ovulation (the latter stage corresponding to late oestrous) and in pregnant mares on Days 14, 21 and 28 of gestation, before recovery of the endometrial biopsy samples and, in pregnant mares, the conceptus. After recovery, the histotroph (~10 μL) was frozen and stored at −80°C.

### Tissue collection

Endometrial biopsies were harvested from mares ($n = 4$ mares per group) during the oestrous cycle on Days 7, 14 and 21 after ovulation (the latter stage corresponding to late oestrous), and in pregnant mares on Days 7, 14, 21 and 28. Endometrial tissue was recovered from the base of one uterine horn using crocodile biopsy forceps; in the case of Day 21 and 28 pregnant mares, biopsy recovery was guided video-endoscopically to ensure recovery of endometrium that had been in apposition to the conceptus. After recovery, endometrial biopsies were washed 10 times in serum-free phosphate-buffered saline (PBS) to remove any contaminants.

Conceptuses were recovered from Day 7 pregnant mares ($n = 4$ conceptuses per group) by standard non-surgical uterine lavage using 3 × 1 L Dulbecco's PBS supplemented with 0.5% (v/v) heat-inactivated fetal bovine serum. Day 14, 21 and 28 conceptuses ($n = 4$ conceptuses per group) were harvested before endometrial biopsy recovery as described previously (Stout and Allen 2002). Briefly a fresh sharpened PTFE cannula was used to puncture the membranes and aspirate yolk sac (Days 14, 21, 28) and allantoic (Day 28) fluids. Following puncture and aspiration, the conceptus membranes were recovered using a sterile, disposable transendoscopic net. After recovery, all conceptuses were washed 10 times in serum-free PBS to remove contaminating maternal cells. Day 7 conceptuses were snap-frozen and stored at −80°C for subsequent RNA extraction. In the case of Day 14 and 21 conceptuses, the blastocyst capsule was removed and the trilaminar

**Table 1. Nucleotide sequences of primers for candidate reference ('housekeeping') genes and target genes used for quantitative real-time polymerase chain reaction**

$T_a$, annealing temperature; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HPRT1*, hypoxanthine phosphoribosyltransferase I; *PGK1*, phosphoglycerate kinase 1; *RPL32*, ribosomal protein 32; *SRP14*, signal recognition particle 14 kDa; *LIF*, leukaemia inhibitory factor; *LIFR*, low-affinity LIF receptor; *IL6ST*, interleukin-6 signal transducer

| Gene | Sequence | $T_a$ (°C) | Amplicon size (bp) | GenBank Accession no. |
|---|---|---|---|---|
| *GAPDH* | F: 5′-AGGCCATCACCATCTTCCAG-3′<br>R: 5′-CCAGCCTTCTCCAAGGTAGT-3′ | 53 | 112 | NM_001163856.1 |
| *HPRTI* | F: 5′-GAGATGTGATGAAGGAGATGG-3′<br>R: 5′-CTTTCCAGTTAAAGTTGAGAGG-3′ | 58 | 232 | XM_005614512.1 |
| *PGK1* | F: 5′-CTGTGGGTGTATTTGAATGG-3′<br>R: 5′-GACTTTATCCTCCGTGTTCC-3′ | 54 | 151 | XM_005614287.1 |
| *RPL32* | F: 5′-TGATACCAATGGAAGTAAGGAG-3′<br>R: 5′-ATAGCAATAGCCACAAAGGAC-3′ | 60 | 201 | XM_001495244.2 |
| *SRP14* | F: 5′-CTGAAGAAGTATGACGGTCG-3′<br>R: 5′-CCATCAGTAGCTCTCAACAG-3′ | 55 | 101 | XM_001503583.3 |
| *LIF* | F: 5′-GGAGTTGTGCCCCTGCTGCTA-3′<br>R: 5′-CGTGGGAAAGGGCGGGAAGTC-3′ | 65 | 264 | XM_003365488.2 |
| *LIFR* | F: 5′-GTTTCCTTAATTCCAGACACTC-3′<br>R: 5′-CAACGTAGCATCTAATTCCC-3′ | 58 | 279 | XM_005604303.1 |
| *IL6ST* | F: 5′-GCACTGTTGATTATTCTCCTG-3′<br>R: 5′-GTTGAAGCATCTTTGGTCCT-3′ | 62 | 258 | XM_005604259.1<br>XM_005604258.1<br>XM_001495796.4<br>XM_005604257.1<br>XM_005604256.1<br>XM_005604255.1 |

omphalopleure–embryonic disc region was divided from the bilaminar trophoblast (BT) using microsurgical scissors; for Day 21 conceptuses, the embryo proper (EP) was also isolated if it showed signs of detaching from the membranes. For Day 28 conceptuses, the chorioallantois (CA) and yolk sac (YS) portions of the membranes and the EP were separated using microsurgical scissors. Each endometrial biopsy, BT, CA or YS recovered was dissected into two pieces. One piece was snap-frozen and stored at −80°C for RNA extraction and the other piece was fixed in 4% paraformaldehyde overnight before being embedded in paraffin in preparation for immunohistochemistry.

### *RNA extraction and cDNA synthesis*

Isolation of total RNA and on-column DNAse digestion was performed using the Invisorb Spin Cell RNA Mini Kit (Invitek, Berlin, Germany) combined with the RNAse-free DNAse set (Qiagen, Valencia, CA, USA). Each sample was lysed in 700 μL lysis buffer and applied to the DNA-binding spin filter. After incubation for 2 min and centrifugation for 2 min at 11 000*g* all at room temperature, the binding filter containing DNA was discarded and the RNA-containing lysate was diluted (1 : 1) with 70% ethanol and pipetted directly onto an RNA-binding filter. After the column had been washed twice with washing buffer, the RNA-binding filter was incubated with RNAse-free DNAse for 15 min at room temperature. After three further washes with washing buffer, the RNA was eluted from the RNA-binding filter with 33 μL RNA elution buffer.

The quantity and quality of total RNA were determined spectrophotometrically using an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA) with an RNA 6000 Nano Labchip kit (Agilent), in accordance with the manufacturer's instructions. Only samples with a RNA Integrity Number (RIN) of 7.5 or greater were used for analysis. Reverse transcription (RT) was performed in a total volume of 40 μL made up of 20 μL sample containing 1000 ng RNA, 8 μL of 5× RT buffer (Invitrogen, Breda, The Netherlands), 16 U RNAsin (Promega, Leiden, The Netherlands), 300 U Superscript II reverse transcriptase (Invitrogen), 1.2 μg random primers (Invitrogen), 10 mM dithiothreitol (Invitrogen) and 0.5 mM of each dNTP (Promega). The mixture was incubated for 5 min at 70°C, 1 h at 42°C and 5 min at 80°C before being stored at −20°C. Minus RT (–RT) blanks were prepared from 10 μL sample containing 500 ng RNA under the same conditions, but in the absence of reverse transcriptase.

### *Quantitative real-time polymerase chain reaction primer design*

The primer pairs for candidate reference (housekeeping) and target genes used for quantitative real-time polymerase chain reaction (PCR) are listed in Table 1. The primer pairs were designed using Primer Designer version 2.0 (Scientific and Educational Software, Cary, NC, US) on the equine coding sequence; where possible, each primer of a pair was located on a separate gene exon. The specificity of the primers was screened *in silico* using NCBI Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed 30 March 2015). A standard sequencing procedure (ABI PRISM 310 Genetic analyzer; Applied Biosystem, Foster City, CA, USA) was used to verify the specificity of the PCR products of each target gene.

### *Quantitative real-time PCR*

For each gene of interest, quantitative real-time PCR was performed on two replicates of cDNA, a single –RT blank and a water blank. Simultaneous quantification of all samples in a 96-well plate was performed using a quantitative real-time PCR detection system (MyIQ Single-Colour Real-Time PCR Detection System; Bio-Rad Laboratories, Veenendaal, The Netherlands). Standard curves were created using 10-fold serial dilutions of known amounts of target gene PCR product, to quantify expression. The quantitative real-time PCR reaction mixture (25 μL) contained 1 μL sample cDNA solution, 0.5 mM of each primer (Isogen Bioscience, Maarssen, The Netherlands) and 12.5 μL IQTMSybr Green Supermix (Bio-Rad Laboratories). Initial DNA denaturation at 95°C for 5 min was followed by 40 cycles consisting of 95°C for 15 s, the primer-specific annealing temperature (see Table 1) for 30 s and 72°C for 45 s. To verify the purity of the product after amplification, melting curves were plotted. The MyIQ analysis program (Bio-Rad Laboratories) was used to analyse quantitative real-time PCR results. Standard curves were produced by plotting the logarithm of the starting amount versus the quantification cycle (Cq) for detection. Only plates with standard curves reaching 0.95 (95%) or higher efficiency were included in the analysis.

### *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis*

To verify the presence of LIF in undiluted histotroph from pregnant (Day 14, 21 and 28 after ovulation) and non-pregnant (Day 14 and 21 after ovulation) mares, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were performed. Equine endometrium was used as control tissue and, for this purpose, 100 μg was lysed in 125 mL RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1% phenylmethylsulphonyl fluoride solution, 1% protease inhibitor solution, 1% sodium orthovanadate solution in Tris-buffered saline (TBS); Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cellular lysis was enhanced by repeated snap-freezing in liquid nitrogen and manual crushing, after which the lysate was centrifuged at 10 000*g* for 5 min at 4°C and the supernatant diluted 1 : 3 in sample buffer (62.5 mM Tris, 2% SDS, 0.1% glycerol, 0.05% β-mercaptoethanol and 0.006% bromophenol blue) and boiled for 5 min. For each sample, 5 μL undiluted histotroph was similarly diluted 1 : 3 in sample buffer and boiled for 5 min; thereafter, the proteins were separated on a 10% SDS–polyacrylamide gel, and transferred to nitrocellulose membranes (Trans-Blot1; Bio-Rad Laboratories). The membranes were rinsed in Tris-buffered saline with 0.05% Tween-20 (TBST; ICN, Aurora, OH, USA) and blocked with 5% non-fat dry milk in TBST (blocking buffer) for 1 h, followed by overnight incubation at 4°C with the first antibody (rabbit polyclonal antibody raised against human LIF; HPA018844; Sigma-Aldrich, St Louis, MO, USA) diluted 1 : 500 in blocking buffer. After three washes with blocking buffer, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (31460; Pierce Biotechnology, Rockford, IL, USA) diluted 1 : 10 000 in blocking buffer. Subsequently, membranes were washed four times in blocking buffer, three times in TBST and once in TBS; this was followed by visualisation of the antibody–protein complex using Immun-Star1 chemiluminescent substrate (Bio-Rad Laboratories) and exposure to X-ray film (Fuji, Düsseldorf, Germany). Bands of interest obtained by western blot analysis were analysed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All band intensities were normalised with respect to the control.

### *Immunohistochemistry*

Immunohistochemical staining was performed using the avidin-biotin-peroxidase complex (ABC–PO) procedure (DAKO, Hamburg, Germany). For each tissue collected, 5-μm sections were deparaffinised and rehydrated in xylene (2 × 5 min) followed by 100% ethanol, 96% ethanol, 70% ethanol and double-distilled water (2 × 3 min each). The sections were then rinsed (3 × 5 min) in 1% Tween 20–PBS (pH 7.6) and endogenous peroxidase activity was blocked by immersing the sections in 1% $H_2O_2$ in methanol for 30 min at room temperature. After washing in 1% Tween 20–PBS (3 × 5 min), sections were incubated with normal goat serum (1 : 10 dilution in PBS; X0907; DAKO) for 15 min at room temperature to reduce non-specific binding before being incubated for 16 h at 4°C with the primary rabbit polyclonal antibody raised against human LIF (1 : 400 dilution; HPA018844; Sigma-Aldrich), human LIFR (1 : 400 dilution; SC-659; Santa Cruz Biotechnology) or human IL6ST (1 : 50 dilution; 06-291; Upstate, Lake Placid, NY, USA). After washing in 1% Tween 20–PBS, slides were incubated with a biotinylated goat-anti-rabbit secondary antibody (1 : 250 dilution; BA-1000; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. The sections were then washed with PBS (3 × 5 min), incubated with ABC/PO complex (Vectastain, Vector Laboratories) for 30 min at room temperature and then washed again in PBS (3 × 5 min). Binding sites were visualised using 3-amino-9-ethylcarbazole (AEC + High Sensitivity; K3469; Dako, Glostrup, Denmark) for 15 min. The slides were then washed for 5 min under running tap water before nuclei were counterstained with Mayer's haematoxylin (30 s). After a final 10 min rinse under running tap water, the sections were sealed under a coverslip using Aquamount Mounting Medium (Polysciences, Eppelheim, Germany). As a negative control, the primary antibody was substituted with rabbit serum blocking solution. The preparations were examined at a magnification of ×200, and photographs were taken using a digital camera connected to a microscope (BX41; Olympus Nederland, Rotterdam, The Netherlands) using Colour View (Soft Imaging System; Olympus Nederland).

### *Statistical analysis*

Quantitative real-time PCR data were analysed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). To obtain continuous, normally distributed datasets, the relative starting quantities were subjected to natural logarithmic transformation. Endometrial biopsy data were analysed using a two-way between-groups ANOVA, followed by a post hoc Tukey test, in order to explore the impact of pregnancy status and stage of

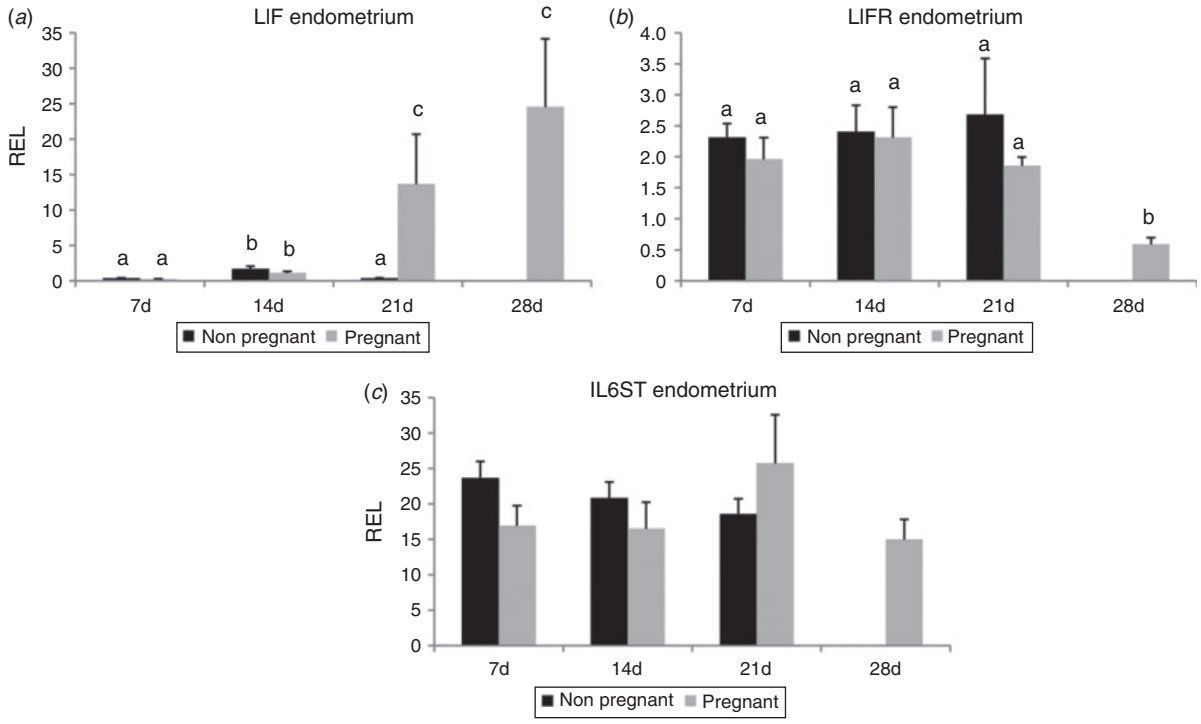


**Fig. 1.** Relative endometrial mRNA expression of (*a*) leukaemia inhibitory factor (LIF), (*b*) low-affinity LIF receptor (LIFR) and (*c*) interleukin-6 signal transducer (IL6ST) during the oestrous cycle and early pregnancy in the mare. 7d, Day 7 after ovulation; 14d, Day 14 after ovulation; 21d, Day 21 after ovulation; 28d, Day 28 after ovulation. Data are the mean ± s.e.m. ($n = 4$ mares per group). Columns with different letters differ significantly ($P < 0.05$). REL, relative mRNA expression.

pregnancy and/or cycle on the relative starting quantity for each target gene. When a significant result for the interaction effect was found, an analysis of simple effects was conducted by running separate one-way ANOVAs. Conceptus gene expression data were analysed using a one-way ANOVA, followed by a post hoc Tukey test in order to evaluate the effect of stage of pregnancy on the relative starting quantities for each target gene. Western blot densitometric data were analysed using a two-way between-groups ANOVA, followed by a post hoc Tukey test to evaluate the effects of pregnancy status and stage of pregnancy and/or cycle. Statistical significance was set at $P < 0.05$.

## Results

### *Selection of reference genes*

To normalise gene expression levels for comparative purposes, the stability of expression (M) of five potential reference genes, namely glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), signal recognition particle 14 kDa (*SRP14*), phosphoglycerate kinase 1 (*PGK1*), ribosomal protein 32 (*RPL32*) and hypoxanthine phosphoribosyltransferase I (*HPRT1*), was determined using Genorm analysis of mRNA expression in all experimental samples, as described by Vandesompele *et al.* (2002). The average M-value was recalculated serially after stepwise exclusion of the least stable gene, and the genes then ranked by expression stability. To evaluate the optimum number of reference genes for normalisation purposes, the effect of stepwise inclusion of less stable genes was then examined. For the endometrium, inclusion of a fourth gene, and for conceptus membranes the inclusion of a third gene, did not significantly affect the normalisation factor (Vandesompele *et al.* 2002), therefore the geometric mean of the expression levels for *GAPDH*, *SRP14* and *RPL32* was used for normalising the starting quantities of the target genes for the endometrial samples and the geometric mean of *GAPDH* and *PGK1* was used to normalise the starting quantities for the conceptus samples. Quantitative real-time PCR revealed mRNA expression for both LIF and both subunits contributing to the LIF receptor (*LIFR* and *IL6ST*) in all endometrial (Fig. 1) and conceptus (Fig. 2) samples. Amplification of the –RT blanks with gene-specific primers did not result in measurable amounts of product for any of the quantitative PCR studies.

### *LIF expression*

Endometrial expression of *LIF* mRNA was influenced by stage of pregnancy and/or cycle ($P < 0.001$; partial Eta squared = 0.809), by pregnancy status ($P < 0.005$; partial Eta squared = 0.337) and by the interaction of these two factors ($P < 0.001$; partial Eta squared = 0.685). In the cycling mare, *LIF* mRNA showed fivefold upregulation ($P < 0.05$) at Day 14 after ovulation compared with oestrus (Day 21 after ovulation; Fig. 1*a*). Similarly, during pregnancy *LIF* mRNA was upregulated fivefold on Day 14 compared with Day 7 after ovulation

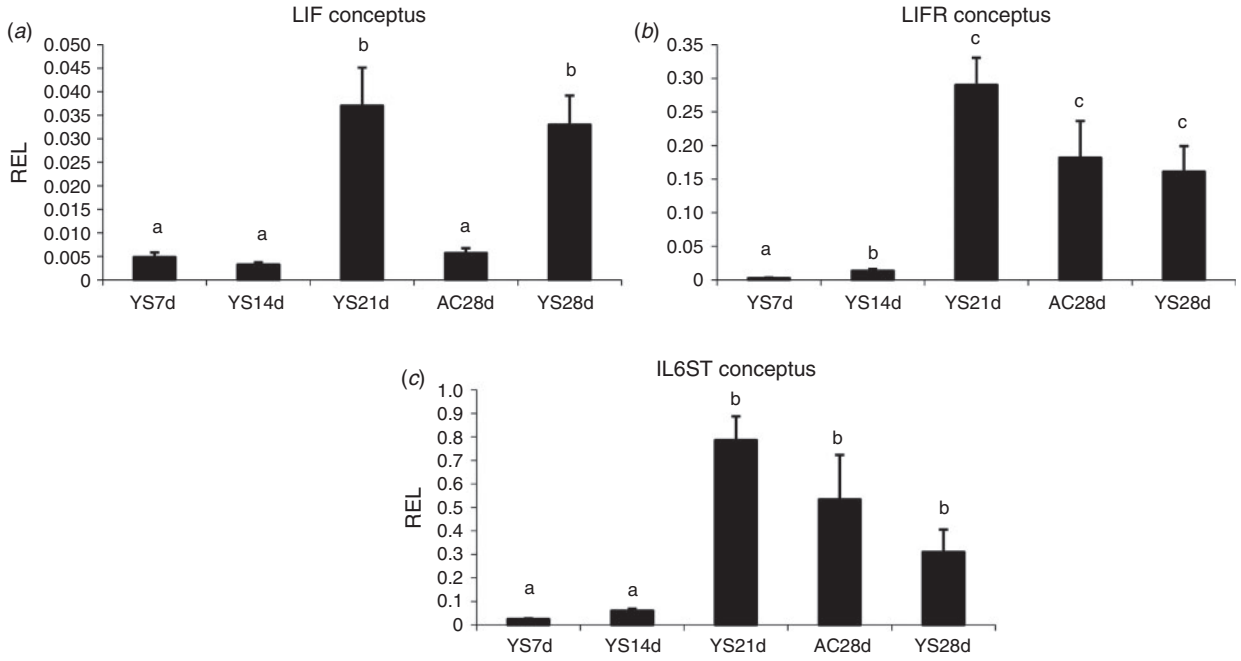


**Fig. 2.** Relative mRNA expression of (*a*) leukaemia inhibitory factor (LIF), (*b*) low-affinity LIF receptor (LIFR) and (*c*) interleukin-6 signal transducer (IL6ST) in equine conceptus membranes. YS7d, Day 7 yolk sac; YS14d, Day 14 yolk sac; YS21d, Day 21 yolk sac; AC28d, Day 28 allantochorion; YS28, Day 28 yolk sac. Data are the mean ± s.e.m. ($n = 4$ conceptuses per group). Columns with different letters differ significantly ($P < 0.05$). REL, relative mRNA expression.

($P < 0.05$; Fig. 1*a*). *LIF* mRNA expression showed a further and more marked increase in expression of 59- and 106-fold on Days 21 and 28 of pregnancy, respectively ($P < 0.005$; Fig. 1*a*). Lagging slightly behind the upregulation in gene expression, the amount of LIF protein present in the histotroph increased significantly between Days 21 and 28 of gestation, whereas no significant changes in LIF protein abundance were detectable in the other stages of the cycle and/or pregnancy studied (Fig. 3). Immunohistochemical staining of formalin-fixed endometrial samples revealed that LIF was localised primarily in the cytoplasm of glandular epithelial cells and superficial stromal cells during both the cycle and early pregnancy. LIF immunoreactivity was low during oestrus and early pregnancy, but increased markedly on Day 28 of pregnancy (Fig. 4).

The expression of *LIF* mRNA in the conceptus membranes increased by a factor of 11 ($P < 0.001$) between Days 14 and 21 of pregnancy (Fig. 2*a*). *LIF* gene expression remained high in the yolk sac membrane of Day 28 conceptuses ($P < 0.001$), whereas Day 28 allantochorion showed a similar level of *LIF* mRNA expression to that of Day 7 and Day 14 trophectoderm (Fig. 2*a*). Immunohistochemical staining of formalin-fixed conceptus membranes revealed that LIF was undetectable in Day 14 trophectoderm and only weakly expressed in the endoderm of Day 21 conceptuses (Fig. 5). By Day 28, conceptus LIF immunoreactivity was more obvious and localised specifically in the mesoderm and endoderm of both allantochorion and yolk sac (Fig. 5).

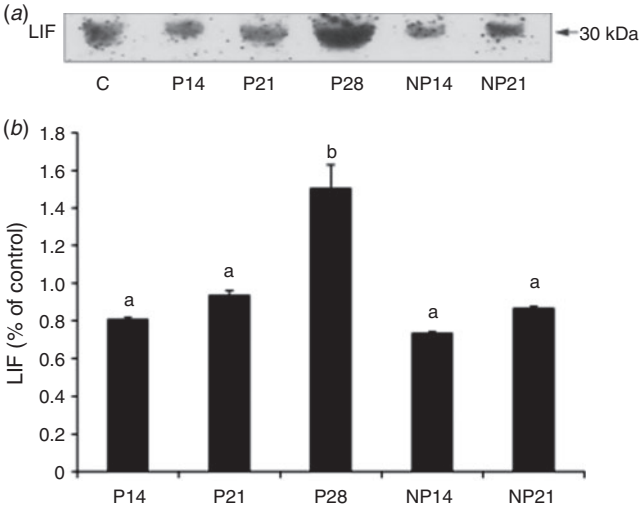


**Fig. 3.** Western blot analysis of leukaemia inhibitory factor (LIF) in equine histotroph (uterine secretions). (*a*) Representative western blot. Bands of ~30 kDa are consistent with glycosylated LIF. Lane 1, positive control (C; equine endometrium); Lanes 2–4, histotroph from Days 14, 21 and 28 of pregnancy (P14, P21 and P28, respectively); Lanes 5, 6, histotroph from Days 14 (dioestrus; NP14) and 21 (oestrus; NP21) of the oestrous cycle. (*b*) Densitometric analysis of LIF abundance in western blots expressed as a percentage of values for corresponding control tissue sample. Data are the mean ± s.e.m. ($n = 3$). Columns with different letters differ significantly ($P < 0.05$).

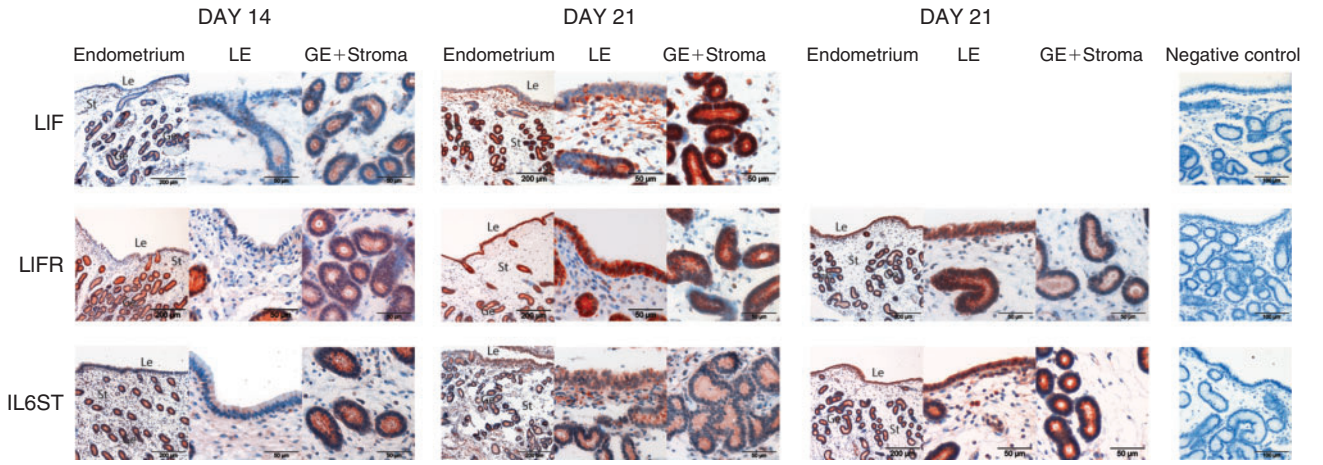


**Fig. 4.** Immunohistochemical localisation of leukaemia inhibitory factor (LIF), low-affinity LIF receptor (LIFR) and interleukin-6 signal transducer (IL6ST) in equine endometrium and negative controls. Sections are counterstained with aqueous haematoxylin. LE, luminal epithelium; GE, glandular epithelium; St, stroma. Note that LIFR and IL6ST are strongly expressed in the cytoplasm of the LE only from Day 21 of pregnancy.

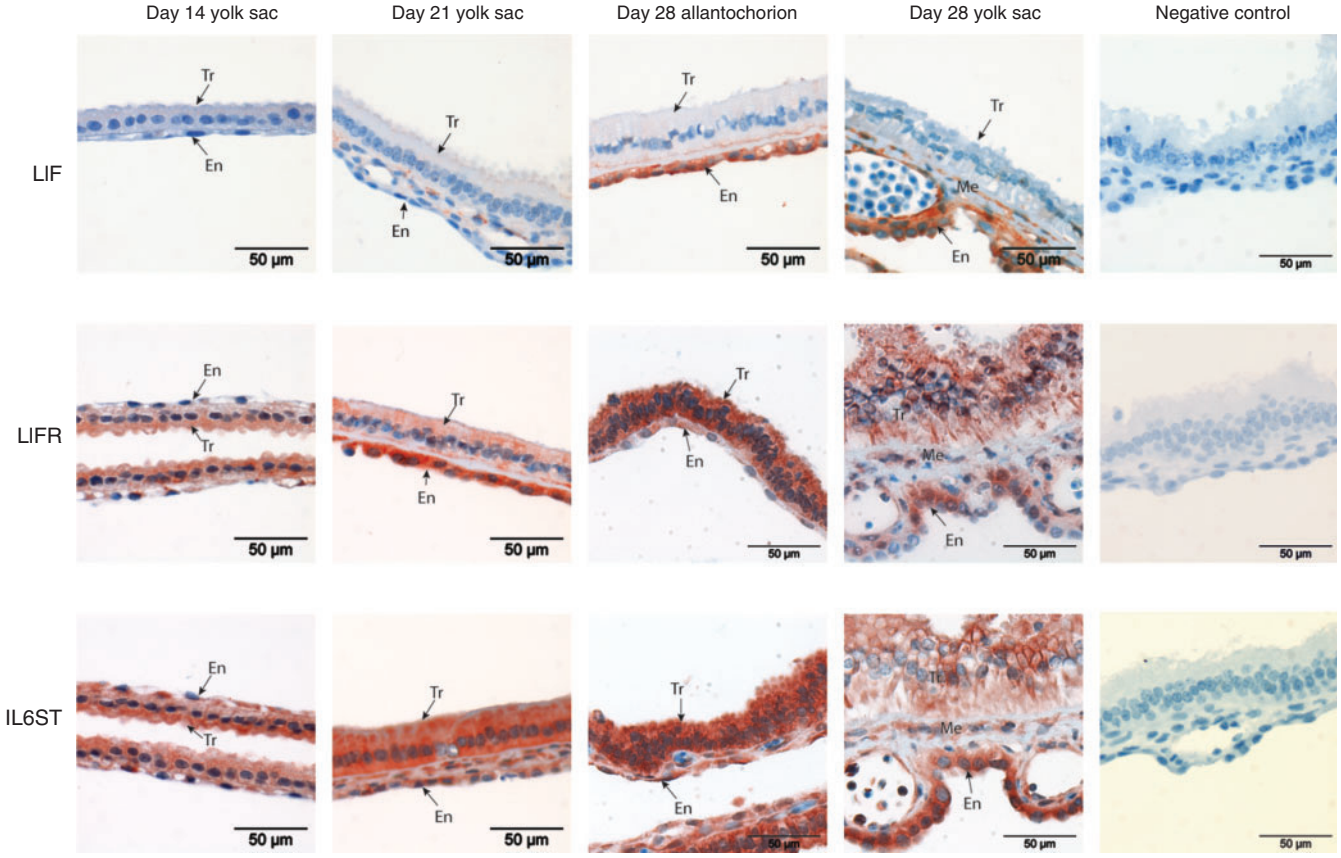


**Fig. 5.** Immunohistochemical localisation of leukaemia inhibitory factor (LIF), low-affinity LIF receptor (LIFR) and interleukin-6 signal transducer (IL6ST) in equine conceptus membranes and negative controls. Sections are counterstained with aqueous haematoxylin. Tr, trophectoderm; En, endoderm; Me, mesoderm.

### *LIFR and IL6ST expression*

Endometrial expression of *LIFR* mRNA was influenced by the stage of the cycle and/or pregnancy ($P < 0.001$; partial Eta squared = 0.56), but not by pregnancy status. *LIFR* gene expression was constant during the cycle and early pregnancy, but exhibited a fourfold downregulation ($P < 0.001$) in endometrium from Day 28 of pregnancy (Fig. 1*b*). *IL6ST* mRNA expression was constant in the endometrium at all stages of the

oestrous cycle and pregnancy examined (Fig. 1*c*). Immunohistochemical staining of formalin-fixed endometrial samples indicated that LIFR and IL6ST were localised specifically in the cytoplasm of glandular epithelial cells during the cycle and on Days 7 and 14 of pregnancy (Fig. 4). However, from Day 21 of pregnancy, both LIFR and IL6ST were also expressed in the cytoplasm of luminal epithelial cells (Fig. 4). Moreover, IL6ST was visible in the cytoplasm of plasma cells. Although IL6ST immunoreactivity appeared constant during the cycle and early pregnancy, LIFR staining appeared to be less intense on Day 28 than Day 21 of pregnancy. LIFR immunoreactivity was constant at all other stages.

In yolk sac, *LIFR* mRNA exhibited fivefold upregulation ($P < 0.01$) on Day 14 compared with Day 7 (Fig. 2*b*). Moreover *LIFR* gene expression showed a further 21-fold increase on Day 21 of pregnancy ($P < 0.001$), and then remained high in both the allantochorion and yolk sac of Day 28 conceptuses (Fig. 2*b*). The expression of *IL6ST* mRNA increased after Day 21 in the conceptus membranes ($P < 0.05$; Fig. 2*c*). Immunohistochemical staining of formalin-fixed conceptus membrane samples demonstrated that LIFR and IL6ST were expressed in the cytoplasm of trophectoderm and endoderm cells at all stages studied (Fig. 5). The mesoderm of Day 21 and Day 28 conceptus membranes also showed cytoplasmic LIFR and IL6ST expression. Both LIFR and IL6ST immunoreactivity appeared to increase after Day 21 of pregnancy.

## Discussion

In the present study we demonstrated that intrauterine LIF expression in the horse increases during the period of blastocyst capsule disintegration, when direct contact between the trophectoderm and endometrial epithelium is first permitted and the apposition and adhesion that characterise implantation can begin in earnest. The upregulation of *LIF* mRNA expression observed in the endometrium during the mid-luteal phase of the oestrous cycle is similar to previous reports in women, where LIF expression is progesterone dependent (Dimitriadis *et al.* 2010). It therefore seems reasonable to speculate that the moderate increase in *LIF* mRNA seen in equine endometrium on Day 14 of both pregnancy and the oestrous cycle is also progesterone dependent and is related to endometrial receptivity to pregnancy (i.e. preparation of the endometrium for its role in the implantation process). Conversely, the more marked upregulation in endometrial LIF mRNA expression detected from Day 21 of pregnancy, and followed by a marked increase in LIF protein abundance in both endometrial cells and the histotroph on Day 28, is clearly pregnancy dependent and, given that it coincides with the loss of continuity of the blastocyst capsule (Days 18–22; Arar *et al.* 2007) and the onset of direct contact between the endometrium and the conceptus trophectoderm, is presumably stimulated by paracrine factors secreted by the trophectoderm cells. Similar to other species, endometrial LIF expression in the mare was localised primarily in the superficial glandular epithelium and was only moderately abundant in the stroma (Modrić *et al.* 2000; Rao *et al.* 2008; Song *et al.* 2009; Dimitriadis *et al.* 2010). It is therefore reasonable to conclude that, as in the sheep (Song *et al.* 2009), the superficial glandular epithelium is the main source of endometrial LIF during early equine pregnancy. As in rodent species, the rabbit, cow and women (Nichols *et al.* 1996; Eckert and Niemann 1998; Chen *et al.* 1999; Lei *et al.* 2004; Rao *et al.* 2008), the preimplantation equine conceptus was also shown to produce LIF. Moreover, the localisation of LIF to the yolk sac suggests a paracrine regulation of embryonic disc development by yolk sac-derived LIF, as has been described in the mouse (Nichols *et al.* 1996).

The difference in expression of *LIFR* and *IL6ST* mRNA in the endometrium of cyclic and pregnant mares could be attributable to the fact that although the LIFR subunit contributes exclusively to the LIF receptor, the IL6ST subunit is incorporated not only into the LIF receptor, but also receptors for other members of the IL-6 family (Heinrich *et al.* 1998). As described in other species, endometrial LIFR and IL6ST were mainly localised in the luminal and glandular epithelium during both the oestrous cycle and early pregnancy (Cheng *et al.* 2001; Song and Lim 2006; Song *et al.* 2009). Interestingly, in the mare, the immunoreactivity for both LIFR and IL6ST translocated from the glandular to the luminal epithelium in the period coincident with blastocyst capsule disintegration (i.e. from Day 21 of gestation); this may indicate autocrine or paracrine regulation of LIF at the conceptus–endometrium interface as soon as apposition between the endometrium and trophectoderm is possible. Similar to women, IL6ST protein is expressed in plasma cells present within the endometrium of mares (Burger *et al.* 2001). The expression of both LIFR and IL6ST in the membranes of peri-implantation horse conceptuses is temporally consistent with a role in the preparation for placentation, as proposed in other species (Bhatt *et al.* 1991; Anegon *et al.* 1994; Charnock-Jones *et al.* 1994; Vogiagis *et al.* 1997; Modrić *et al.* 2000). LIF has been reported to enhance trophectoderm outgrowth and stimulate both trophoblast cells and epithelial endometrial cells to adhere to extracellular matrix in mouse and humans (Cai *et al.* 2000; Tapia *et al.* 2008; Marwood *et al.* 2009). Although the implantation process in the horse (non-invasive) differs considerably to that in both mice and women (invasive), the initial interactions between endometrial epithelium and trophectoderm (i.e. modification of surface extracellular matrix to promote apposition and adhesion) are well conserved between species and modes of implantation (Bazer *et al.* 2010). That the pattern of LIF expression shows clear similarities between the mare and species with an invasive mode of implantation suggests that LIF is primarily involved in stimulating conceptus development and preparing both endometrial epithelium and trophectoderm for their roles in apposition and adhesion; however, it is also possible that LIF plays a role in preparing the endometrium and/or trophectoderm for invasion of either the entire conceptus (rodents, women) or the chorionic girdle cells (mare).

In conclusion, the expression of LIF in equine endometrium increases during the luteal phase of the oestrous cycle and during the course of early pregnancy; an even greater increase is seen in the endometrium, conceptus membranes and histotroph in the period of glycoprotein capsule attenuation and loss of continuity (Days 18–22 of gestation). Because contemporaneous upregulation of the expression of LIFR and IL6ST was also observed in the trophoblast, we propose that LIF plays an important role in

both the (progesterone-dependent) development of endometrial receptivity for implantation and in stimulating trophoblast–endometrium apposition and adhesion in the period immediately following dissolution of the blastocyst capsule, as well as in preparation for either placentation and/or chorionic girdle cell invasion.

## Acknowledgements

The authors thank W. K. Hendriks, J. de Rijk and F. Mosel for help with the collection of tissue samples and B. Vriend and D. Paris for their help designing and testing the quantitative real-time PCR primers.

## References

Allen, W. R. (2001). Fetomaternal interactions and influences during equine pregnancy. *Reproduction* **121**, 513–527. doi:10.1530/REP.0.1210513

Allen, W. R., Brown, L., Wright, M., and Wilsher, S. (2007). Reproductive efficiency of Flatrace and National Hunt thoroughbred mares and stallions in England. *Equine Vet. J.* **39**, 438–445. doi:10.2746/042516407X1737581

Anegon, I., Cuturi, M. C., Godard, A., Moreau, M., Terqui, M., Martinat-Botte, F., and Soulillou, J. P. (1994). Presence of leukaemia inhibitory factor and interleukin 6 in porcine uterine secretions prior to conceptus attachment. *Cytokine* **6**, 493–499. doi:10.1016/1043-4666(94)90076-0

Arar, S., Chan, K. H., Quinn, B. A., Waelchli, R. O., Hayes, M. A., Betteridge, K. J., and Monteiro, M. A. (2007). Desialylation of core type 1 O-glycan in the equine embryonic capsule coincides with immobilization of the conceptus in the uterus. *Carbohydr. Res.* **342**, 1110–1115. doi:10.1016/J.CARRES.2007.02.016

Auernhammer, C. J., and Melmed, S. (2000). Leukemia-inhibitory factor: neuroimmune modulator of endocrine function. *Endocr. Rev.* **21**, 313–345.

Ball, B. A. (1988). Embryonic loss in mares. Incidence, possible causes, and diagnostic considerations. *Vet. Clin. North Am. Equine Pract.* **4**, 263–290.

Bazer, F. W., Wu, G., Spencer, T. E., Johnson, G. A., Burghardt, R. C., and Bayless, K. (2010). Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol. Hum. Reprod.* **16**, 135–152. doi:10.1093/MOLEHR/GAP095

Bazer, F. W., Spencer, T. E., Johnson, G. A., and Burghardt, R. C. (2011). Uterine receptivity to implantation of blastocysts in mammals. *Front. Biosci. (Schol. Ed.)* **S3**, 745–767. doi:10.2741/S184

Bhatt, H., Brunet, L. J., and Stewart, C. L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc. Natl Acad. Sci. USA* **88**, 11 408–11 412. doi:10.1073/PNAS.88.24.11408

Burger, R., Guenther, A., Bakker, F., Schmalzing, M., Bernand, S., Baum, W., Duerr, B., Hocke, G. M., Steininger, H., Gebhart, E., and Gramatzki, M. (2001). Gp130 and ras mediated signaling in human plasma cell line INA-6: a cytokine-regulated tumor model for plasmacytoma. *Hematol. J.* **2**, 42–53. doi:10.1038/SJ.THJ.6200075

Cai, L. Q., Cao, Y. J., and Duan, E. K. (2000). Effects of leukaemia inhibitory factor on embryo implantation in the mouse. *Cytokine* **12**, 1676–1682. doi:10.1006/CYTO.2000.0758

Carnevale, E. M., Ramirez, R. J., Squires, E. L., Alvarenga, M. A., Vanderwall, D. K., and McCue, P. M. (2000). Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. *Theriogenology* **54**, 965–979. doi:10.1016/S0093-691X(00)00405-2

Charnock-Jones, D. S., Sharkey, A. M., Fenwick, P., and Smith, S. K. (1994). Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J. Reprod. Fertil.* **101**, 421–426. doi:10.1530/JRF.0.1010421

Chen, H.-F., Shew, J.-Y., Ho, H.-N., Hsu, W.-L., and Yang, Y.-S. (1999). Expression of leukemia inhibitory factor and its receptor in preimplantation embryos. *Fertil. Steril.* **72**, 713–719. doi:10.1016/S0015-0282(99)00306-4

Cheng, J.-G., Chen, J. R., Hernandez, L., Alvord, W. G., and Stewart, C. L. (2001). Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proc. Natl Acad. Sci. USA* **98**, 8680–8685. doi:10.1073/PNAS.151180898

Clarke, C. L., and Sutherland, R. L. (1990). Progestin regulation of cellular proliferation. *Endocr. Rev.* **11**, 266–301. doi:10.1210/EDRV-11-2-266

Dimitriadis, E., Menkhorst, E., Salamonsen, L. A., and Paiva, P. (2010). Review: LIF and IL11 in trophoblast–endometrial interactions during the establishment of pregnancy. *Placenta* **31**, S99–S104. doi:10.1016/J.PLACENTA.2009.12.027

Eckert, J., and Niemann, H. (1998). mRNA expression of leukaemia inhibitory factor (LIF) and its receptor subunits glycoprotein 130 and LIF-receptor-β in bovine embryos derived *in vitro* or *in vivo*. *Mol. Hum. Reprod.* **4**, 957–965. doi:10.1093/MOLEHR/4.10.957

Filant, J., and Spencer, T. E. (2014). Uterine glands: biological roles in conceptus implantation, uterine receptivity and decidualization. *Int. J. Dev. Biol.* **58**, 107–116. doi:10.1387/IJDB.130344TS

Heinrich, P. C., Behrmann, I., Müller-Newen, G., Schaper, F., and Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* **334**, 297–314.

Hilton, D. J. (1992). LIF: lots of interesting functions. *Trends Biochem. Sci.* **17**, 72–76. doi:10.1016/0968-0004(92)90505-4

Kenney, R. M. (1978). Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a note on early embryonic death. *J. Am. Vet. Med. Assoc.* **172**, 241–262.

Kimber, S. J. (2005). Leukaemia inhibitory factor in implantation and uterine biology. *Reproduction* **130**, 131–145. doi:10.1530/REP.1.00304

Lei, T., Yang, Z. Q., Xia, T., Gan, L., Chen, X. D., Yuan, J. H., and Zhu, Y. (2004). Stage-specific Expression of leukaemia inhibitory factor and its receptor in rabbit pre-implantation embryo and uterine epithelium during early pregnancy. *Reprod. Domest. Anim.* **39**, 13–18. doi:10.1046/J.1439-0531.2003.00469.X

Marwood, M., Visser, K., Salamonsen, L. A., and Dimitriadis, E. (2009). Interleukin-11 and leukemia inhibitory factor regulate the adhesion of endometrial epithelial cells: implications in fertility regulation. *Endocrinology* **150**, 2915–2923. doi:10.1210/EN.2008-1538

Modrić, T., Kowalski, A. A., Green, M. L., Simmen, R. C., and Simmen, F. A. (2000). Pregnancy-dependent expression of leukaemia inhibitory factor (LIF), LIF receptor-beta and interleukin-6 (IL-6) messenger ribonucleic acids in the porcine female reproductive tract. *Placenta* **21**, 345–353. doi:10.1053/PLAC.1999.0493

Morris, L. H. A., and Allen, W. R. (2002). Reproductive efficiency of intensively managed thoroughbred mares in Newmarket. *Equine Vet. J.* **34**, 51–60. doi:10.2746/042516402776181222

Nichols, J., Davidson, D., Taga, T., Yoshida, K., Chambers, I., and Smith, A. (1996). Complementary tissue-specific expression of LIF and LIF-receptor mRNAs in early mouse embryogenesis. *Mech. Dev.* **57**, 123–131. doi:10.1016/0925-4773(96)00531-X

Rao, R. P., Fischer, B., and Seshagiri, P. B. (2008). Embryo–endometrial expression of leukemia inhibitory factor in the golden hamster (*Mesocricetus auratus*): increased expression during proestrous and window of implantation stages. *Reprod. Fertil. Dev.* **20**, 440–449. doi:10.1071/RD07154

Song, H., and Lim, H. (2006). Evidence for heterodimeric association of leukemia inhibitory factor (LIF) receptor and gp130 in the mouse uterus for LIF signaling during blastocyst implantation. *Reproduction* **131**, 341–349. doi:10.1530/REP.1.00956

Song, G., Satterfield, M. C., Kim, J., Bazer, F. W., and Spencer, T. E. (2009). Progesterone and interferon tau regulate leukemia inhibitory factor receptor and IL6ST in the ovine uterus during early pregnancy. *Reproduction* **137**, 553–565. doi:10.1530/REP-08-0437

Spencer, T. E., Johnson, G. A., Bazer, F. W., Burghardt, R. C., and Palmarini, M. (2007). Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. *Reprod. Fertil. Dev.* **19**, 65–78. doi:10.1071/RD06102

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F., and Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76–79. doi:10.1038/359076A0

Stout, T. A. (2006). Equine embryo transfer: review of developing potential. *Equine Vet. J.* **38**, 467–478. doi:10.2746/042516406778400529

Stout, T. A., and Allen, W. R. (2002). Prostaglandin E(2) and F(2 alpha) production by equine conceptuses and concentrations in conceptus fluids and uterine flushings recovered from early pregnant and dioestrous mares. *Reproduction* **123**, 261–268. doi:10.1530/REP.0.1230261

Tapia, A., Salamonsen, L. A., Manuelpillai, U., and Dimitriadis, E. (2008). Leukemia inhibitory factor promotes human first trimester extravillous trophoblast adhesion to extracellular matrix and secretion of tissue inhibitor of metalloproteinases-1 and -2. *Hum. Reprod.* **23**, 1724–1732. doi:10.1093/HUMREP/DEN121

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, research00341–research0034.11. doi:10.1186/GB-2002-3-7-RESEARCH0034

Vogiagis, D., Fry, R. C., Sandeman, R. M., and Salamonsen, L. A. (1997). Leukaemia inhibitory factor in endometrium during the oestrous cycle, early pregnancy and in ovariectomized steroid-treated ewes. *J. Reprod. Fertil.* **109**, 279–288. doi:10.1530/JRF.0.1090279