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Ovarian steroids do not impact bovine endometrial cytokine or chemokine responses to *E. coli* or LPS *in vitro*

Short title: Endometrial innate immunity and ovarian steroids

João P E Saut[1,2*], Gareth D Healey[1], Alan M Borges[1,3], I Martin Sheldon[1]

[1]Institute of Life Science, College of Medicine, Swansea University, Swansea, UK

[2]Faculty of Veterinary Medicine, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil

[3]Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

***Corresponding author:**
Dr. João P E Saut
Faculty of Veterinary Medicine, Federal University of Uberlândia, Uberlândia, Campus Umuarama - Bloco 2T - Av. Pará, 1720 - Bairro Umuarama, Uberlândia, Minas Gerais, 38400-902, Brazil.
Tel.: 00 55 34 32182228
E-mail: jpsaut@famev.ufu.br

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Abstract
The risk of bacterial infection of the endometrium causing uterine disease in cattle is increased in the progesterone-dominated luteal phase of the ovarian cycle, whilst oestrogens or oestrous are therapeutic or protect against disease. The first line of defence against bacteria such as *Escherichia coli* that cause inflammation of the endometrium is the innate immune system, which recognises bacterial lipopolysaccharide (LPS). The present study tested the hypothesis that cyclic variation in ovarian hormone concentrations alters innate immune responses within the bovine endometrium. *Ex vivo* organ cultures of endometrium, and *in vitro* cultures of endometrial epithelial and stromal cells, and peripheral blood mononuclear cells, all mounted inflammatory responses to *E. coli* or LPS, with secretion of inflammatory mediators IL-1β, IL-6 and IL-8, and increased expression of mRNA encoding *IL1B*, *IL6*, *IL8* and *CCL5*. However, these inflammatory responses, typical of innate immunity, were not affected by the stage of ovarian cycle in which the endometrium was collected for organ culture, or by exogenous oestradiol or progesterone. Although a dexamethasone positive control reduced inflammation stimulated by *E. coli* or LPS, treatment with oestradiol or progesterone, or inhibitors of oestradiol or progesterone nuclear receptors, did not affect endometrial cell or peripheral blood mononuclear cell secretion of IL-1β, IL-6 or IL-8, or *IL1B*, *IL6*, *IL8* and *CCL5* gene expression. In conclusion, the stage of oestrous cycle or ovarian steroids did not modulate the innate immune response in the bovine endometrium *in vitro*.

Introduction
Microbial infections of the uterus are a common cause of infertility, abortion, pre-term labour and clinical disease of humans and animals (Turner, et al. 2012, Wira, et al. 2005). In dairy cows, postpartum infection rates reach > 90% after parturition, with clinical disease evident in nearly half of these cows. Disease of the uterus may persist for several weeks, is refractory to current treatments, and leads to infertility (Sheldon, et al. 2009). As a result, uterine disease of dairy cows is of economic importance, costing the EU dairy industry €1.4 billion per year (Sheldon, et al. 2009). *Escherichia coli* are an important cause of pathology in the endometrium (Sheldon, et al. 2010); with infection often preceding infection with *Trueperella pyogenes* and anaerobic bacteria (Sheldon, et al. 2002). Furthermore, infection with *E. coli* is associated with negative effects on the ovary, hypothalamic-pituitary axis, and animal health and welfare (Williams, et al. 2007). The endometrium forms an essential barrier to infection of the uterus. Cytokines and chemokines orchestrate the recruitment and activation of immune cells to combat invading pathogens (Wira, et al. 2005). Responses to microbial infection depend on pattern recognition receptors, such as the Toll-like receptors (TLRs), which are expressed by the cells of the endometrium (Herath, et al. 2006, Herath, et al. 2009a, Sheldon and Bromfield 2011). In particular, endometrial epithelial and stromal cell responses to *E. coli* infection are mediated by TLR4, which binds lipopolysaccharide (LPS) leading to secretion of the chemokine IL-8 and the cytokine IL-6 (Cronin, et al. 2012).

The endometrium undergoes physiological changes under the control of the ovarian steroids oestradiol and progesterone to create an environment suitable for pregnancy (Lewis 2003, Wira, et al. 2005). These steroids also have an impact on endometrial disease. During the follicular phase of the oestrous cycle, when oestradiol concentrations are high, the endometrium is more resistant to infection, whilst the progesterone-dominated luteal phase of the oestrous cycle is associated with a predisposition to development of disease (Lewis 2003, 2004, Rowson, et al. 1953). Despite the clear effect of ovarian cycle on uterine disease progression, mechanistic data for the immune polarising effects of oestradiol and progesterone are less apparent. However, differences in uterine cellular profiles have been noted. Cells harvested from the uterine lumen around the time of ovulation secrete higher concentrations of cytokines and chemokines compared with cells harvested during the luteal phase of the oestrous cycle (Fischer, et al. 2010). In some studies, ovarian steroids were associated with changes in neutrophil function (Roth, et al. 1983); whereas in other studies, there were no consistent differences in peripheral leukocyte populations or their function (Subandrio and

Noakes 1997, Subandrio, et al. 2000, Winters, et al. 2003). The inconsistency of leukocyte population differences and neutrophil functional changes, suggest that steroid control of uterine disease progression may be the product of altered endometrial cell responses. Exogenous oestradiol and progesterone alter prostaglandin secretion *in vivo* in cows, sheep and pigs (Del Vecchio, et al. 1992, Seals, et al. 2002, Wulster-Radcliffe, et al. 2003). *In vitro*, exogenous ovarian steroids reduce the secretion of prostaglandins by bovine epithelial and stromal cells stimulated with LPS (Herath, et al. 2006). Therefore, we aimed to test whether stage of oestrous cycle or exogenous ovarian steroids might impact the innate immune response in the bovine endometrium using *ex vivo* studies to avoid confounding effects of humoral factors and adaptive immunity *in vivo*.

The present study tested the hypothesis that cyclical variation in ovarian hormone concentrations alter cytokine and chemokine responses in bovine endometrial *ex vivo* organ cultures (EVOCs) and purified cell populations challenged with LPS or *E. coli*. Two main approaches were used. Firstly, inflammatory responses to *E. coli* or LPS were examined in tissues collected from animals at different stages of the oestrous cycle. Secondly, tissues and cells were treated with exogenous oestradiol and progesterone, or treated with inhibitors of the oestradiol or progesterone receptors. Comparisons were made to the glucocorticoid dexamethasone, which is an established modulator of innate immune responses (Kern, et al. 1988, Waage and Bakke 1988)

Materials and methods

Organ and cell culture

Uteri with no gross evidence of genital disease or microbial infection were collected over a ten-month period from postpubertal mixed-breed beef heifers or dairy cows within 15 min of slaughter at a commercial slaughterhouse, as part of the routine operation of the slaughterhouse. Cattle up to 120 days post partum were not used to avoid confounding experiments due to the presence of ubiquitous bacterial contamination and disruption of the epithelium, which is typical of the puerperal endometrium (Herath, et al. 2009b, Wathes, et al. 2009). The beef heifers (n = 174) were twenty to twenty six months old, reared on extensive grassland and had never been pregnant or inseminated. Dairy cows that were pregnant, as determined when the uterine horns were opened (see below), were excluded from the study. The stage of reproductive cycle was determined by examination of ovarian morphology and vasculature, as described previously in detail (Ireland, et al. 1979); and by the measurement of hormones in peripheral blood. In accordance with these criteria, stage I is defined as days 1–4 of the oestrous cycle; stage II, days 5–10; stage III, days 11–17, and stage IV as days 18–20. Only animals that had gross evidence of ovarian cyclic activity were included. To further evaluate the stage of ovarian cycle, blood samples were collected from the animal carcass at the time of uteri collection, allowed to clot at room temperature, and then centrifuged at 2000 x g for 15 min to separate the serum, which was then aliquoted into 1.5 ml eppendorf tubes and frozen at -80°C until used for progesterone analysis (see below). Within the present study animals from stages IV and I of the oestrous cycle were grouped together, since this represents the follicular phase when serum progesterone concentration is < 1 ng/ml.

The uteri were kept on ice for approximately 1 h until further processing at the laboratory. Endometrial tissue for *ex vivo* organ culture (EVOC) was collected from the contralateral horn, unless otherwise stated, and the intercaruncular areas of the endometrium, except for comparison of responses between intercaruncular and caruncular tissue, using sterile 8 mm-diameter biopsy punches (Stiefel Laboratories Ltd, High Wycome, UK), as previously described (Borges, et al. 2012). Tissues were cultured in 24-well plates (TPP, Trasadingen, Switzerland) containing 2 ml complete medium per well, comprised of: Phenol red-free Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) containing 10% heat inactivated, double charcoal-stripped, fetal bovine serum (FBS; Biosera, East Sussex, UK). The EVOC treatments (see below) were initiated within 4 h of

slaughter, and maintained in a humidified, 5% CO_2 in air atmosphere incubator at 37°C, with supernatants collected 6 h, 24 h or 48 h later.

Endometrial cells were isolated as described previously (Cronin, et al. 2012, Turner, et al. 2014). The epithelial and stromal cells were cultured in complete medium and plated at 1×10^5 cells/ml in 24-well plates (TPP). The purity of epithelial and stromal cell populations was confirmed by cell morphology and flow cytomteric analysis of cytokeratin and vimentin expression, respectively (Fortier, et al. 1988, Turner, et al. 2014).

The isolation and culture of peripheral blood mononuclear cells (PBMCs) was performed as described (Amos, et al. 2014, Herath, et al. 2007).Cells were seeded into 24-well plates at 1×10^6 cells/well in 1 ml of complete medium and medium changed every two days for until cells exhibited characteristic macrophage morphology (Steinman and Cohn 1973). The cell population phenotype, which was CD14+, CD45+ and MHC class II+, was confirmed by flow cytometry as previously described (Herath, et al. 2007, Price, et al. 2013).

Experimental design

Treatments

Cultures of *E. coli* (isolate MS499) obtained from an animal with persistent uterine disease, and identified as an endometrial pathogenic *E. coli* (Goldstone, et al. 2014, Sheldon, et al. 2010), were grown overnight in Luria-Bretani medium (Sigma-Aldrich). Bacteria were re-suspended to 1×10^8 colony forming units (CFU)/ml in sterile PBS (Life Technologies Ltd, Paisley, UK), followed by centrifugation at 6000 x g for 10 min at 4°C. After washing, bacteria were diluted to 1×10^3 CFU/ml in complete medium ready for experimental use. Ultrapure LPS from *E. coli* 0111:B4 was obtained from Invivogen (Toulouse, France). Ovarian and glucocorticoid steroids (Oestradiol, E2758; progesterone, P8783; dexamethasone, D4902) and the steroid receptor antagonists (MPP dihydrochloride hydrate, M7068; mifepristone, M8046) were obtained from Sigma-Aldrich Ltd. Ovarian and glucocorticoid steroids were prepared by dissolving 1 mg of the steroid in 1 ml absolute ethanol. Stock solutions were prepared at 20 µg/ml in complete medium. Final concentrations of oestradiol, progesterone and dexamethasone were prepared by further dilutions in complete medium. The final concentration of ethanol within tissue or cell cultures was equal to or less than 1 part in 200,000. MPP dihydrochloride hydrate was prepared by dissolving 20 mg in 1 ml dimethyl sulfoxide. A stock solution was prepared at 1 mg/ml (1.85 mM) in complete medium. The final concentration of MPP dihydrochloride hydrate (100 nM) was prepared by further dilution in complete medium. The final concentration of dimethyl sulfoxide within tissue of cell cultures was 1 part in 370,000. Mifepristone was prepared by dissolving 10 mg in 1 ml absolute ethanol. A stock solution was prepared at 1 mg/ml (2.32 mM) in complete medium. The final concentration of mifepristone (100 nM) was prepared by further dilution in complete medium. The final concentration of ethanol within tissue of cell cultures was 1 part in 232,000. All treatments were performed in complete medium that did not contain antibiotics to ensure bacteria were alive and replicating.

Validation of innate immune responses of endometrial ex vivo organ cultures

To compare endometrial innate immune responses between beef heifers and dairy cows, EVOCs were prepared from beef heifer (n = 9) and dairy (n = 7) cow uteri that were within the early-luteal phase of the oestrous cycle. Comparison of EVOCs using endometrial tissue from intercaruncular (n = 4) and caruncular (n = 4) zones of the endometrium was performed using early luteal phase beef heifer uteri. Endometrial EVOCs were treated with control medium or medium containing 1 µg/ml LPS or 1×10^3 CFU/ml *E. coli* for 24 h. Comparison of EVOCs using endometrial tissue from the horn ipsilateral (n = 13) and contralateral (n = 29) to the active CL structure was performed using early luteal phase beef heifer uteri. Endometrial EVOCs were treated with control medium or medium

containing 1 μg/ml LPS for 24 h. After treatment, supernatants were collected and stored at -20°C for analysis of IL-1β, IL-6 and IL-8 by ELISA. The EVOC tissues were weighed, and stored in 0.5 ml TRI Reagent® at -20°C until RNA extraction and analysis of *IL1B*, *IL6* and *IL8* mRNA expression by qPCR.

Uterine innate immune responses and stage of oestrous cycle

To evaluate the effect of the stage of oestrous cycle, tissues from 55 beef heifers were divided into three groups by examination of ovarian morphology (Ireland, et al. 1979) and retrospective serum progesterone analysis (see below): Follicular phase was defined by ovarian stage (Ireland *et al* 1979 - stage IV and I) with serum progesterone concentration < 1 ng/ml (n = 6); early-luteal phase (Ireland *et al* 1979 - stage II) with serum progesterone concentration 1 to 2 ng/ml (n = 10); and mid-luteal phase (Ireland *et al* 1979 - stage III) with serum progesterone concentration > 2 ng/ml (n = 39). Endometrial EVOCs from each group were treated with control medium or medium containing 1 μg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for 6 h or 24 h. After treatment, supernatants were collected and stored at -20°C for analysis of IL-1β, IL-6, IL-8 and PGE_2 by ELISA. The EVOC tissues were weighed, and stored in 0.5 ml TRI Reagent® at -20°C until RNA extraction and analysis of *IL1B*, *IL6, IL8* and *CCL5* mRNA expression by qPCR.

Steroids and innate immune responses within ex vivo organ cultures

Seventy six beef heifers were used to evaluate the effect of ovarian and glucocorticoid steroids on innate immune responses within the bovine endometrium. Endometrial EVOCs were divided into two groups according to retrospective analysis of serum progesterone concentration: group 1 with serum progesterone concentration < 2ng/ml (n = 20); group 2 with serum progesterone concentration > 2 ng/ml (n = 56). Endometrial EVOCs were pre-treated for 24 h with control medium or medium containing 3 pg/ml oestradiol, 5 ng/ml progesterone or 5 ng/ml dexamethasone. The concentration of oestradiol and progesterone reflect serum concentrations around the time of ovulation and during the luteal phase of the oestrous cycle, respectively (Jimenez-Krassel, et al. 2009, Scully, et al. 2014, Sheldon, et al. 2002). The concentration of dexamethasone used was based upon the recommended potency range from the manufacturer (4 to 500 ng/ml) and a previous publication, with the aim being to use a minimal effective dose (Kern, et al. 1988). After 24 h, EVOCs were challenged with control medium or medium containing 1 μg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids. After challenge, supernatants were collected and stored at -20°C for analysis of IL-1β, IL-6 and IL-8 by ELISA. The EVOC tissues were weighed, and stored in 0.5 ml TRI Reagent® at -20°C for analysis of *IL1B*, *IL6*, *IL8* and *CCL5*. In addition, the expression of *ESR1* and *PGR* mRNA was determined by qPCR to ensure the tissue remained responsive to exogenous steroids for the 48 h treatment period.

Steroids and cellular innate immune responses

To evaluate the impact of ovarian and glucocorticoid steroids on *in vitro* cellular innate immune responses, endometrial epithelial and stromal cells, and PBMCs, collected from four beef heifers were pre-treated for 24 h with control medium or medium containing 3 pg/ml oestradiol, 5 ng/ml progesterone or 5 ng/ml dexamethasone. After 24 h, cells were challenged with control medium or medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. After challenge, supernatants were collected and stored at -20°C for analysis of IL-6 and IL-8 by ELISA. Additionally, endometrial stromal cells from three beef heifers were pre-treated for 24 h with control medium or medium containing 1 to 30 pg/ml oestradiol, 1 to 30 ng/ml progesterone or 5 ng/ml dexamethasone. After 24 h, cells were challenged with control medium or medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. After challenge, supernatants were collected and stored at -20°C for analysis of IL-6 and IL-8 by ELISA. Cell survival was assessed by the mitochondria-dependent reduction of 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, as previously described (Mosmann 1983). Briefly, supernatants were removed and replaced with fresh complete medium containing 0.5 mg/ml MTT before being incubated with the

cells at 37°C in a humidified, 5% CO_2 in air incubator for 1 h. The medium was then removed and the cells washed with sterile PBS prior to lysis with dimethyl sulfoxide and measurement of the optical density at 570 nm using a microplate reader (POLARstar Omega; BMG Labtech, Offenburg, Germany). The correlation between MTT OD 570 measurements and the number of live cells was confirmed using trypan blue exclusion and counting the number of live cells using a haemocytometer.

Steroid receptor antagonists and endometrial innate immune responses

To further explore the impact of steroids on immunity in the endometrium, their actions were inhibited using antagonists for their nuclear receptors. Endometrial EVOCs from 23 beef heifers in the luteal phase of the oestrous cycle were pre-treated for 24 h with control medium or medium containing 5 ng/ml progesterone, 3 pg/ml oestradiol or 5 ng/ml dexamethasone. Pre-treatments were performed in the presence or absence of the oestrogen receptor alpha (ERα) antagonist MPP dihydrochloride hydrate (MPP, 100 nM) (Sun, et al. 2002) or the progesterone/glucocorticoid receptor (PR/GR) antagonist mifepristone (100 nM) (Siemieniuch, et al. 2010, Skinner, et al. 1999). After 24 h, the EVOCs were challenged with control medium or medium containing 1 µg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids and/or antagonists. After challenge, supernatants were collected and stored at -20°C for analysis of IL-1β, IL-6 and IL-8 by ELISA, and EVOC tissues were weighed.

Enzyme immune assays

Concentrations of IL-1β, IL-6 and IL-8 in EVOC and cell culture supernatants were measured in duplicate by ELISA according to the manufacturer's instructions [Bovine IL-1β Screening Set (ESS0027; ThermoFisher Scientific, Perbio Science UK Ltd, Cramlington, UK); Bovine IL-6 Screening Set (ESS0029; ThermoFisher Scientific); Human CXCL8/IL-8 DuoSet (DY208; R&D Systems Europe Ltd., Abingdon, UK)]. The human CXCL8/IL-8 DuoSet has previously been validated for the measurement of bovine IL-8 (Rinaldi, et al. 2008). To take into account differences between the weights of EVOC tissues, concentrations are reported as picogram per milligram of tissue. Serum progesterone concentrations were determined using a Progesterone Enzyme Immunoassay (Ridgeway Research Ltd, St Briavels, UK), according to the manufacturer's instructions. The inter-assay and intra-assay coefficients of variation were all less than 12% and 7%, respectively; the limits of detection were 12.5 pg/mL for IL-1β, 75.0 pg/mL for IL-6, 5.7 pg/mL for IL-8, and 0.1 ng/mL for progesterone.

Gene expression analysis

Gene expression analysis was performed according to MIQE guidelines (Bustin, et al. 2009). Total RNA was isolated from EVOC tissues by homogenising the tissue in 2 ml tubes containing 0.5 ml TRI Reagent® (Sigma-Aldrich) and lysing matrix D (MP Biomedicals, Cambridge, UK) at 6.0 m/sec for 2 min. After homogenization, tubes were centrifuged at 12,000 × g for 10 min, the supernatants transferred to fresh 2 ml eppendorf tubes, and RNA extraction from TRI Reagent® then performed according to the manufacturer's instructions. Reverse transcription of 1 µg mRNA was performed in a 20 µl reaction using the QuantiTect Reverse Transcription kit (Qiagen, Manchester, UK) according to the manufacturer's instructions.

Quantitative PCR (qPCR) for *IL1B, IL8, CCL5, ESR1, GAPDH* and *ACTB* was performed by multiplex probe-based PCR, and comprised two panels of primer/probe combinations (panel 1 = *IL1B, IL8*, and *GAPDH*; panel 2 = *CCL5*, *ESR1* and *ACTB*), which simultaneously measured cDNA for each target gene. PCR primers and probes were designed using Eurofins MWG Operon qPCR primer/probe design software (https://ecom.mwgdna.com/services/webgist/dual_probe_design?usca_p=t) and validated by BLAST analysis against the *Bos taurus* (taxid:9913) Refseq mRNA database. Primers/probes were obtained from Eurofins MWG Operon, Ebersberg, Germany. Multiplex qPCR was performed in 10 µl reactions comprising 1 × QuantiFast Multiplex PCR master mix (Qiagen) with primers and probes

added in nuclease-free water to a final concentration of 0.4 µM and 0.2 µM respectively and 2 µl of cDNA. Thermal cycling parameters were: 1 cycle of 95°C for 5 min followed by 50 cycles of 95°C for 15 sec and 60°C for 30 sec.

Quantitative PCR for *IL-6* and *PGR* was performed by SYBR green based PCR because existing primers/methods for these genes were already present in the lab. PCR primers were designed using Eurofins MWG Operon qPCR primer/probe design software (https://ecom.mwgdna.com/services/webgist/dual_probe_design?usca_p=t) and validated by BLAST analysis against the *Bos taurus* (taxid:9913) Refseq mRNA database. *PGR* and *IL6* primers were obtained from Eurofins MWG Operon and Sigma-Aldrich, respectively. SYBR-based PCR was performed in a 25 µl reaction comprising 1 x QuantiFast SYBR green PCR master mix (Qiagen) with primers added in nuclease-free water to a final concentration of 0.4 µM and 2 µl of cDNA. Thermal cycling parameters were: 1 cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 60 sec.

All primers and probes used are detailed in Table 1. The expression of each gene was normalised against the geometric mean of the reference genes *GAPDH* and *ACTB*, which were invariant across treatment groups (Vandesompele, et al. 2002), and the relative quantification method employed to quantify target gene mRNA within samples (Nolan, et al. 2006). To generate standard curves, total RNA extracted from EVOC tissues that had been treated with 1 µg/ml LPS for 24 h, was reverse transcribed to cDNA, as described. Ten-fold serial dilutions of this reference cDNA were prepared (neat to 1 x 10^{-5}) in nuclease-free water (Qiagen). For each sample, target and reference gene mRNA abundance was determined from the appropriate standard curve (quantification cycle, C_q). Changes in mRNA abundance between samples were then determined from the ratio of the target gene C_q to reference gene C_q.

Data analysis

Statistical analyses were performed using SAS version 8.0 with the animal as the experimental unit. Initially the data were tested for homogeneity, followed by analysis using General Linear Model multiplex analysis of variance (GLM-ANOVA) using Dunett's pair-wise multiple comparison t-test for individual group comparisons. Gene data are presented as dot plots, protein data are presented as histograms, data are presented as mean with standard error (SEM) and $P < 0.05$ was considered statistically significant.

Results

Endometrial EVOCs from beef heifers and dairy cows respond similarly to LPS and *E. coli*

To validate the utility of the EVOC system, selected cytokine and chemokine responses were compared between EVOCs from beef heifer and dairy cow endometrium. Endometrial EVOCs from beef heifers and dairy cows accumulated more IL-1β, IL-6 and IL-8 following challenge with LPS or *E. coli* compared with control medium (Fig. 1A-C: $P < 0.0001$). There was also increased *IL1B*, *IL6* and *IL8* mRNA expression in response to challenge with LPS or *E. coli* (Fig. 1D-F: $P < 0.0001$). However, there was no significant difference in the protein or mRNA responses to LPS or *E. coli* between EVOCs from beef heifers and dairy cows.

To further validate the use of EVOCs, inflammatory responses to *E. coli* and LPS were compared between caruncular and intercaruncular EVOCs collected from beef heifer endometrium. Endometrial EVOCs from both areas secreted more IL-1β, IL-6 and IL-8 in response to challenge with LPS or *E. coli* compared with control medium (Fig. 1G-I: $P < 0.05$). However, more IL-1β, IL-6 and IL-8 was secreted from intercaruncular EVOCs compared with caruncular EVOCs following challenge with LPS ($P < 0.05$), and more IL-1β and IL-6 was secreted following challenge with *E. coli* ($P < 0.05$).

Finally, inflammatory responses to LPS were compared between endometrial EVOCs collected from the horn ipsilateral and contralateral to the active CL structure. Endometrial EVOCs from both horns secreted more IL-6 in response to challenge with LPS compared with control medium (Fig. 1K: $P < 0.001$), and there was a trend for increased IL-1β and IL-8. Importantly, however, there were no significant differences in responses to LPS between EVOCs from the ipsilateral or contralateral horns.

Endometrial innate immune responses and stage of oestrous cycle

To investigate the role of the oestrous cycle in modulating innate immunity, responses to challenge with LPS or *E. coli* for 6 h or 24 h were tested using EVOCs of intercaruncular endometrium collected from beef animals at different stages of the oestrous cycle. As expected, cows in the mid-luteal phase had more progesterone present in their serum (8.51 + 0.75 ng/ml: $P < 0.001$) compared with cows in the follicular (0.08 + 0.07 ng/ml) or early luteal (0.5 + 0.21 ng/ml) phases.

Following a 6 h challenge with LPS or *E. coli,* EVOCs accumulated more IL-6 ($P < 0.0001$), compared with control medium, and after a 24 h challenge with LPS or *E. coli*, more IL-1β, IL-6 and IL-8 ($P < 0.0001$) (Fig. 2). Mid-luteal phase cow EVOCs accumulated more IL-6 ($P < 0.05$), but not IL-1β or IL-8, in response to a 6 h challenge with LPS compared with follicular and early luteal phase cow EVOCs (Fig. 2A-C). There were no significant differences between stages of oestrous cycle following a 6 h challenge with *E. coli*. Mid-luteal phase cow EVOCs accumulated less IL-1β ($P < 0.05$) compared with early-luteal phase cow EVOCs following a 24 h challenge with *E. coli*, but there was no difference in IL-6 or IL-8 secretion (Fig. 2D-F). There were no significant differences between stages of oestrous cycle for EVOCs challenged with LPS for 24 h. To further explore the impact of stage of the oestrous cycle on inflammatory responses in the endometrium, the EVOC data were combined (Follicular, n = 19; Early-luteal, n = 43; Mid-luteal, n = 22). Treatment with LPS or *E. coli* increased the accumulation of IL-6 compared with control (305.7 ± 13.1, 243.4 ± 12.4, 93.1 ± 13.9 pg/ml, respectively; ANOVA, $P < 0.001$). However, there was no significant effect of stage of cycle ($P = 0.23$) or the interaction between treatment and stage ($P = 0.88$).

Challenge of EVOCs with *E. coli* or LPS for 6 h or 24 h also increased the expression of *IL1B, IL6, IL8* and *CCL5* (Fig. 3: $P < 0.05$). However, there were no consistent differences in mRNA expression amongst the different stages of oestrous cycle measured for any of the four genes examined at 6 h or 24 h (Fig. 3).

Ovarian steroids and endometrial responses to LPS or *E. coli*

In the absence of an effect of the stage of oestrous cycle on the inflammatory response, an alternative approach was examined to test if ovarian steroids regulate endometrial innate immunity, by treating EVOCs with exogenous steroids prior to challenge with LPS or *E. coli*. The EVOCs were retrospectively divided into two groups, based on serum progesterone concentration: < 2 ng/ml (follicular) and > 2 ng/ml (luteal). As ovarian hormones regulate the *ESR1* and *PGR* genes (Kimmins and MacLaren 2001), to confirm the responsiveness of the EVOC to steroid treatment, the impact of oestradiol, progesterone and the glucocorticoid dexamethasone on *ESR1* and *PGR* mRNA expression was measured. Within EVOCs of follicular phase endometrial tissue, 48 h treatment with oestradiol increased the expression of *ESR1* (Fig. 4A: $P < 0.05$) and *PGR* (Fig. 4C: $P < 0.05$) mRNA compared with the control, and progesterone significantly decreased the expression of *PGR* (Fig. 4C: $P < 0.05$) mRNA, whilst dexamethasone had no effect (Fig. 4A, C). The same pattern of change in expression of *PGR* mRNA was measured in EVOCs from luteal phase cows (Fig. 4D: $P < 0.05$), but none of the steroids had any significant effect on *ESR1*.

As previously, irrespective of steroid treatment EVOCs responded to challenge with LPS by accumulating IL-6 and IL-8; and, accumulated IL-6, IL-8 and IL-1β in response to challenge with *E. coli* (Fig. 5: $P < 0.05$). However, pre-treatment of EVOCs with either oestradiol or progesterone for 24 h

had no significant effect on responses to subsequent challenge with LPS or *E. coli*, and there was no effect of the prior hormone concentration in the animals. Although, dexamethasone reduced the accumulation of IL-1β in EVOCs prior to, and following challenge with LPS or *E. coli* (Fig. 5A, B: $P < 0.05$). As previously, challenge of EVOCs with LPS or *E. coli* increased the expression of *IL1B*, *IL6*, *IL8* and *CCL5* mRNA compared with control medium (Fig. 6: $P < 0.001$). However, pre-treatment with oestradiol or progesterone had no effect on mRNA abundance in response to these challenges, and again this was irrespective of the prior hormone concentration in the animals. In contrast, pre-treatment with dexamethasone reduced the expression of *IL1B* (Fig. 6A, B: $P < 0.05$) and *IL8* (Fig. 6 E, F: $P < 0.05$) in EVOCs prior to, and following challenge with LPS or *E. coli*, and *CCL5* (Fig. 6 G, H: $P < 0.05$) following challenge with LPS or *E. coli*, compared with control medium.

To determine whether the lack of effect of ovarian steroids on endometrial innate immune responses was unique to the EVOCs, experiments were also conducted using pure populations of endometrial cells. Endometrial epithelial cells, stromal cells and PBMCs accumulated IL-6 (Fig. 7A-C) and IL-8 (Fig. 7 D-F) in response to challenge with LPS for 24 h ($P < 0.001$). However, pre-treatment with oestradiol or progesterone for 24 h had no significant effect on IL-6 or IL-8 production of each of the three cell types. Although, pre-treatment with dexamethasone reduced the accumulation of IL-6 in epithelial cells (Fig. 7A: $P < 0.05$), and IL-8 in stromal cells and PBMCs (Fig. 7E, F: $P < 0.05$).

However, different concentrations of exogenous steroid could modulate cellular responses, so endometrial stromal cells were treated with a range of concentrations of oestradiol or progesterone, prior to challenge with LPS. Stromal cells were used for two reasons: firstly, stromal cells are more responsive to LPS than epithelial cells, and the increased dynamic range gave the best chance of observing and effect. Secondly, epithelial cells are lost during and after parturition exposing the underlying stromal cells to bacterial infection (Archbald, et al. 1972). Pre-treatment of endometrial stromal cells with 1 to 30 pg/ml oestradiol (Fig. 8A, C), or 1 to 30 ng/ml progesterone (Fig. 8B, D) for 24 h, did not modulate IL-6 or IL-8 responses to LPS during subsequent challenge.

Ovarian steroid receptor antagonists and endometrial responses to LPS or *E. coli*

To examine whether steroid nuclear receptor function modulates endometrial innate immunity, EVOCs were pre-treated with the oestrogen receptor alpha antagonist MMP or the progesterone/glucocorticoid receptor antagonist mifepristone, with or without the appropriate steroid present. After the 24 h pre-treatment, EVOCs were challenged with control medium or medium containing LPS or *E. coli*. Endometrial EVOCs accumulated IL-1β, IL-6 and IL-8 in response to challenge with LPS and IL-1β and IL-6 following challenge with *E. coli* (Fig. 9: $P < 0.0001$). Pre-treatment of EVOCs for 24 h with oestradiol and/or MPP had no effect on endometrial responses to challenge with LPS or *E. coli* (Fig. 9A, D, G). Pre-treatment for 24 h with progesterone and/or mifepristone also had no significant effect on endometrial responses to challenge with LPS or *E. coli* (Fig. 9B, E, H). However, pre-treatment with dexamethasone reduced ($P < 0.05$) the accumulation of IL-1β in response to challenge with *E. coli*, and importantly, pre-treatment with dexamethasone and mifepristone blocked the IL-1β inhibiting effect of dexamethasone (Fig. 9C: $P < 0.05$).

Discussion

In vivo, there is clear evidence for a protective effect of oestradiol or oestrous against infection of the uterus, and of a disease-promoting effect for progesterone or the luteal phase of the oestrous cycle (Del Vecchio, et al. 1992, Lewis 2004, Rowson, et al. 1953). Although, several explanations for these effects have been explored previously, the mechanistic explanations are elusive, particularly in relation to leukocyte population differences and neutrophil function (Subandrio and Noakes 1997, Subandrio, et al. 2000, Winters, et al. 2003). Thus, we reasoned that ovarian steroids might modulate innate immune responses in the endometrium. However, in the present study the stage of oestrous cycle did not influence the cytokine or chemokine response of *ex vivo* endometrial tissue to *E. coli* or LPS at the gene or the protein level. Furthermore, exogenous ovarian steroids did not modulate the innate immune response by endometrial tissue or cells. Finally, even blocking the nuclear receptors for oestradiol or progesterone did not impact the inflammatory response to *E. coli* or LPS. We conclude that ovarian steroids have little effect on *in vitro* inflammatory responses associated with innate immunity in the bovine endometrium.

The use of EVOCs maintains the architecture of the cells in the tissue, and retains an imprint of the stage of oestrous cycle of the animal. Using EVOCs also avoids potential confounders of *in vivo* studies, including humoral factors, effects of nutrition, and adaptive immune responses, enabling exploration of the impact of steroids in the localized tissue and cells of the endometrium, independent of the whole animal response. In the present study, endometrial EVOCs from beef heifers were a good surrogate for tissues from dairy cows, producing similar cytokine and chemokine responses to *E. coli* and LPS. Furthermore, using tissue and cells from beef heifers removed potential confounders in dairy cows, such as insemination, pregnancy, previous uterine disease, and lactation. Yet the increased cytokine and chemokine secretion, and increased mRNA expression following challenge of EVOCs with *E. coli* or LPS mirror the changes during disease *in vivo* (Herath, et al. 2009b, Sheldon, et al. 2009). Additionally, EVOCs collected from the horn ipsilateral or contralateral to the active corpus luteum were equally responsive to LPS, suggesting that the inflammatory response is not modulated by differing concentration gradients of hormone across the two horns. This view is supported by gene array analyses, which report hundreds of differentially expressed genes in the endometrium of luteal versus follicular phase animals, but very few genes differ in expression between the horn ispsilateral and contralateral to the corpus luteum, and those that do have very low ratios (Bauersachs, et al. 2005, Shimizu, et al. 2010). However, EVOCs incorporating tissue from intercaruncular areas of the endometrium were more responsive to challenge with LPS or *E. coli* than caruncular tissue. With over 1100 differentially expressed genes between intercaruncular and caruncular tissue, including several inflammation and immune regulating genes (Mansouri-Attia, et al. 2009), use of tissues from the intercaruncular zones was an important optimization step.

Central to the response to bacterial challenge is the detection of pathogen associated molecular patterns by TLRs, and in particular for *E. coli* infection, binding of LPS by TLR4. Endometrial epithelial and stromal cells also express TLRs, including TLR4, and produce IL-6 and IL-8 following challenge with LPS (Herath, et al. 2006, Sheldon and Roberts 2010). In the present study, *E. coli* and LPS stimulated the accumulation of IL-1β and IL-8 by 24 h. The kinetics of IL-6 production likely reflects the roles of IL-6 in the early response to infection such as leukocyte recruitment, B-lymphocyte development, antibody secretion by plasma cells , and the regulation of acute-phase proteins. Interleukin-8, a potent chemo-attractor and activator of neutrophils and T-lymphocytes, is secreted by monocytes, lymphocytes, fibroblasts, epithelial and endothelial cells (Mukaida 2000, Schaefer, et al. 2004). Interleukin-1β is secreted predominantly by monocytes following inflammasome activation and stimulates the production of additional pro-inflammatory cytokines, such as IL-6 (van de Veerdonk, et al. 2011), and chemokines such as IL-8, which recruit more immune cells, and promote phagocytosis and bacterial clearance (Petrilli, et al. 2007).

The most striking observations in the present study were that endometrial tissue and cell responses to challenge with *E. coli* or LPS were not influenced by the stage of oestrous cycle, or by the addition of exogenous oestradiol or progesterone. First we found that the stage of oestrous cycle did not affect innate immune responses of endometrial EVOCs challenged with LPS or *E. coli*. So, we considered whether separating out the cellular populations would uncover steroid-responsive effects, using differential regulation of *ESR1* and *PGR* mRNA to verify that the endometrial cells were responsive to exogenous oestradiol and progesterone. However, ovarian steroids had no effect on separated endometrial cell or PBMC responses to challenge with LPS or *E. coli*, and similarly, inhibition of oestrogen receptor alpha or progesterone receptor had no effect on innate immune responses; although, inhibiting the glucocorticoid receptor inhibited dexamethasone related inflammatory modulation. One could argue that the initial staging of the oestrous cycle may have been erroneous. However, more than 150 animals were used across the studies, peripheral plasma progesterone concentrations were used to verify the stage of cycle, and the variance across groups was small irrespective of the stage of cycle. It could also be argued that higher steroid concentrations in the uterine tissue, compared to the peripheral plasma, might effectively modulate inflammatory responses (Einer-Jensen, et al. 1989, McCracken, et al. 1984, Weems, et al. 1988). However, extended dose range experiments showed no effect on inflammatory responses to LPS, and EVOC tissue had been exposed to native uterine steroid concentrations. Taken together, these data suggest that there is neither a direct effect of the ovarian steroids on innate immunity nor is oestrous cyclic regulation of ovarian steroid receptor expression likely to impact innate immunity.

In vivo, there is a clear oestrous-dependant effect on basal mRNA expression of cytokines and chemokines such as *IL1B*, *IL8* and *CXCL5* in cells collected from the uterine lumen (Fischer, et al. 2010). So, how do the present study's negative results *in vitro* fit into the whole animal effects? Firstly, there may be an innate immune effect mediated by regulatory molecules not investigated in this study. *In vitro*, exogenous ovarian steroids reduce the synthesis of prostaglandin $F2_{\alpha}$ and prostaglandin E_2 in endometrial cells (Herath, et al. 2006). Other classes of molecules, such as antimicrobial peptides, lipoxins or resolvins could also be examined. Secondly, alteration of the adaptive immune response would have a significant effect on disease outcome. The presence of ovarian steroid receptors on immune cells suggests the possibility of their regulation, and there is evidence from humans that ovarian steroids have a significant impact on disease outcome (Rodriguez-Garcia, et al. 2013a, Rodriguez-Garcia, et al. 2013b, Waage, et al. 1990, Wira, et al. 2005). Thirdly, there may be an indirect effect of ovarian steroids on innate or adaptive immunity. Indeed, in the present study dexamethasone reduced IL-1β, IL-6 and IL-8 secretion, together with *IL1B*, *IL6*, *IL8* and *CCL5* mRNA responses to challenge with *E. coli* or LPS. In addition, the GR antagonist mifepristone attenuated the suppressive effect of dexamethasone on IL-1β secretion following *E. coli* challenge. Kuse *et al* recently demonstrated a regulatory effect of ovarian steroids on *NR3C1* expression within the bovine endometrium. As a function of stage of the oestrous cycle, *NR3C1* expression within the bovine endometrium was greater during the mid-luteal phase when progesterone concentrations are high, compared with other phases of the oestrous cycle, and the glucocorticoid cortisol more strongly suppressed $PGF_{2\alpha}$ production during the mid-luteal phase than during the follicular phase. The addition of progesterone to cultured endometrial epithelial cells also increased expression of *NR3C1*, whilst oestradiol reduced expression levels (Kuse, et al. 2013). A future approach might also probe single cell responses to PAMPs and steroids since although the present study has shown no response to ovarian steroids within large populations of cells *in vitro*, individual cells may respond. Indeed, such an approach recently revealed the production of the lymphosteroid pregnenolone by Th2 T cells, which is associated with immunosuppression, inhibiting Th cell proliferation and B cell immunoglobulin class switching (Mahata, et al. 2014).

In conclusion, there was no effect of the stage of oestrous cycle, exogenous ovarian steroids, or inhibiting their nuclear receptors on key cytokine and chemokine responses to *E. coli* or LPS in endometrial tissues or cells. The lack of effect of ovarian steroids challenges the central dogma that steroids suppress immunity across species.

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Table 1:
Quantitative PCR primers and probes used for gene expression analysis.

Gene	Primers/probes (5' – 3')
IL1B	*Forward:* TCCTATTCTCTCCAGCCA *Reverse:* AGCCTCAAATAACAGCTCATTC *Probe:* FAM-ATGGCAACCGTACCTGAACCCATCAA-BHQ1
IL8	*Forward:* CACATTCCACACCTTTCCAC *Reverse:* CCTTCTGCACCCACTTTTC *Probe:* Hex-GGAAACGAGGTCTGCCTAAACCCCAA-BHQ1
GAPDH	*Forward:* ATTCCACCCACGGCAAGTTC *Reverse:* TCCATCGTCCACCGCAAATGCTTCT *Probe:* Cy5-GCAGAGAACGGGAAGCTCGTCATCAATGGAA-BBQ650
CCL5	*Forward:* CTTTGCCTATATCTCCCGCC *Reverse:* TCTCGCACCCACTTCTTCTC *Probe:* FAM-CAGCAGTTGTCTTTATCACCAGGAAGAAGCGCCA-BHQ1
ACTB	*Forward:* AAGAAAAAGGGTGTAACGCAG *Reverse:* TCCATCGTCCACCGCAAATGCTTCT *Probe:* LC705-ATTCCACCCACGGCAAGTTC-BBQ650
ESR1	*Forward:* ACTCCTCCTCATCCTCTCTC *Reverse:* CACCACGTTCTTGCACTTC *Probe:* Hex-GGCACATGAGCAACAAAGGCATGGA-BHQ1
IL6	*Forward:* ATGACTTCTGCTTTCCCTACCC *Reverse*: GCTGCTTTCACACTCATCATTC
PGR	*Forward:* CGTGGAGGGGGCGTATTCCG *Reverse*: CGGGGCCAAAGAGGCACCAA

Figure legends

Figure 1
Ex vivo organ culture responses to LPS or *E. coli*. Endometrial EVOCs were collected from the intercarulcular areas (contralateral horn) of early luteal beef heifer (Open bars or ○) and dairy cow (Closed bars or ■) endometrium, and challenged with control medium or medium containing 1 µg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for 24 h. Supernatants were collected and analysed for IL-1β, IL-6 and IL-8 by ELISA (A – C: Beef heifers, n = 9; dairy cows, n = 7) and *IL1B*, *IL6* and *IL8* mRNA expression by qPCR (D – F; Beef heifers, n = 4; dairy cows, n = 4). Endometrial EVOCs collected from the caruncular (Closed bars, n = 4) and intercaruncular (Open bars, n = 4) areas of early luteal beef heifer endometrium (contralateral horn) were challenged with control medium or medium containing 1 µg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for 24 h. Supernatants were collected and analysed for IL-1β, IL-6 and IL-8 by ELISA (G – I). Endometrial EVOCs collected from the intercaruncular areas of the ipsilateral (Open bars, n = 13) and contralateral (Closed bars, n = 13) horns of early luteal beef heifer endometrium were challenged with control medium or medium containing 1 µg/ml LPS for 24 h. Supernatants were collected and analysed for IL-1β, IL-6 and IL-8 by ELISA (J – L).Data are presented as mean (SEM), protein data as histograms; qPCR data as dot plots with the horizontal bar representing the mean and vertical bars SEM. Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison t-test, values differ between groups: *P < 0.05.

Figure 2
Ex vivo organ culture responses are not affected by the stage of oestrous cycle. Endometrial EVOCs collected from the intercaruncular areas of beef heifer endometrium (contralaterial horn) at different stage of the oestrous cycle were challenged with control medium or medium containing 1 µg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for 6 (A – C: Follicular, open bars , n = 3; early luteal, grey bars, n = 10, mid luteal, closed bars, n = 20) or 24 h (D – F: Follicular, open bars, n = 6, early luteal, grey bars, n = 10, mid luteal, closed bars, n = 39). Supernatants were collected and analysed for IL-1β, IL-6 and IL-8 by ELISA. Data are presented as mean (SEM). Data were analysed by GLM multivariate ANOVA and Dunnett's pairwise multiple comparison t-test, values differ between groups: *P < 0.05.

Figure 3
Gene expression within *ex vivo* organ cultures is not affected by the stage of oestrous cycle. Endometrial EVOCs collected from the intercaruncular areas of beef heifer endometrium (contralateral horn) at different stages of the oestrous cycle were treated with control medium or medium containing 1 µg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for 6 or 24 h. For 6 h treatment: Folliclar (●), n = 3, early luteal (□), n = 4, mid luteal(△), n = 10. For 24 h treatment: Folliclar (●), n = 3, early luteal (□), n = 4, mid luteal (△), n = 9. The EVOC tissues were collected and total RNA extracted for analysis of *IL1B* (A, B), *IL6* (C, D), *IL8* (E, F) and *CCL5* (G, H) mRNA expression by qPCR. Data are presented in dot plots with the horizontal bar representing the mean and vertical bars SEM. Data were analysed by GLM mutlivariate ANOVA and Dunnett's pairwise multiple comparison t-test, values differ between groups: *P < 0.05, and each dot represents an individual animal..

Figure 4
Ovarian steroid receptor (*ESR1* and *PGR*) expression in *ex vivo* organ cultures is regulated by progesterone and oestradiol but not by dexamethasone. Follicular (A, C) or luteal (B, D) phase endometrial EVOCs from the intercaruncular areas of beef heifer endometrium (contralateral horn) were treated for 48 h with control medium or medium containing 5 ng/ml progesterone, 3 pg/ml oestradiol or 5ng/ml dexamethasone. The EVOC tissues were collected and total RNA extracted for analysis of *ESR1* (A, B) and *PGR* (C, D) mRNA expression by qPCR. For follicular EVOCs: Control, n = 6, oestradiol, n = 3, progesterone, n = 3, dexamethasone, n = ; for luteal EVOCs: control, n = 10, oestradiol, n = 4, progesterone, n = 4, dexamethasone, n = 6. Data are presented in dot plots with the horizontal bar representing the mean and vertical bars SEM. Data were analysed by ANOVA

using Dunett's pairwise multiple comparison t-test, values differ from control: *P < 0.05, and each dot represents an individual animal.

Figure 5

Exogenous ovarian steroids do not regulate *ex vivo* organ culture responses to LPS or *E. coli.* Follicular (A, C, E) and luteal phase (B, D, F) endometrial EVOCs from the intercaruncluar areas of beef heifer endometrium (contralateral horn) were pre-treated for 24 h with control medium (Open bar) or medium containing 5 ng/ml progesterone (Chequered bar), 3 pg/ml oestradiol (Striped bar) or 5 ng/ml dexamethasone (Closed bar). After 24 h, the EVOCs were challenged with control medium or medium containing 1 µg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids. Supernatants were collected and analysed for IL-1β (A, B), IL-6 (C, D) and IL-8 (E, F) by ELISA. Data are presented as mean (SEM). Data were analysed by GLM multivariate ANOVA and Dunett's pairwise multiple comparison t-test, values differ between groups: *P < 0.05. Number of animals (n) is indicated in the figure.

Figure 6

Ex vivo organ culture mRNA expression is regulated by dexamethsone, but not by the ovarian steroids. Follicular (A, C, E, G) or luteal phase (B, D, F, H) endometrial EVOCs from the intercaruncular areas of beef heifer endometrium (contralateral horn) were pre-treated for 24 h with control medium (●) or medium containing 5 ng/ml progesterone (△), 3 pg/ml oestradiol (□) or 5 ng/ml dexamethasone (■). The EVOCs were then challenged with control medium or medium containing 1 µg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids. The EVOC tissues were collected and total RNA extracted for analysis of *IL1B* (A, B), *IL6* (C, D)*, IL8* (E, F) and *CCL5* (G, H) mRNA expression by qPCR. For follicular EVOCs: control, n = 6, oestradiol, n = 5, progesterone, n = 5, dexamethasone, n = 3. For Luteal EVOCs: control, n = 10, oestradiol, n = 6, progesterone. n = 6, dexamethasone, n = 4. Data are presented in dot plots with the horizontal bar representing the mean and vertical bars SEM. Data were analysed by GLM multivariate ANOVA and Dunett's pairwise multiple comparison t-test, values differ between groups: *P < 0.05, and each dot represents an individual animal.

Figure 7

Ovarian steroids do not regulate endometrial cell responses to LPS. Endometrial epithelial (A, D) and stromal (B, E) cells from the ipsilateral horn of early luteal phase beef herifer endometrium, and peripheral blood derived mononuclearcells (PBMCs; C, F) from beef heifers were pre-treated for 24 h with control medium (Open bar) or medium containing 5 ng/ml progesterone (Chequered bar), 3 pg/ml oestradiol (Striped bar) or 5 ng/ml dexamethasone (Closed bar). After 24 h, the cells were challenged with control medium or medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. Supernatants were collected and analysed for IL-6 (A – C) and IL-8 (D – F) by ELISA. Data are presented as mean (SEM). Data were analysed by GLM multivariate ANOVA and Dunett's pairwise multiple comparison t-test, values differ from control: *P < 0.05. Number of animals, n = 4.

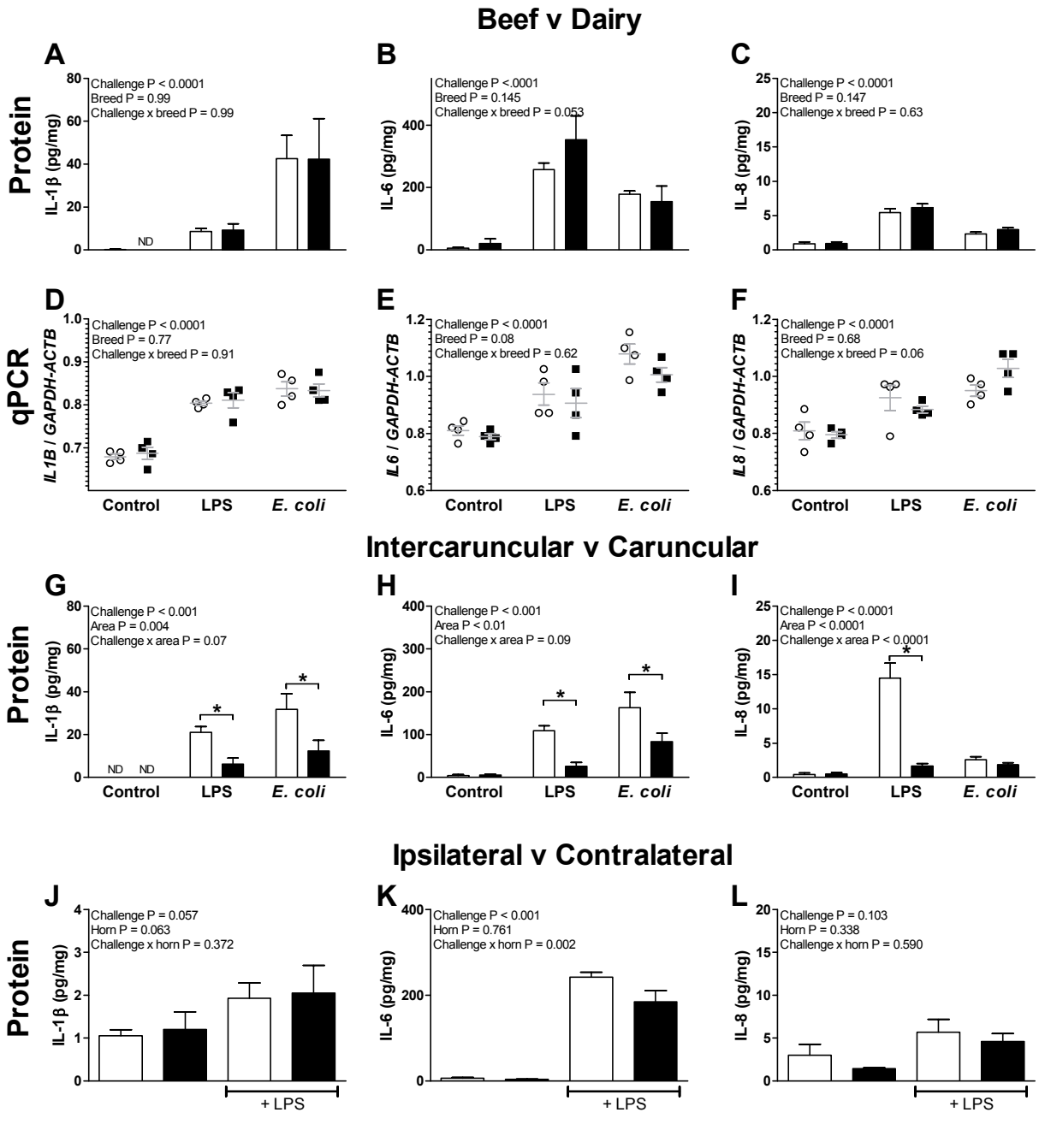
Figure 8

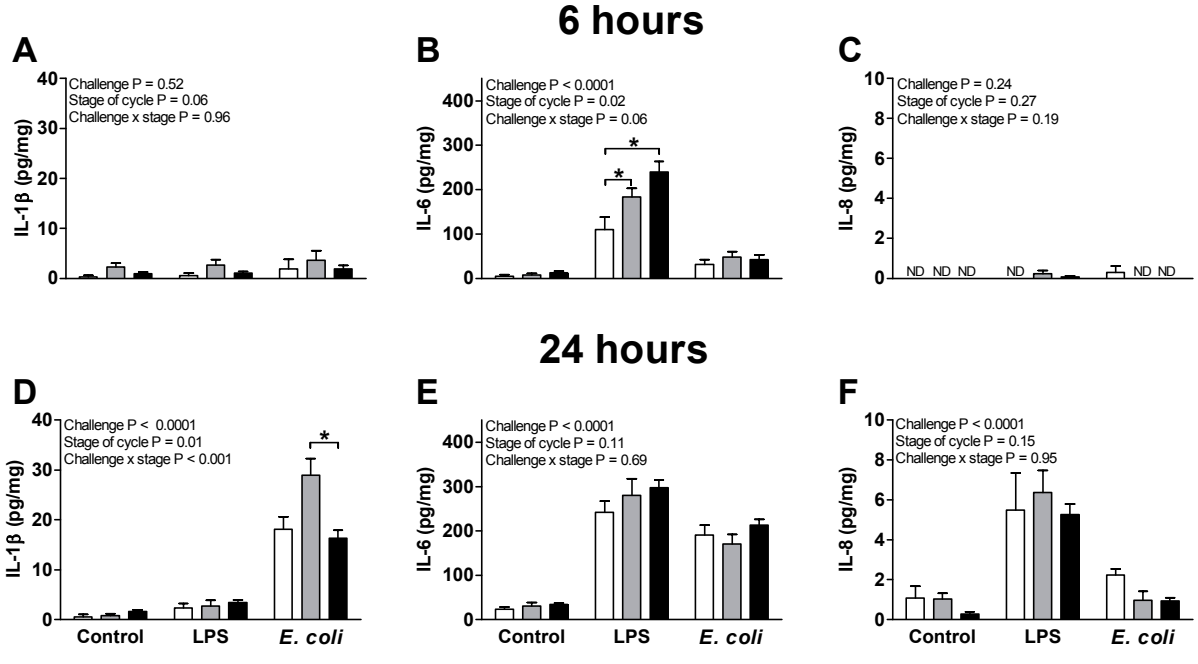
High concentrations of ovarian steroids do not regulate endometrial stromal cell responses to LPS. Endometrial stromal cells from the ipsilateral horn of early luteal phase beef heifer endometrium were pre-treated for 24 h with control medium or medium containing 1 to 30 pg/ml oestradiol (A, C, E), 1 to 30 ng/ml progesterone (B, D, F) or 5 ng/ml dexamethasone. After 24 h, the cells were challenged with control medium or medium containing 100 ng/ml LPS for a further 24 h in the presence of the steroids. Supernatants were collected and analysed for IL-6 (A, B) and IL-8 (C, D) by ELISA. Cell viability was determined by MTT assay (E, F). Data are presented as mean (SEM). Data

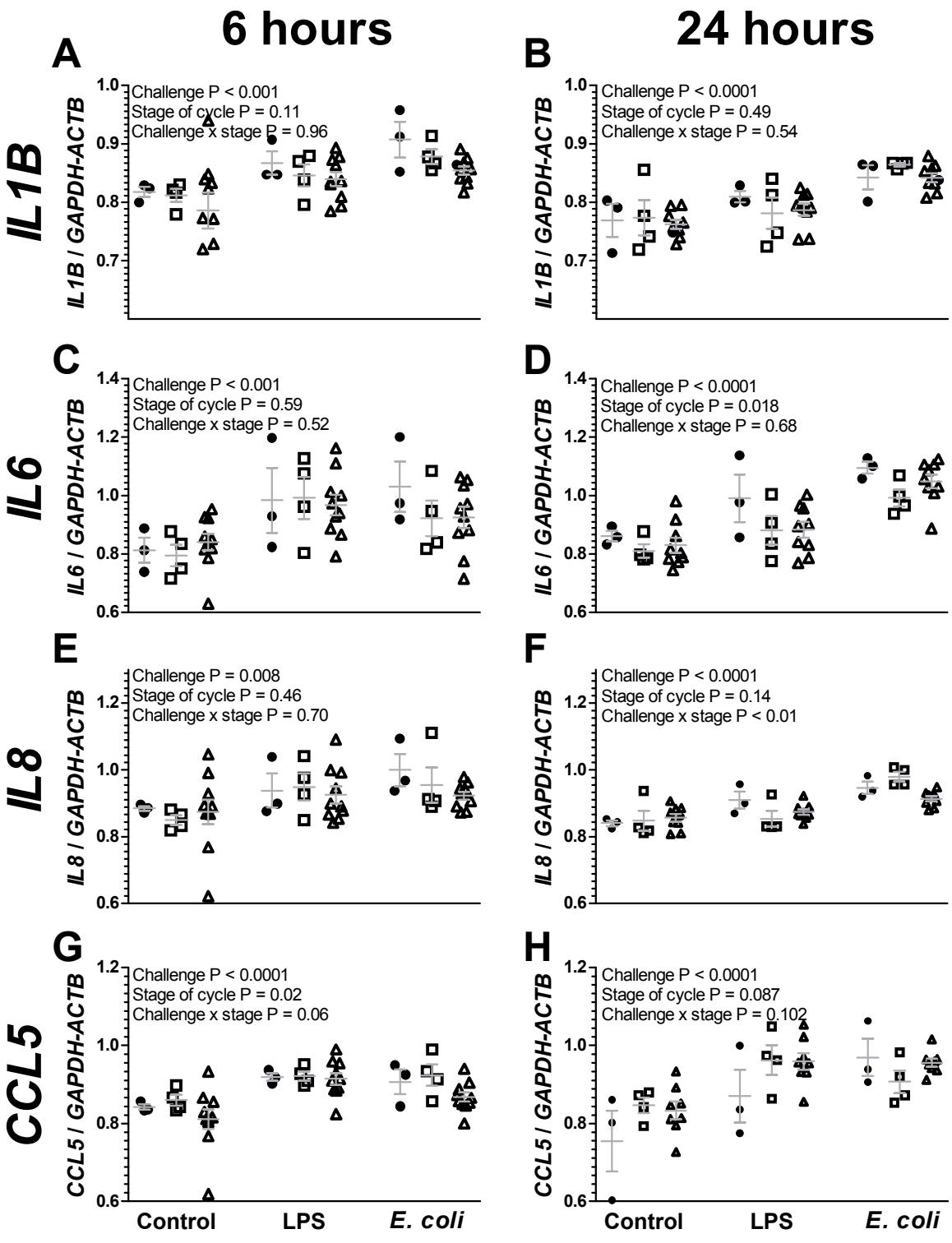
were analysed by ANOVA and Dunett's pairwise multiple comparison t-test, values differ from LPS: *P < 0.05. Number of animals, n = 3.

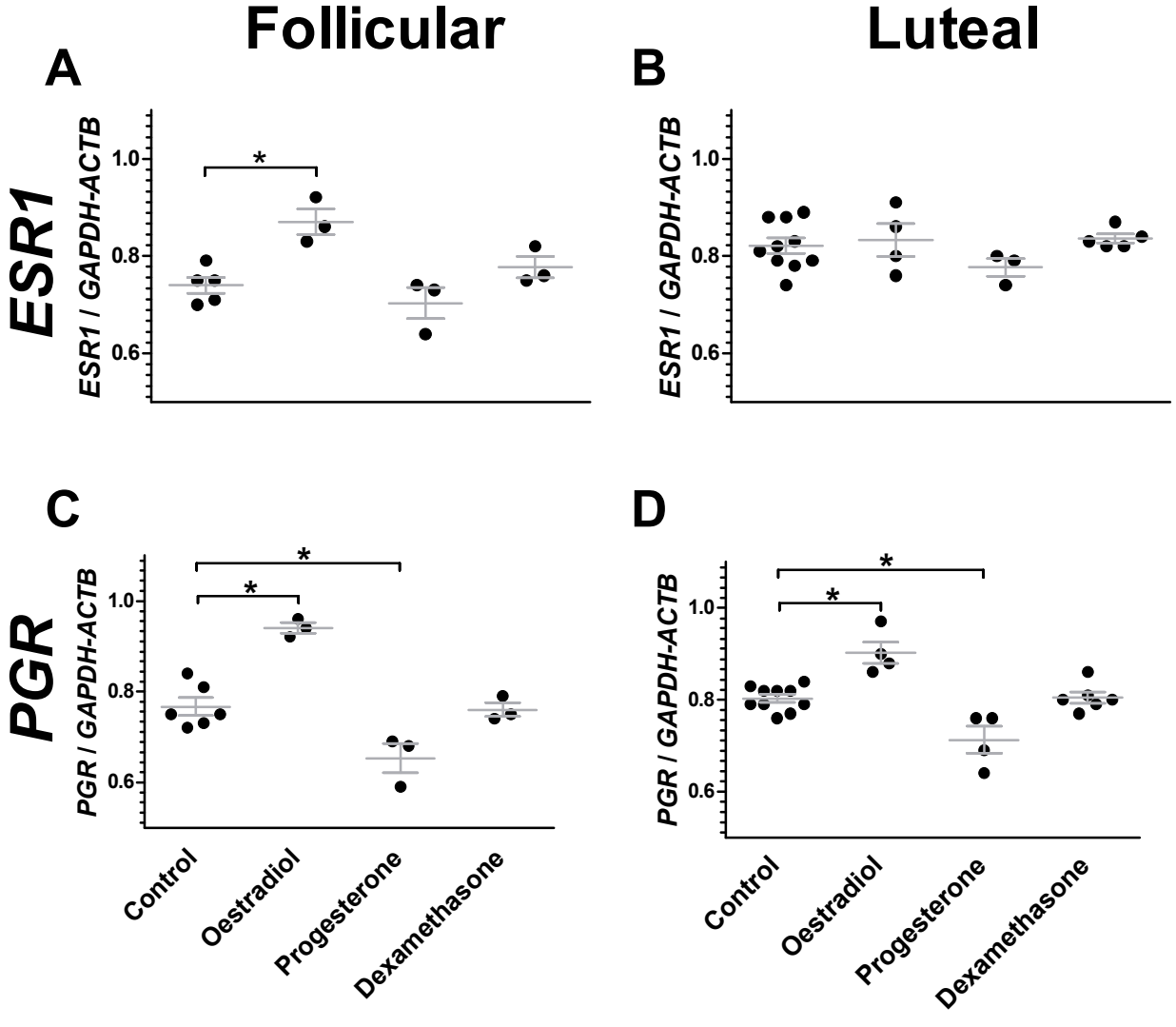
Figure 9

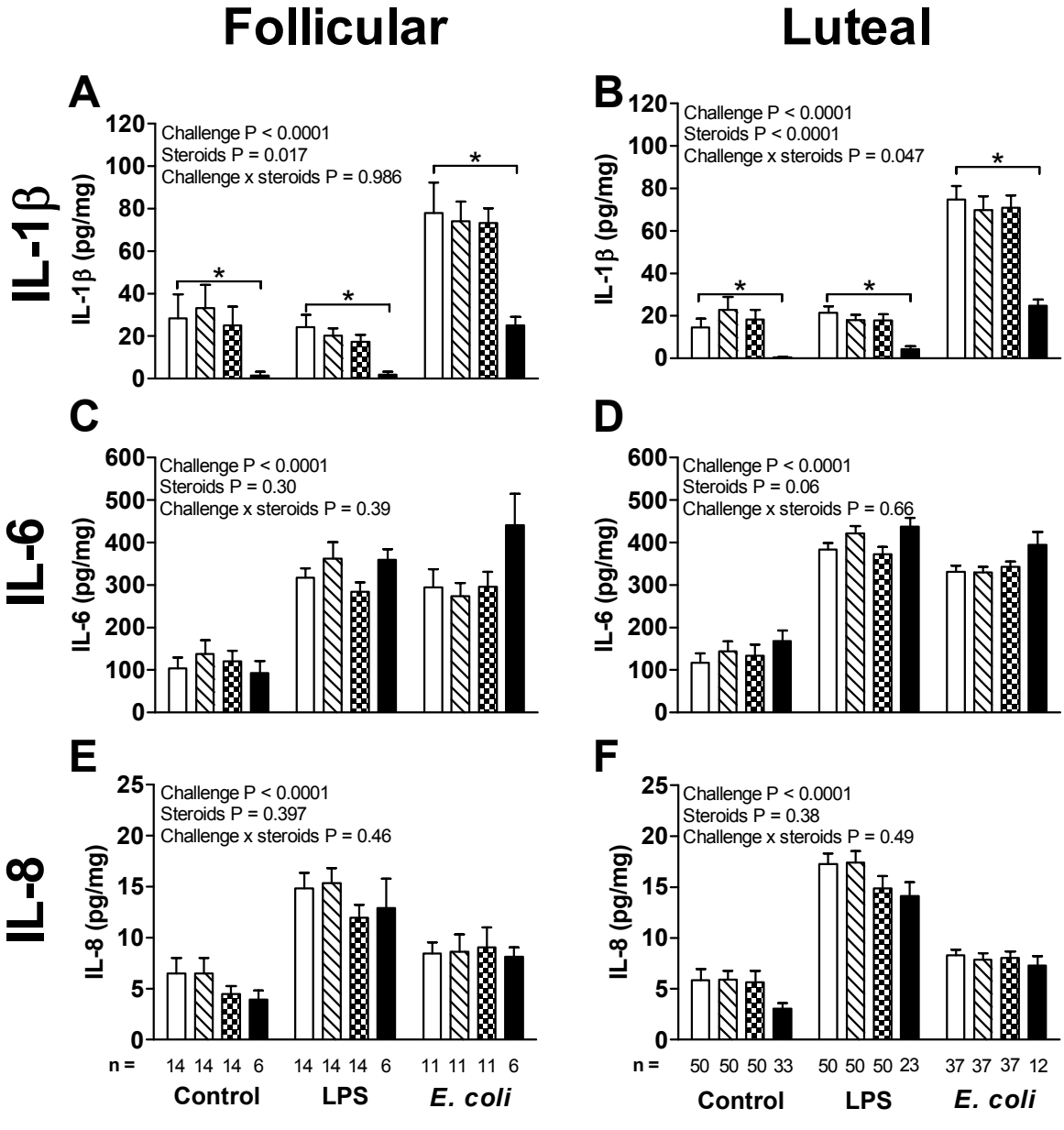
Competitive inhibition of ovarian nuclear receptors within *ex vivo* organ cultures. Endometrial EVOCs were harvested from the intercaruncular areas of early luteal phase beef heifer endometrium (contralateral horn), and treated for 24 h with control medium or medium containing 3 pg/ml oestradiol and/or 100nM MPP (A, D, G), 5 ng/ml progesterone and/or 100 nM mifepristone (B, E, H) or 5 ng/ml dexamethasone and/or 100nM mifepristone (C, F, I). After 24 h EVOCs were challenged with control medium of medium containing 1 μg/ml LPS or 1 x 10^3 CFU/ml *E. coli* for a further 24 h in the presence of the steroids/antagonists. Supernatants were collected and analysed for IL-1β (A – C), IL-6 (D – F) and IL-8 (G – I) by ELISA. Data are presented as mean (SEM). Data were analysed by two-way ANOVA and Dunett's pairwise multiple comparison, values differ between groups: *P < 0.05. Number of animals (n) is indicated within the figure.

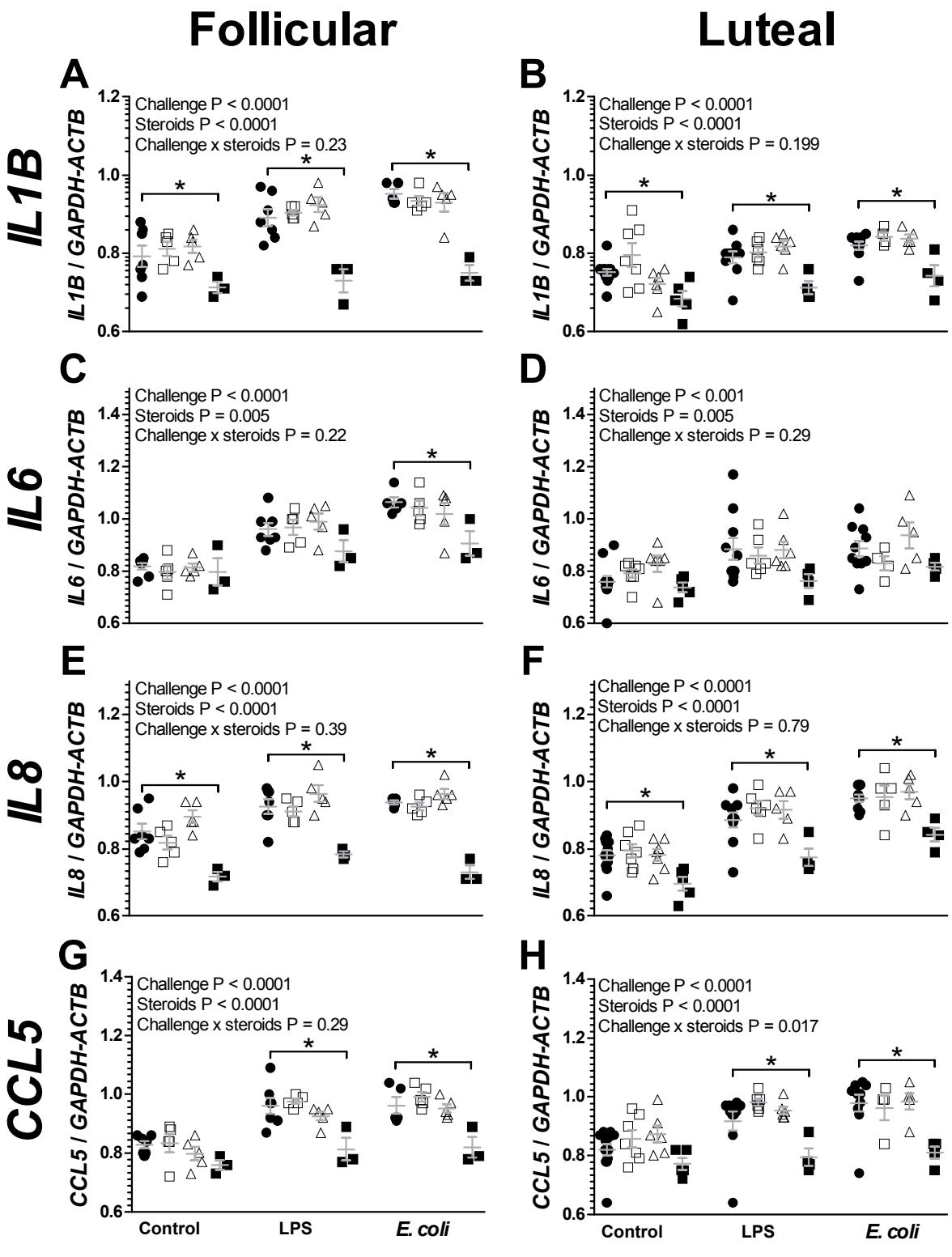

Beef v Dairy
A
B
C
Protein
IL-1β (pg/mg)
Challenge P < 0.0001
Breed P = 0.99
Challenge x breed P = 0.99
ND
IL-6 (pg/mg)
Challenge P <.0001
Breed P = 0.145
Challenge x breed P = 0.053
IL-8 (pg/mg)
Challenge P < 0.0001
Breed P = 0.147
Challenge x breed P = 0.63
D
E
F
qPCR
IL1B / GAPDH-ACTB
Challenge P < 0.0001
Breed P = 0.77
Challenge x breed P = 0.91
IL6 / GAPDH-ACTB
Challenge P < 0.0001
Breed P = 0.08
Challenge x breed P = 0.62
IL8 / GAPDH-ACTB
Challenge P < 0.0001
Breed P = 0.68
Challenge x breed P = 0.06
Control
LPS
E. coli
Intercaruncular v Caruncular
G
H
I
Protein
IL-1β (pg/mg)
Challenge P < 0.001
Area P = 0.004
Challenge x area P = 0.07
ND
ND
IL-6 (pg/mg)
Challenge P < 0.001
Area P < 0.01
Challenge x area P = 0.09
IL-8 (pg/mg)
Challenge P < 0.0001
Area P < 0.0001
Challenge x area P < 0.0001
Control
LPS
E. coli
Ipsilateral v Contralateral
J
K
L
Protein
IL-1β (pg/mg)
Challenge P = 0.057
Horn P = 0.063
Challenge x horn P = 0.372
IL-6 (pg/mg)
Challenge P < 0.001
Horn P = 0.761
Challenge x horn P = 0.002
IL-8 (pg/mg)
Challenge P = 0.103
Horn P = 0.338
Challenge x horn P = 0.590
+ LPS

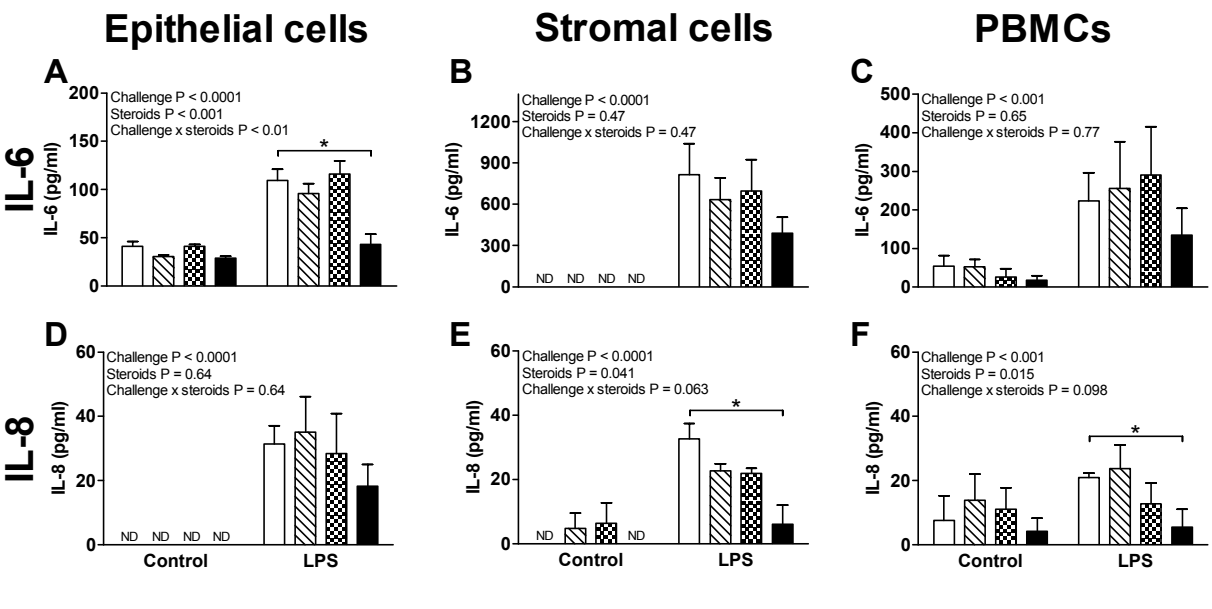

6 hours
A
Challenge P = 0.52
Stage of cycle P = 0.06
Challenge x stage P = 0.96
IL-1β (pg/mg)
B
Challenge P < 0.0001
Stage of cycle P = 0.02
Challenge x stage P = 0.06
IL-6 (pg/mg)
C
Challenge P = 0.24
Stage of cycle P = 0.27
Challenge x stage P = 0.19
IL-8 (pg/mg)
ND ND ND ND ND ND
24 hours
D
Challenge P < 0.0001
Stage of cycle P = 0.01
Challenge x stage P < 0.001
IL-1β (pg/mg)
E
Challenge P < 0.0001
Stage of cycle P = 0.11
Challenge x stage P = 0.69
IL-6 (pg/mg)
F
Challenge P < 0.0001
Stage of cycle P = 0.15
Challenge x stage P = 0.95
IL-8 (pg/mg)
Control
LPS
E. coli

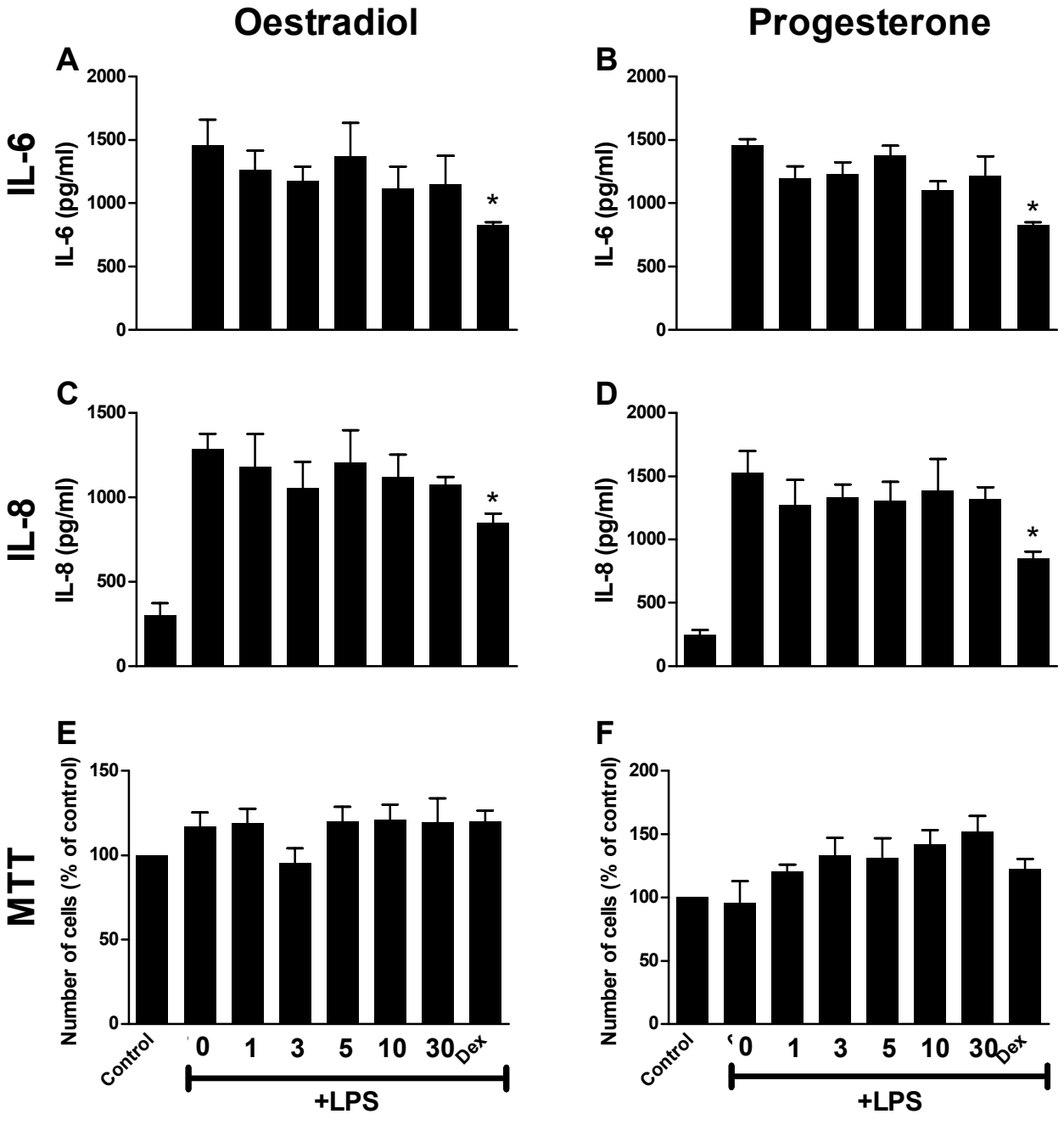

6 hours
24 hours
A
B
C
D
E
F
G
H
IL1B
IL6
IL8
CCL5
IL1B / GAPDH-ACTB
IL6 / GAPDH-ACTB
IL8 / GAPDH-ACTB
CCL5 / GAPDH-ACTB
Challenge P < 0.001
Stage of cycle P = 0.11
Challenge x stage P = 0.96
Challenge P < 0.0001
Stage of cycle P = 0.49
Challenge x stage P = 0.54
Challenge P < 0.001
Stage of cycle P = 0.59
Challenge x stage P = 0.52
Challenge P < 0.0001
Stage of cycle P = 0.018
Challenge x stage P = 0.68
Challenge P = 0.008
Stage of cycle P = 0.46
Challenge x stage P = 0.70
Challenge P < 0.0001
Stage of cycle P = 0.14
Challenge x stage P < 0.01
Challenge P < 0.0001
Stage of cycle P = 0.02
Challenge x stage P = 0.06
Challenge P < 0.0001
Stage of cycle P = 0.087
Challenge x stage P = 0.102
Control
LPS
E. coli

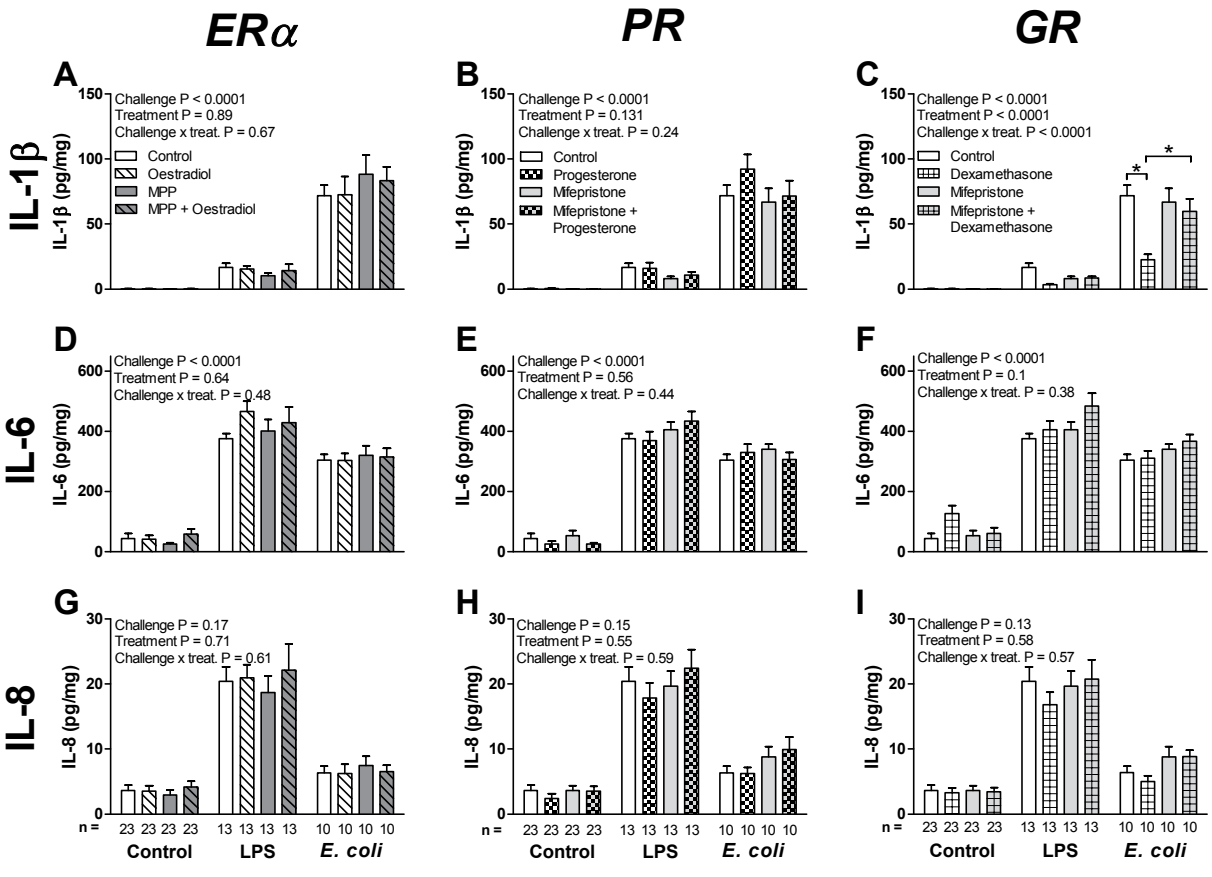

Follicular
Luteal
A
B
C
D
ESR1
PGR
ESR1 / GAPDH-ACTB
PGR / GAPDH-ACTB
1.0
0.8
0.6
*
Control
Oestradiol
Progesterone
Dexamethasone


Follicular
Luteal
IL-1β
IL-6
IL-8
A
B
C
D
E
F
IL-1β (pg/mg)
IL-6 (pg/mg)
IL-8 (pg/mg)
Challenge P < 0.0001
Steroids P = 0.017
Challenge x steroids P = 0.986
Challenge P < 0.0001
Steroids P < 0.0001
Challenge x steroids P = 0.047
Challenge P < 0.0001
Steroids P = 0.30
Challenge x steroids P = 0.39
Challenge P < 0.0001
Steroids P = 0.06
Challenge x steroids P = 0.66
Challenge P < 0.0001
Steroids P = 0.397
Challenge x steroids P = 0.46
Challenge P < 0.0001
Steroids P = 0.38
Challenge x steroids P = 0.49
n = 14 14 14 6 14 14 14 6 11 11 11 6
n = 50 50 50 33 50 50 50 23 37 37 37 12
Control
LPS
E. coli


Follicular
Luteal
A
B
C
D
E
F
G
H
IL1B
IL6
IL8
CCL5
IL1B / GAPDH-ACTB
IL6 / GAPDH-ACTB
IL8 / GAPDH-ACTB
CCL5 / GAPDH-ACTB
Challenge P < 0.0001
Steroids P < 0.0001
Challenge x steroids P = 0.23
Challenge P < 0.0001
Steroids P < 0.0001
Challenge x steroids P = 0.199
Challenge P < 0.0001
Steroids P = 0.005
Challenge x steroids P = 0.22
Challenge P < 0.001
Steroids P = 0.005
Challenge x steroids P = 0.29
Challenge P < 0.0001
Steroids P < 0.0001
Challenge x steroids P = 0.39
Challenge P < 0.0001
Steroids P < 0.0001
Challenge x steroids P = 0.79
Challenge P < 0.0001
Steroids P < 0.0001
Challenge x steroids P = 0.29
Challenge P < 0.0001
Steroids P < 0.0001
Challenge x steroids P = 0.017
Control
LPS
E. coli


Epithelial cells
Stromal cells
PBMCs
A
B
C
D
E
F
IL-6
IL-8
IL-6 (pg/ml)
IL-8 (pg/ml)
Challenge P < 0.0001
Steroids P < 0.001
Challenge x steroids P < 0.01
Challenge P < 0.0001
Steroids P = 0.47
Challenge x steroids P = 0.47
Challenge P < 0.001
Steroids P = 0.65
Challenge x steroids P = 0.77
Challenge P < 0.0001
Steroids P = 0.64
Challenge x steroids P = 0.64
Challenge P < 0.0001
Steroids P = 0.041
Challenge x steroids P = 0.063
Challenge P < 0.001
Steroids P = 0.015
Challenge x steroids P = 0.098
ND
Control
LPS
*


Oestradiol
Progesterone
A
B
C
D
E
F
IL-6
IL-8
MTT
IL-6 (pg/ml)
IL-8 (pg/ml)
Number of cells (% of control)
2000
1500
1000
500
0
150
100
50
200
*
Control
0
1
3
5
10
30
Dex
+LPS


ERα
PR
GR
A
B
C
D
E
F
G
H
I
IL-1β
IL-6
IL-8
IL-1β (pg/mg)
IL-6 (pg/mg)
IL-8 (pg/mg)
Challenge P < 0.0001
Treatment P = 0.89
Challenge x treat. P = 0.67
Control
Oestradiol
MPP
MPP + Oestradiol
Challenge P < 0.0001
Treatment P = 0.131
Challenge x treat. P = 0.24
Control
Progesterone
Mifepristone
Mifepristone + Progesterone
Challenge P < 0.0001
Treatment P < 0.0001
Challenge x treat. P < 0.0001
Control
Dexamethasone
Mifepristone
Mifepristone + Dexamethasone
*
*
Challenge P < 0.0001
Treatment P = 0.64
Challenge x treat. P = 0.48
Challenge P < 0.0001
Treatment P = 0.56
Challenge x treat. P = 0.44
Challenge P < 0.0001
Treatment P = 0.1
Challenge x treat. P = 0.38
Challenge P = 0.17
Treatment P = 0.71
Challenge x treat. P = 0.61
Challenge P = 0.15
Treatment P = 0.55
Challenge x treat. P = 0.59
Challenge P = 0.13
Treatment P = 0.58
Challenge x treat. P = 0.57
n = 23 23 23 23 13 13 13 13 10 10 10 10
Control
LPS
E. coli