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Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation

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ABSTRACT

In cattle, elevated concentrations of circulating progesterone (P4) in the immediate post-conception period have been associated with an advancement of conceptus elongation, an associated increase in interferon-tau production and higher pregnancy rates. Low P4 has been implicated as a causative factor in the low pregnancy rates observed in dairy cows. The aims of this study were (1) to describe the changes that occur in the bovine endometrial transcriptome during the estrous cycle, (2) to determine how elevated P4 affects the temporal pattern of gene expression in the endometrium of cyclic heifers, (3) to determine if the expression of these genes is altered in heifers with low P4 and (4) to determine the consequences of low P4 for conceptus development following embryo transfer. The main findings were that 1) relatively few differences occurred in endometrial gene expression during the early luteal phase of the estrous cycle under normal concentrations of P4 (Day 5 versus Day 7) but comparison of endometria from more distant stages of the luteal phase (Day 7 versus Day 13) revealed large transcriptional changes; 2) exogenous supplementation of P4, leading to elevated concentrations from Day 3 to Day 8, considerably altered the expression of a large number of genes at all stages of the luteal phase; 3) induction of low circulating P4 altered the normal temporal changes that occured in the expression of these genes, mainly by delaying their expression; 4) this delay in gene expression was, in part, due to delayed down regulation of the PGR from the LE and GE, and 5) the altered endometrial gene expression induced by low P4 was associated with a reduced capacity of the uterus to support conceptus development after embryo transfer on Day 7. In conclusion, the present study provides clear evidence for a temporal change in the transcriptomic signature of the

bovine endometrium which is sensitive to the concentrations of circulating P4 in the first few days after estrus and which can, under conditions of low progesterone, lead to an suboptimal uterine environment and a reduced ability to support conceptus elongation.

INTRODUCTION

The steroid hormone progesterone (P4) plays a key role in the reproductive events associated with the establishment and maintenance of pregnancy. Elevated concentrations of circulating P4 in the immediate post-conception period are associated with an advancement of conceptus elongation [1-3], an associated increase in interferon-tau production [4, 5] and higher pregnancy rates in cattle and sheep [6-8]. In cattle, approximately 40% of conception loss is estimated to occur in the period from Days 8 to 16 of pregnancy (Day 0 = ovulation) [9]; a substantial proportion of this loss may be attributable to inadequate circulating P4 concentrations and the subsequent downstream consequences on endometrial gene expression [10] and histotroph secretion into the uterine lumen [11].

Preparation of the uterine endometrium for embryo attachment and implantation in all studied mammals, including ruminants, involves carefully orchestrated spatio-temporal alterations in transcriptomic profiles. In both cyclic and pregnant animals, similar changes occur in endometrial gene expression up to initiation of conceptus elongation, suggesting that the default mechanism in the uterus is to prepare for an expect pregnancy [12]. This is strengthened by the fact that it is possible to transfer an embryo to a synchronous uterus seven days after estrus and establish higher rate of pregnancy, as is routine in commercial embryo transfer. It is only in association with maternal recognition of pregnancy, which occurs on approximately Day 16 in cattle, that significant changes in the transcriptomic profile are detectable between cyclic and pregnant endometria [12], when the endometrium responds to increasing concentrations of interferon-tau secreted by the filamentous conceptus. Elevated P4 advances the transcriptomic changes which normally occur

during pregnancy resulting in enhanced conceptus elongation [13]. Interestingly, we have shown that the embryo does not have to be present during the period of P4 elevation in order to benefit from it, supporting the concept that the positive effect on conceptus growth is mediated via P4-induced changes in the endometrial transcriptome [14].

Low P4 concentrations have been implicated as a causative factor in the low pregnancy rates observed in dairy cows [15]. Recent evidence from our group has shown that lower circulating concentrations of P4 in post partum dairy cows are associated with an impaired ability of the oviduct/uterus to support embryo development compared with that of dairy heifers [16]. To truly demonstrate the importance of P4 for embryo survival, a model in which low P4 concentrations can be maintained and that parallels the endogenous output of P4 from the CL, is required. We recently reported that administration of a prostaglandin F2α (PGF2α) analogue on Days 3, 3.5, and 4 postestrus induces reduced circulating P4 concentrations during the early luteal phase in cattle [17]. However, the mechanism of action of low P4 concentrations on reduced embryo/conceptus development and survival are not known. The use of this model of low P4, that is independent of the many confounding factors that affect pregnancy rates in dairy cows, can facilitate the understanding of the mechanisms of action of low P4 on conceptus development. Thus, it is hypothesized that in heifers with induced low P4, there would sequential modulations of the endometrial transcriptome leading to a suboptimal uterine environment incapable of supporting normal conceptus development. The specific objectives were (1) to describe the changes that occur in the endometrial transcriptome during the estrous cycle, (2) to determine how elevated P4 affects the temporal pattern of gene expression in the endometrium of cyclic heifers, (3) to determine if the expression of

these genes is altered in heifers with low P4 and (4) to determine the consequences of low P4 for conceptus development following embryo transfer.

MATERIALS AND METHODS

All experimental procedures involving animals were licensed by the Department of Health and Children, Ireland, in accordance with the Cruelty to Animals Act (Ireland 1897) and the European Community Directive 86/609/EC and were sanctioned by the Animals Research Ethics Committee, University College Dublin, Ireland. Unless otherwise stated all reagents were sourced from Sigma-Aldrich, Dublin, Ireland.

Experiment 1

The aim of Experiment 1 was to describe global changes in the endometrial transcriptome during the luteal phase of the bovine estrous cycle. This study was carried out as part of a larger study identifying changes in the endometrial transcriptome under varying conditions of pregnancy and/or P4 status. Only data relating to cyclic heifers will be presented here. The experimental model used has been previously described [3, 13]. Briefly, estrus was synchronized in crossbred-beef heifers (n=263) using an 8-day CIDR treatment (controlled internal drug release device, 1.94 g P4; InterAg, Hamilton, New Zealand) with an intramuscular injection of a PGF2α analogue (Estrumate, Shering-Plough Animal Health, Hertfordshire, UK; 2 ml, equivalent to 0.5 mg cloprostenol) one day before CIDR removal. Only those heifers which came into standing estrus (Day 0; n=210) within a narrow window (36 to 48 h after CIDR removal) were used. One-third of these heifers were randomly assigned to be non-inseminated cyclic heifers. In order to elevate circulating

concentrations of P4 approximately one-half of the heifers received a P4-releasing intravaginal device (PRID, containing 1.55 g P4, CEVA Animal Health Ltd, Chesham, UK) on Day 3 of the estrous cycle. For the current study, this generated two treatment groups: (i) cyclic heifers with high P4 and (ii) cyclic heifers with normal P4. Blood samples were taken from all heifers twice daily from Days 0 to 7 after estrus and once daily thereafter until slaughter to characterize the post-ovulatory changes in concentrations of P4 in serum; these profiles have been previously published [3] and confirm that the PRID treatment did, as expected, significantly increase P4 concentrations between Day 3.5 and Day 8. Heifers were slaughtered on either Day 5 (n=16), 7 (n=15), 13 (n=12) or 16 (n=11), of the luteal phase of the estrous cycle.

Tissue collection

Within 30 min of slaughter the reproductive tract of each heifer was recovered and placed on ice. The uterine horn ipsilateral to the corpus luteum (CL) was flushed with 20 ml of phosphate-buffered saline containing 5% fetal calf serum. Strips of endometrial tissue (~300 mg) were removed from the ipsilateral horn and immediately immersed in 1:5 w/v of RNAlater. Endometrial samples were stored at 4°C for 24 h. Excess RNAlater was removed, samples were placed into new RNase/DNase free tubes and stored at -80°C until RNA extraction.

RNA extraction and microarray hybridization

RNA extraction and subsequent microarray analysis was carried out as described previously [13] on endometria from cyclic heifers with high (H) or normal (N) P4 on Days 5, 7, 13 and 16 of the estrous cycle (n=5 animals per treatment per

time point; n=40 microarrays in total). Total RNA was extracted from 100 mg of endometrial homogenate using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. On column DNase digestion and RNA cleanup was performed using the Qiagen mini kit (Qiagen, Crawley, West Sussex, UK) and RNA quality and quantity was assessed using the Agilent bioanalyser (Agilent Technologies, Santa Clara, CA, USA).

All microarray protocols were carried out by Almac Diagnostics Ltd, (Craigavon, Armagh, Northern Ireland) as previously described [13]. Briefly, 2 µg of total RNA was converted to cDNA via first and second strand synthesis using the GeneChip® Expression 3'-Amplification One-Cycle cDNA Synthesis kit and the GeneChip® Eukaryotic PolyA RNA Control Kit. This double stranded cDNA was cleaned-up using the GeneChip® Sample Cleanup Module. Biotin labeled cRNA was synthesized using the GeneChip® Expression 3'-Amplification in vitro transcription (IVT) labeling kit and unincorporated NTPs were removed using the GeneChip® Sample Cleanup Module. cRNA quality was assessed using an Eppendorf biophotometer and the Agilent 2100 bioanalyzer. Twenty five micrograms of cRNA was fragmented by incubation with 5X Fragmentation buffer and RNase-free water at 94°C for 35 min. The quality of the 35-200 base fragments generated for hybridization was assessed using the Agilent 2100 bioanalyzer. Once the yield was adjusted, 15 mg of fragmented cRNA was made up in hybridization cocktail as per the Affymetrix technical manual corresponding to a 49-format (standard)/64-format array. The hybridization cocktail was added to the GeneChip® Bovine Genome Array and hybridized for 16 h at 45°C, washed and stained and scanned using the GeneChip® Scanner 3000.

Experiment 2

The aim of Experiment 2 was to determine how the temporal changes identified in Experiment 1 were affected by low P4 concentrations. The estrous cycles of an additional batch of cross-bred beef heifers were synchronized as described in Experiment 1 (with the exception that the CIDRs containing 1.38 g P4 were sourced from Pfizer Animal Health, Ireland) and only those observed in standing estrus (n=40) were used. All heifers were randomly assigned to one of two treatments, (i) cyclic heifers with normal P4 (n=12) and (ii) cyclic heifers with low P4 (n=28). To achieve low P4 concentrations heifers received 3 intramuscular injections of 15 mg of a PGF2α analogue (Estrumate) on Day 3, 3.5 and 4 of the estrous cycle as previously described [17]. A greater number were assigned to the low P4 group as previous studies have shown that a proportion of animals either do not respond to the PGF2α administration or undergo luteolysis [17]. All heifers were blood sampled daily from estrus until slaughter on either Day 7 or Day 13 of the estrous cycle to monitor P4 concentrations. These days were chosen to coincide with the timing of the down-regulation of the PGR in the luminal epithelium, a key event required for the normal temporal regulation of gene expression in the endometrium [18].

Tissue collection and serum P4 analysis

At slaughter all reproductive tracts were processed as described for Experiment 1 above. Additionally, for immunohistochemistry, whole uterine cross sections were removed from the tip of each ipsilateral uterine horn and immediately preserved in 10% buffered formalin for 24 h. Samples were then processed by dehydration through a series of ascending concentrations of ethanol, cleared in xylene and mounted in paraffin wax blocks. Serum P4 concentrations were determined using

the solid phase RIA Coat-A-Count progesterone kit (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as previously described (Beltman et al 2010 RFD paper RD09302, in press) The intra-assay CVs were 16.3%, 9.9% and 8.3%, and inter-assay CVs were 16.5%, 10.1% and 8.3% for low, medium and high quality control serum pools, respectively. The assay sensitivity was 0.03 ng progesterone/ml of serum. Animals in the low P4 group that did not respond to PGF2α administration or those which underwent luteolysis were not used. This generated n=5 animals per time-point for control heifers and n=7 animals per time-point for heifers with low P4.

RNA isolation and Quantitative Real-time PCR (Q-RT-PCR) analysis

Quantitative real-time PCR (Q-RT-PCR) was performed on endometrial tissues for genes with the largest P4-induced fold change differences on Days 5 and 7 of the estrous cycle, as determined in Experiment 1 (Table 1) and on other candidate genes known from our previous studies to contribute to an optimal uterine environment [19]. RNA was extracted as described for Experiment 1. Five micrograms of total RNA was converted to cDNA using Superscript III and random hexamers as per manufacturer's instructions. cDNA was then quantified and diluted to give a final concentration of 10 ng/μl. All primers were designed using Primer Express Software and Q-RT-PCR was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions, consisting of 50 ng of cDNA, primer concentrations as per Table 2 and 10 μl Sybrgreen mastermix (Applied Biosystems), were carried out in duplicate with a final reaction volume of 20 μl made up with RNase DNase-free H$_2$O. All reactions were carried out under the following cycling conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 sec and 60°C for 1 min with the inclusion of a dissociation curve to ensure

specificity of amplification. A standard curve was included for each gene of interest as well as for the normalizer gene to obtain primer efficiencies.

Protein localization by immunohistochemistry

Localization of protein for the progesterone receptor (PGR) was carried out using immunohistochemistry as previously described [20] using antibodies against both the A and B isoforms (PGR-AB) or just the B isoform (PGR-B) (Laboratory Instruments and Service Centre, Ashbourne, Ireland). Briefly, 5 μm sections of uterine horn were cut from paraffin embedded blocks, mounted on glass slides coated with 98% 3-Aminopropyl triethoxy-silane (APES), and dried overnight at 56˚C. Unless otherwise stated all washes were carried out with 0.05 M Tris-buffered saline (TBS, pH 7.7) twice for 5 min at room temperature in a humid chamber. All slides were deparaffinized with xylene, rehydrated through a series of graded ethanol washes (70-100% ethanol) and antigen retrieval performed (slides were heated for 20 min in 0.01 M sodium citrate buffer, pH 6.0 and cooled for 20 min). Endogenous peroxidise was then blocked, (30 min in a 1% hydrogen peroxide in methanol solution) and non-specific binding inhibited by incubating the slides with 2% normal rabbit serum in 0.05 M TBS v/v for 30 min. Slides were then incubated with their respective primary antibodies for the optimized time and concentrations (PGR-AB 1:250 dilution; PGR-B 1:200 dilution at 4˚C overnight; [20]). After overnight incubation, all slides were washed and incubated for a further 45 min with a rabbit anti-mouse polyclonal biotinylated secondary antibody at a 1:100 dilution. All slides were then washed, incubated for 30 min with avidin-biotin complex (Vectastain Elite ABC Kit, Vector Labs, Peterborough, UK) and washed again. The 3,3'-Diaminobenzidine tetrahydrochloride (DAB) chromogen substrate was then added to

the slides for 10 min, flushed with distilled water for 7 min and dehydrated through a series of increasing ethanol concentrations (70-100%; 5 min) and subsequently cleared in two successive xylene containers for 10 min each. Finally, all slides were mounted using DPX (AGB Scientific Limited, Dublin, Ireland) and observed under 10X magnification. Intensity of staining of the luminal epithelium (LE), superficial glands (SG), deep glands (DG), stroma (STR) and myometrium (MYO) was scored by two independent observers.

Experiment 3

To test the hypothesis that low circulating P4 was associated with reduced conceptus elongation, *in vitro* derived blastocysts (n = 20) were transferred into recipient heifers with normal or low circulating P4. To test the hypothesis that a modified uterine environment induced by low circulating P4 decelerates post-hatching conceptus elongation, Day 7 blastocysts produced in vitro (n = 20) , in the absence of P4, were transferred to synchronized recipient heifers with normal (n = 9) or low (n = 11) P4 concentrations. Cross bred beef heifers of similar average age (2.2 ± 0.23 years) and weight (484 ± 8.58 kg) were synchronized using a CIDR device in conjunction with PGF2□□administration as described for Experiment 1. Low P4 was achieved by administration of PGF2□ on Days 3, 3.5 and 4 of the estrous cycle as described in Experiment 2 and was confirmed in blood samples that were collected once per day from the onset of estrus until slaughter on Day 14. Samples collected on Day 6 were analyzed for P4 concentrations on the day of collection using a room temperature rapid version of the assay described for Experiment 2 to ensure the selected recipients n = 11 had low P4 concentrations within the required range.

To ensure the production of low P$ concentrations were physiological and to due reduced blood flow from the CL, all heifers were scanned on Day 3, 5 and 6 post estrus using a portable Voluson I scanner (GE Healthcare, Chalfont St. Giles, UK)) with a linear (RSP6–16 MHz) transducer. Blood flow around the CL was measured using Doppler color flow. All Doppler images were 'frozen' and stored for subsequent analysis using the software Pixel Flux® (Chameleon-Software, Leipzig, Germany). This program was used to evaluate the area of color pixels (cm^2) in the CL as a semi quantitative measurement of luteal blood flow. Serum P4 concentrations were measured as described above. The intra-assay coefficients of variation for low, medium and high quality control serum pools were 8.5, 4.8 and 4.9%, and inter-assay CV (n = 3) for the same samples were 10, 4.9 and 5%, respectively.

Following slaughter, the reproductive tracts of heifers were recovered and transported back to the laboratory where both uterine horns were flushed with 40 ml PBS supplemented with 1% fetal calf serum to recover the conceptuses. The presence and number of conceptuses were determined using a stereomicroscope and their dimensions were measured in a petri dish over a transparent graduated grid (1 mm graduations). All CL were dissected out of the ovaries and weighed.

Data analyses

For analysis of the array data, raw signal intensities were read into R and pre-processed using functions of both affy and GCRMA packages of the BioConductor project [21]. Correspondence analysis [22] was performed to determine the greatest source of variation in the tissue samples. Lists of differentially expressed genes (DEGs) were identified using the Limma package [23] employing linear modeling and an empirical Bayes framework to shrink the variance of measurements on each

probe set. A modified t-test was then carried out and all *p* values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate method. Lists of DEGs for the various comparisons were chosen on the basis of an adjusted *p*-value of <0.05. For gene ontology (GO) overrepresentation analysis, a *p*-value of <0.01 was used as the cut-off point. Analysis of the GO terms was performed using the GOstats package of Bioconductor [24]. The chip probes were first filtered as outlined in GOstats vignette providing a 'gene universe' that represents the expressed genes exhibiting some variability across all experimental conditions. Filtering reduced the amount of false positives resulting from the analysis i.e., GO terms marked as statistically significant when in truth they are not. For each list of significant probes generated from the microarray analysis (DEGs), a conditional hyper-geometric statistical test was performed using a cut-off *p*-value of 0.01. This selected the over-represented GO nodes, i.e., those associated with the probe list more than would be expected by chance based on the 'gene universe', while taking into account the structural relationship between terms in the GO graph.

Differences in serum P4 concentrations between groups were analysed by ANOVA. CL blood flow was analysed using a repeated measures model that was developed using the MIXED procedure in SAS 9.1. Differences in mean conceptus length, width, area and number were analysed by Student's t-test [25]. Similarly, differences in gene expression, using the relative standard curve method and differences in protein intensities were analysed using a Student's t-test.

RESULTS

Experiment 1

To identify those genes altered by elevated P4 on a specific day of the estrous cycle, the gene expression in endometria of cyclic heifers with high and normal P4 within Days 5, 7, 13 and 16 was compared. Relatively small numbers of genes were altered by high P4 on a given day of the estrous cycle (36 on Day 5, 910 on Day 7, 81 on Day 13 and 716 on Day 16). On Day 5 of the estrous cycle, the expression of *DGAT2, TIPARP, DKK1* and *LOC782061* (similar to AKR1C1 protein) were altered to the greatest extent (Figure 1, Supplementary Table 1A). On Day 7, elevated P4 altered the expression of 910 genes in total (Figure 1, Supplementary Table 1B) with the expression of *CYP26*, *DGAT2*, and *ASGR2* increased and *FABP3*, *PRC1* and *SLC2A5* decreased to the greatest degree.

In contrast to the differences in expression on a given day, a large number of genes were temporally regulated (i.e., changed between successive days). In particular, the longer the time interval between days the greater the number of DEGs (e.g., for the high P4 group, Day 5 vs Day 7: 3652 probe sets; Day 7 vs Day 13: 6411 probe sets; Figure 1). Between Day 5 and Day 7 of the estrous cycle heifers with normal P4 had negligible differences in endometrial gene expression (7 DEGs) compared with those with elevated P4 in which the expression of 3654 genes was altered (Supplementary Table 2). These genes, altered by high P4, were represented in all GO SLIM categories including those involved in transport, protein transport and transcription (Figure 2A). When Day 7 and 13 endometria were compared, despite considerable differences in gene expression in both groups, P4 supplementation altered considerably more genes than the equivalent normal P4 heifers (6411 vs 3969, respectively) which represented more genes in each of the GO SLIM categories with the exception of cell cycle, protein modification and biosynthetic process (Supplementary Table 3; Figure 2B). Finally, analysis of the differences between Day

13 and Day 16 of the luteal phase of the estrous cycle revealed few DEGs in heifers with normal (160 DEGs) or high P4 (85 DEGs; Supplementary Table 3).

Experiment 2

Repeated administration of PGF2α to heifers resulted in significantly lower concentrations of serum P4 compared with untreated control heifers on all days studied (P<0.05; Figure 3).

The expression of *ASGR2*, *DKK1*, and *MGC137099* was significantly lower on Day 7 and *SLC2A5* expression was significantly higher in heifers with low P4 compared with controls (P<0.05; Figure 4A). In contrast, on Day 13, *ASGR2* and *DGAT2* expression increased in the low P4 group and *FABP3* expression was significantly decreased compared with controls (P<0.05; Figure 4B). Low P4 did not affect the expression of *CTP26*, or *PRC1* on either day examined (P>0.05). Given that *DGAT2* and *FABP3* expression was delayed in low P4 heifers expression of genes involved in their respective pathways was characterised.

Analysis of genes involved in the *FABP3* pathway indicated that *PPARA*, *PPARD, RXRA* and *RXRB* displayed similar expression patterns with lower expression on Day 7 in low P4 heifers compared with controls (P<0.05; Figure 5A). Only *PPARA* and *PPARD* were significantly altered on Day 13 by low P4 with an increase in expression compared with controls (P<0.05; Figure 5B). *RXRG* was not detectable in the endometrium and *PPARG* was not affected by the P4 status of the heifers (P>0.05).

For the genes involved in the *DGAT2* pathway, *AGPAT1* and *AGPAT5* expression was higher on Day 7 in low P4 heifers compared with controls but on Day 13 only *AGPAT1* expression was significantly higher compared with controls.

(P<0.05; Figure 5A and 5B, respectively). There was no significant effect of P4 concentration on the expression of *GPAT*, *DGKA*, *MOGAT1*, *MOGAT2*, *AGPAT 3, AGPAT 4* or *AGPAT 6* members of the DGAT2 pathway (P>0.05; data not shown).

To identify if low P4 concentrations affected the expression of the PGR and genes whose expression is altered in association with the loss of the PGR from the LE and GE [19] the expression of these genes using Q-RT-PCR was examined as well as localising the PGR protein by immunohistochemistry. *PGR* mRNA was more abundant (P < 0.05) in heifers with low P4 on Day 7 compared with controls but was not different on Day 13 (Figure 6A and 6B). Expression of *ANPEP* and *LPL* was lower (P<0.05) on Day 7 in heifers with low P4 compared with controls (Figure 6A and B). In contrast, by Day 13, *ANPEP* and *LPL* expression increased while *CTGF* expression decreased (P<0.05) in the low P4 group compared with controls.

In heifers with normal and low P4, both isoforms of the PGR localized to the LE, SG, DG, STR and MYO on Day 7 of the estrous cycle with a complete loss of PGR in the LE and SG on Day 13 in both groups (Figure 7A). Analysis of intensity of staining revealed significantly more PGR in the LE and SG of heifers with low P4 on Day 7 compared with their normal P4 counterparts (Figure 7B) indicating a delay in the normal loss of this receptor from these tissues.

Experiment 3

Recipient heifers in the low P4 group had lower (P<0.05) P4 concentrations from Day 5 to day of slaughter compared with concentrations of P4 in control recipients (Figure 8A). Moreover, low P4 recipients displayed reduced blood flow on the CL on Day 6 compared with that on CLs of control heifers (P<0.05; Figure 8B).

Recovery rate of expanded conceptuses on Day 14 was higher from normal P4 heifers (9.6 ± 1.7) compared with those recovered from recipients with low P4 (5.4 ± 1.7) although not statistically significant (P<0.09). Mean conceptus length and area were significantly reduced (P<0.05) in conceptuses recovered from low P4 recipients compared with control recipients (Figure 9A and 9B).

DISCUSSION

In the current study, the hypothesis that alterations in circulating P4 concentrations (to induce both high and low circulating P4) in heifers alters the normal sequential changes which occur in the endometrial transcriptome leading to an altered uterine environment with consequences for normal conceptus development. The main findings were that 1) only minor differences occur in endometrial gene expression profiles of heifers when short time intervals were compared during the early luteal phase under normal concentrations of P4 (Day 5 versus Day 7) but much larger differences were identified when endometria from more distant stages of the luteal phase (Day 7 versus Day 13) were compared; 2) exogenous supplementation of P4 that elevated concentrations from Day 3 to Day 8, noticeably altered the expression of a large number of genes at all stages of the luteal phase; 3) induction of low P4 concentrations in serum altered the normal temporal changes that occurred in the expression of genes in the endometrium, mainly by delaying their expression; 4) this delay in temporal changes in endometrial gene expression was, in part, due to delayed down regulation of the PGR from the LE and GE, and 5) the altered endometrial gene expression induced by low P4 was associated with a reduced capacity of the uterus to support conceptus development after multiple embryo transfer on Day 7.

Insertion of a PRID on Day 3 after estrus elevated serum P4 concentrations by approximately 1-2 ng/ml (well within physiological ranges). This led to the altered expression of over 3,500 genes between two time points relatively close together (Day 5 v Day 7). Of these P4 induced genes, over 1,000 were also differentially expressed between Day 7 and 13 in animals with normal P4, indicating that elevated P4 advanced endometrial gene expression in cyclic heifers consistent with our previous observations in pregnant heifers [13]. When endometria from given days were compared relatively few genes were altered by elevated P4 on Day 5 but, by Day 7 (when serum P4 concentrations were significantly elevated for four days [3]), a significant divergence in gene expression in the endometrium was apparent. Moreover, the genes with the largest fold change differences may contribute to the composition of the histotroph. For example, both *DGAT2* and *FABP3* are known to be involved in triglyceride synthesis and transport (discussed below in further detail). *CYP26*A is known to be involved in retinoic acid (RA) metabolism predominantly through eliminating RA [26]. The balance of RA concentration in the uterine environment has been well characterised and the modulation of RA concentrations during pregnancy has been well established [27-30]. *ASGR2* encodes for a protein that, in conjunction with *ASGR1*, forms ASGR which is involved in mediating the endocytosis of serum glycoproteins [31] and may well play a role in trafficking glycoproteins into the uterine lumen. The expression of members of glucose and fructose transporters in the uterine endometrium of ruminants and their increased expression associated with increased P4 and interferon-tau has been recently documented [19, 32]. *SLC2A5* is a facultative fructose transporter [33] known to be expressed in fast cleaving embryos to enhance fructose uptake for nucleotide synthesis [34]. The down regulation of *SLC2A5* in the uterine endometrium may

ensure that available fructose is being utilized by the embryo and not the uterine endometrium.

The main control device that ensures the sequential modulation of endometrial gene expression is circulating concentrations of P4; primarily the role P4 plays in down-regulating its own receptor from the LE and GE. Our previous studies identified that the expression pattern of a number of genes in pregnant heifers is concomitant with the early down-regulation of the PGR and that elevated P4 alters when and for how long these genes are expressed [13, 20]. In the present study a similar panel of genes that were altered by high P4 in endometria of cyclic heifers were identified. We aimed to address the question of how the expression of these genes differed in heifers with low P4 (Table 2). In the low P4 model a number of the transcripts studied displayed a delay in their expression pattern e.g. in heifers with normal or high P4 concentrations, the expression of a given gene was high on Day 7, whereas in heifers with low P4 the expression of the same gene was very low or not detectable. From Experiment 1, the expression of *DGAT2* and *ASGR2* was high on Day 7 in heifers with high and *FABP3* expression was low on Day 7 in heifers with high P4 compared with controls. However, in Experiment 2, *DGAT2* and *ASGR2* expression was high and *FABP3* expression was low on Day 13 in heifers with low P4 only i.e. the normal expression pattern of genes on Day 7 is only detectable on in heifers with low P4 on Day 13 of the estrous cycle. The fact that not all genes identified in the microarray study displayed this same delay in expression in the low P4 model indicated that either these genes were not directly regulated by P4 or their expression patterns were not critically dependent on sustained circulating concentrations of P4.

Despite the importance of P4 for the establishment and maintenance of pregnancy in mammals, paradoxically, endometrial epithelia cease expressing PGR

before implantation in all mammals studied (Bazer *et al.* 2009); this loss of PGR is a key checkpoint allowing the switch on of genes necessary for continued conceptus development. Analysis of genes previously identified as altered in association with the loss of PGR [19] and therefore important for providing an appropriate uterine environment revealed that in the low P4 model their expression levels were either suboptimal on the appropriate day of the estrous cycle (*ANPEP*, *LPL* on Day 7; *CTGF* on Day 13) or their down regulation was not complete at the appropriate time (*ANPEP* and *LPL* on Day 13). *ANPEP* is a peptidase that cleaves neutral amino acids from peptides [35] and its expression is affected by P4 in both cattle and humans [19, 36]. *ANPEP* may be involved in preventing the cleavage of proteins secreted into the uterine lumen or may be associated with allowing increased neutral amino acids into the uterine lumen both of which would alter histotroph composition. *LPL* is involved in delivering TAG to tissues [37] and may supply the uterine histotroph with TAG, an energy source the embryo can use at this stage of development [38]. CTGF, a protein involved in cell proliferation, migration and adhesion was previously identified as up-regulated in the uterine endometrium during the luteal phase of the estrous cycle/ early pregnancy in many species and was detected in the lumen of the uterus [2, 39-42]. Both elevated P4 and IFNT concentrations increase the expression of endometrial *CTGF* in cattle and sheep [2, 19] indicating that it may be involved in proliferation of the conceptus trophectoderm, thus, in animals where high P4 was induced, *CTGF*-induced trophectoderm proliferation may contribute to advanced conceptus development. It is clear from the current data that low P4 concentrations caused a disruption in the temporal regulation of these genes previously identified as required for an optimum uterine environment due, in part, to delayed down regulation of the PGR from the LE on Day 7 of the estrous cycle in heifers with low P4. Exposure of

the uterine endometrium to sustained concentrations of P4 induced the loss of PGR from the LE and GE allowing the tightly regulated temporal changes in endometrial gene to occur [18, 43]. Our data suggest that in heifers with low P4, the sustained concentrations of P4 required to induce the down regulation of the PGR from the LE and GE were not sufficient at Day 7 leading to elevated abundance of the PGR protein in low P4 heifers. This would lead to a delay in the temporal changes of genes required to provide an optimal uterine environment (e.g. *ANPEP*, *CTGF*, *LPL*) independent of the presence of a developing embryo/ conceptus. The loss of the PGR from the LE does occur by Day 13 in heifers with both normal and low P4 . This was not surprising given that, in this model of low P4, the postovulatory rise in P4, although delayed, does still occur.

Induction of low circulating concentrations of progesterone by administration of PGF2α on Day 3, 3.5 and 4 led to a significant reduction in CL blood flow on Day 6 supporting the use of this model as an effective method to obtain low P4 concentrations from heifers whilst still maintaining an output of P4 from the CL that parallels normal physiological events. Furthermore, CL size at slaughter on Day 14 was not different between heifers with low and normal progesterone, supporting the previous hypothesis that the low progesterone induced in this model was due to a change in vascularisation of the CL rather than CL size *per se* [17]. To prove low P4 has a functional effect on the endometrium, thereby affecting conceptus development, in vitro produced blastocysts were transferred to the uteri of recipient heifers with normal or low P4 using a multiple embryo transfer model previously validated with animals induced to have elevated P4 [14]. Twenty Day 7 blastocysts were transferred per recipient and all animals were slaughtered on Day 14 to assess conceptus development. As indicated previously, Day 14 was chosen for slaughter as it

represents the initiation of elongation in cattle, a critical checkpoint in development. In addition, previous personal experience has shown that in the case of multiple transfers of embryos, separating rapidly elongating conceptuses after Day 14 can be extremely difficult. Lastly, it is known that very little embryonic loss occurs after Day 14 in cattle [44] and thus survival to Day 14 represents a useful measure of developmental competence. Recovery rate on Day 14 was lower in the low P4 heifers compared with controls and the mean conceptus length and area were also significantly reduced in the presence of low P4 indicating a functional consequence for conceptus development.

The potential implications of circulating P4 on the composition of oviduct and uterine fluid are interesting. Hugentobler et al., [45] characterized the effects of changes in systemic P4 on amino acid, ion and energy substrate composition of oviduct and uterine fluids on Days 3 and 6, respectively, of the estrous cycle. Infusion of P4 had no effect on oviduct fluid secretion rate; however, uterine fluid secretion rate was lowered. Progesterone increased uterine glucose, decreased oviduct sulphate and to a lesser extent oviduct sodium but had no effect on any of the ions in the uterus. The most marked effect of P4 was on oviductal amino acid concentrations with a 2-fold increase in glycine, while in the uterus only valine was increased. These data provide evidence of P4 regulation of oviduct amino acid concentrations in cattle and may partly explain the differences in embryo development observed in Experiment 3.

In conclusion, the present study provides clear evidence for a temporal change in the transcriptomic signature of the bovine endometrium which is sensitive to the concentrations of circulating progesterone in the first few days after estrus and which

can, under conditions of low progesterone, lead to an suboptimal uterine environment and a reduced ability to support conceptus elongation..

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Numbers of differentially expressed genes that were temporally regulated in the endometrium of cyclic beef heifers with normal (CN) or high (CH) concentrations of circulating progesterone. Numbers indicate the number of different genes for each comparison. Venn diagrams indicate the number of common and unique genes that change across time between treatments.

Figure 2. Bar graph representing GO slim terms and numbers of differentially expressed genes associated with each term between Day 5 and 7 (A), Day 7 and 13 (B) and Day 13 and 16 (C) in the endometrium of cyclic beef heifers with normal (CN, black bars) or high (CH, open bars) concentrations of circulating progesterone. GO slim terms are only included when five or more genes are associated with the term in at least one category.

Figure 3. Progesterone profiles for control heifers with normal circulating progesterone (CN) and those induced to have low progesterone by repeated administration of PGF2α (CL) (Experiment 2).

Figure 4. Q-RT-PCR analysis of 8 candidate genes identified from Experiment 1 in the endometrium of cyclic beef heifers with normal (CN, black bars) or low (CL, open bars) concentrations of circulating progesterone on Day 7 (A) and Day 13 (B) of the estrous cycle. An asterisk (*) indicates a significant difference (P<0.05).

Figure 5. Q-RT-PCR analysis of 7 candidate genes involved in the *DGAT2* and *FABP3* pathways in the endometrium of cyclic beef heifers with normal (CN, black bars) or low (CL, open bars) concentrations of circulating progesterone on Day 7 (A) and Day 13 (B) of the estrous cycle. An asterisk (*) indicates a significant difference (P<0.05).

Figure 6. Q-RT-PCR analysis of 5 candidate genes, the expression of which has been shown previously to be important in an optimal uterine environment [19, 20], in the endometrium of cyclic beef heifers with normal (CN, black bars) or low (CL, open bars) concentrations of circulating progesterone on Day 7 (A) and Day 13 (B) of the estrous cycle. An asterisk (*) indicates a significant difference (P<0.05).

Figure 7. (A) Graphical representation of the intensity of PGR-AB protein isoform localized within the luminal epithelium (LE) (B) or superficial glands SG) (C) in uterine cross sections of cyclic heifers with normal (CN, black bars) or low (CL, open bars) P4 on Days 7 and 13 of the estrous cycle. (B) Representative images depicting the localization of PGR-AB protein in the luminal epithelium (LE), superficial glands (SG) and stroma (STR) of the bovine endometrium on Days 7 and 13 of the estrous cycle of heifers with normal (CN) or low (CL) P4.

Figure 8. (A) Mean serum progesterone concentrations ± SEM (ng/ml) in control heifers (n = 9) and those administered PGF2α on Days 3, 3.5 and 4 post estrus (n = 11) to induce low progesterone. Treatment with PGF2α resulted in a significant decrease in progesterone concentrations in treated animals from Day 5 (24 h after last PGF2α injection) until slaughter on Day 14, indicated by an asterisk (*, P<0.05). (B)

Blood flow intensity and representative images through a CL on Day 6 of the estrous cycle in a heifer induced to have low P4 (left) compared with a control heifer (right) measured by color Doppler ultrasound.

Figure 9. Mean (±S.E.M.) Day 14 embryo dimensions (A) and representative images of Day 14 elongating embryos following transfer of Day 7 blastocysts to synchronized recipient heifers with normal or low circulating progesterone and recovery at slaughter on Day 14.

Table 1. Entrez gene symbol and gene description for genes altered to the greatest extent by high P4 on Day 5 and Day 7 of the estrous cycle and used for Q-RT-PCR analysis in heifers with low P4 (in Experiment 2). Fold change (high P4 compared with normal P4 heifers) and significance ($P<0.05$) are given for each gene as determined by microarray analysis and validated in an independent sample set by quantitative realtime PCR.

Entrez gene ID	Gene Description	Day of cycle	Experiment 1: Affymetrix Microarray	
			Fold change (High P4)	P-value
DKK1	dickkopf homolog 1	5	-3.06	<0.05
DGAT2	diacylglycerol O-acyltransferase homolog 2	5	2.94	<0.05
DGAT2	diacylglycerol O-acyltransferase homolog 2	7	3.65	<0.01
CYP26	cytochrome P450, family 26, subfamily A, polypeptide 1	7	3.78	<0.05
ASGR2	asialoglycoprotein receptor 2	7	3.32	<0.01
MGC137099	hepatitis A virus cellular receptor 1 N-terminal domain containing protein	7	2.58	<0.01
FABP3	fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)	7	-3.03	<0.01
PRC1	protein regulator of cytokinesis 1	7	-3.07	<0.01

| SLC2A5 | solute carrier family 2 (facilitated glucose/fructose transporter), member 5 | 7 | -3.58 | <0.05 |

Table 2. Primers for all genes

To be added

Figure 1
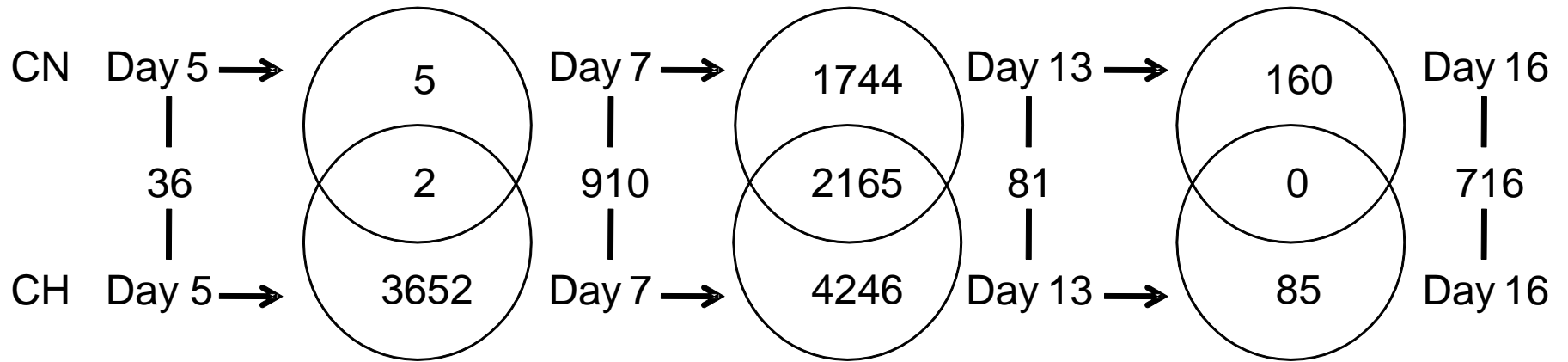
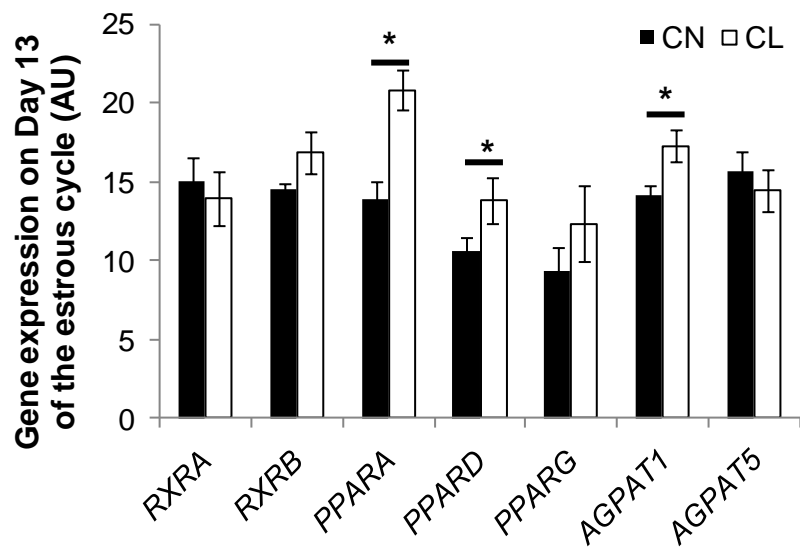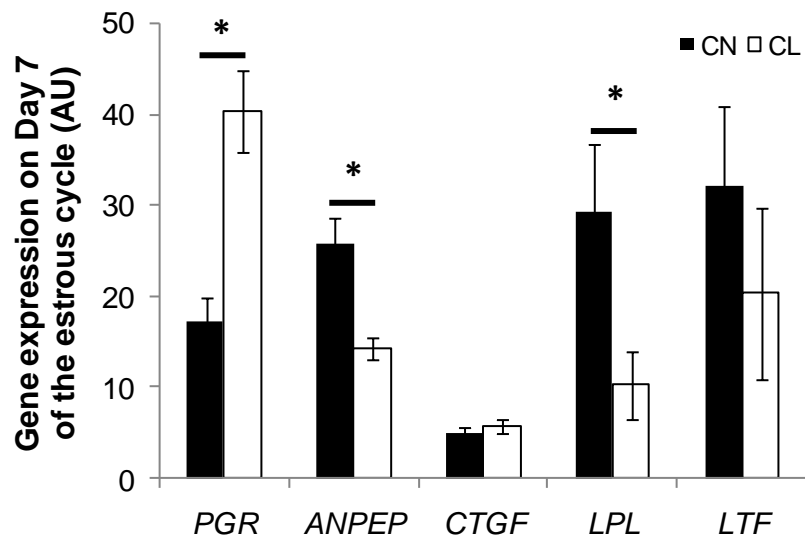Figure 2 (A)
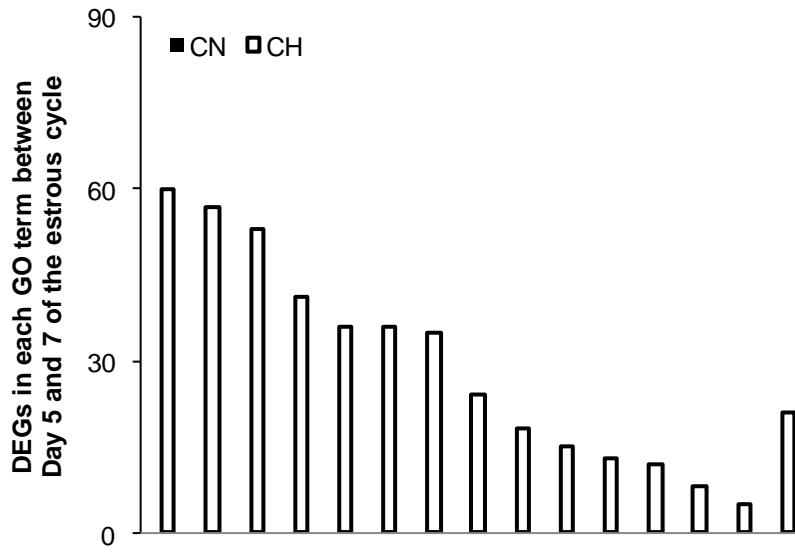
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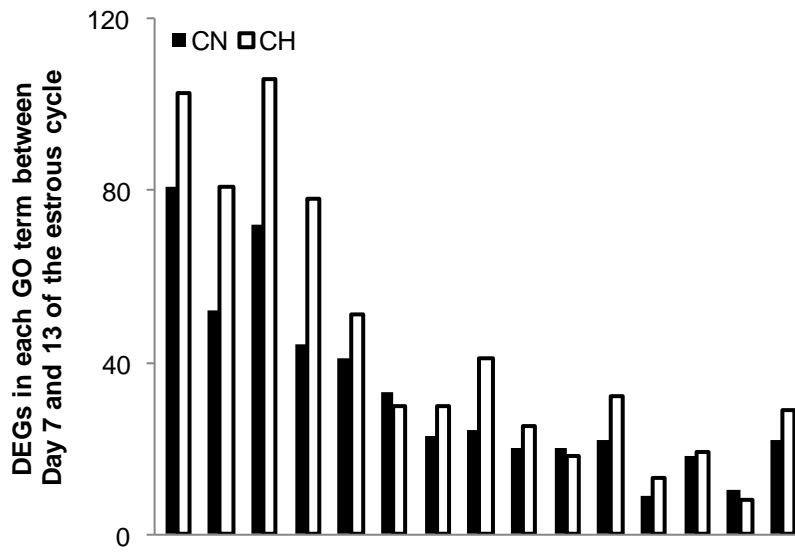
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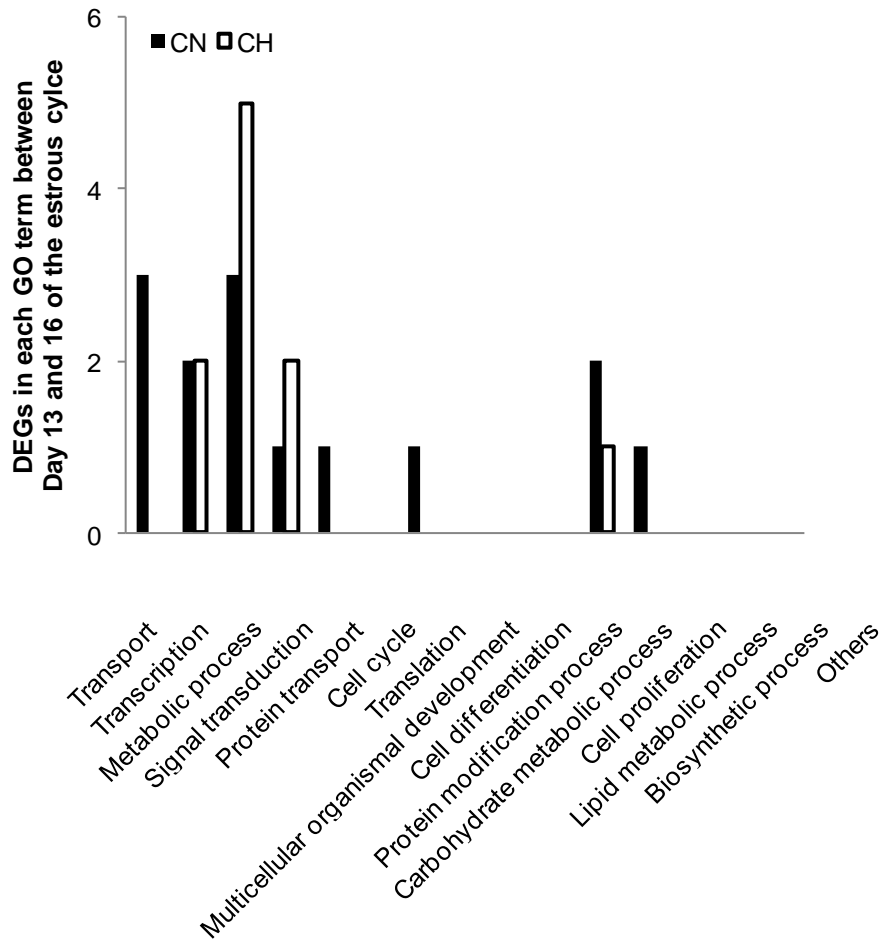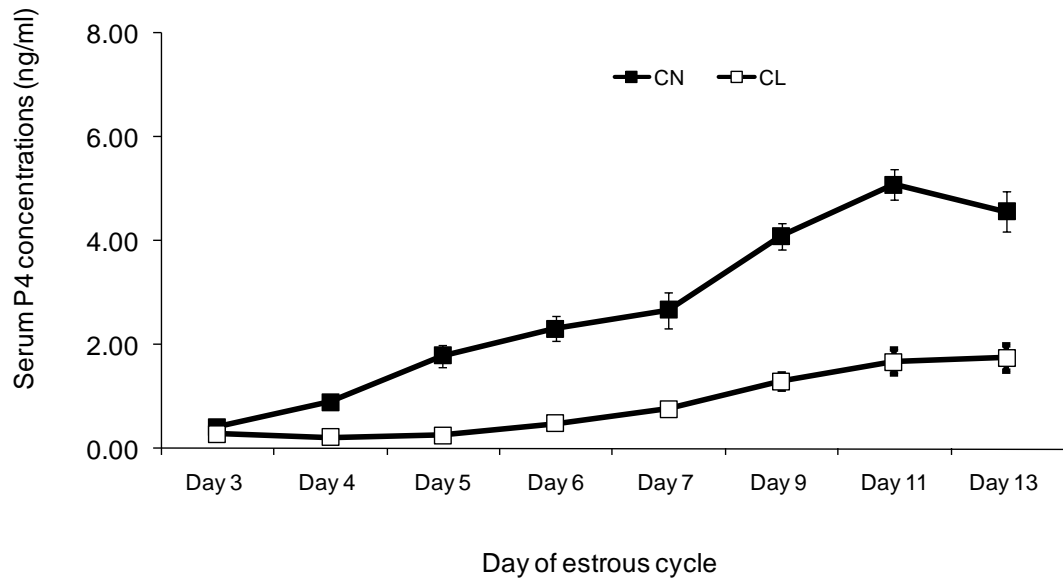
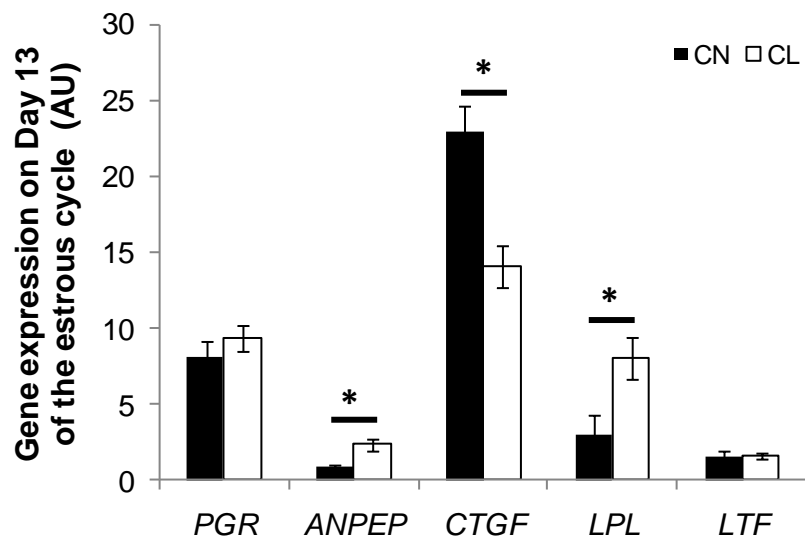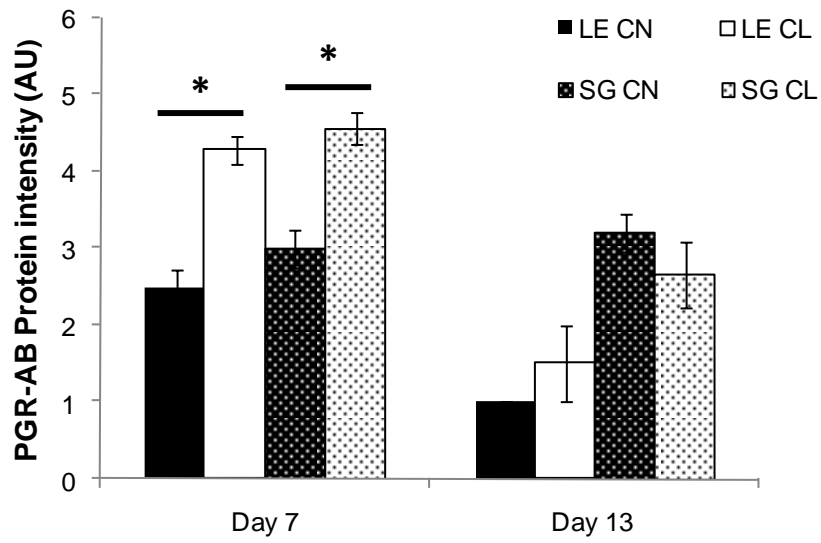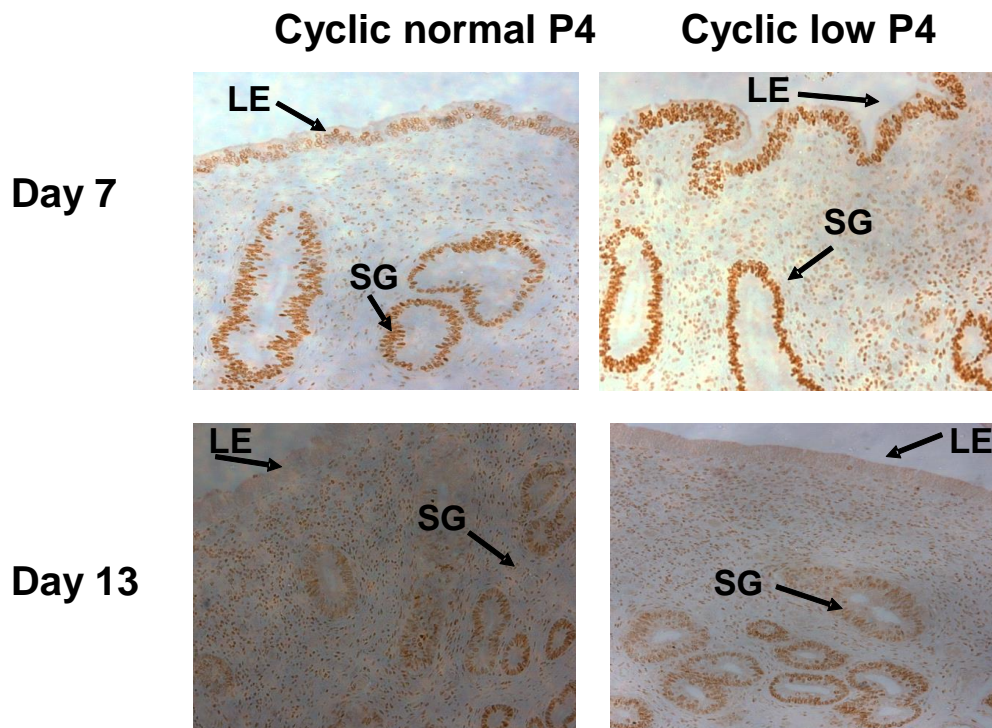Figure 3.

Figure 4

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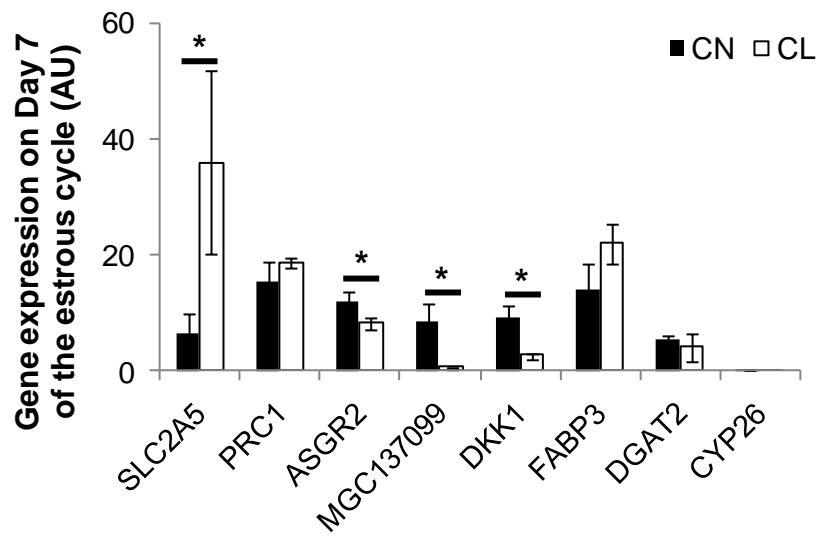
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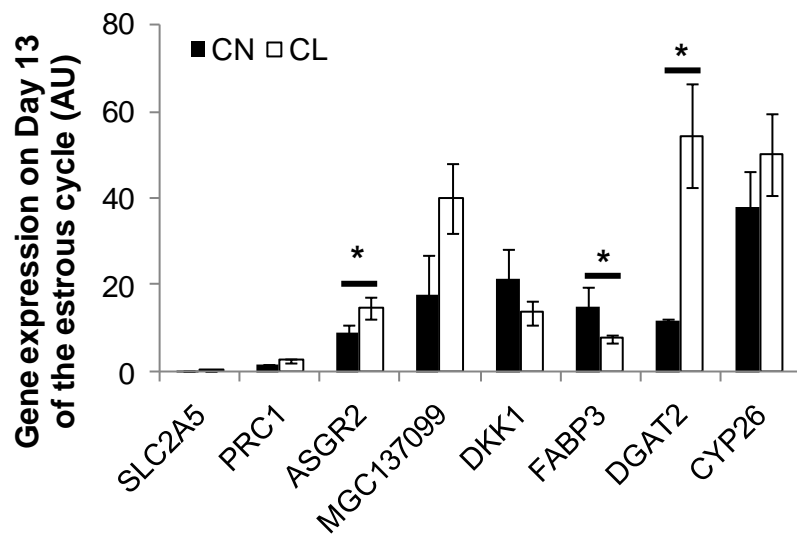
Figure 5

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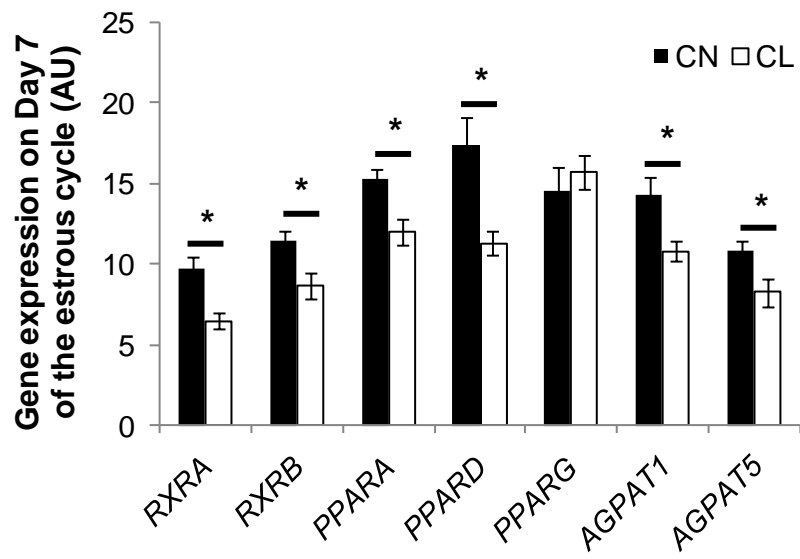
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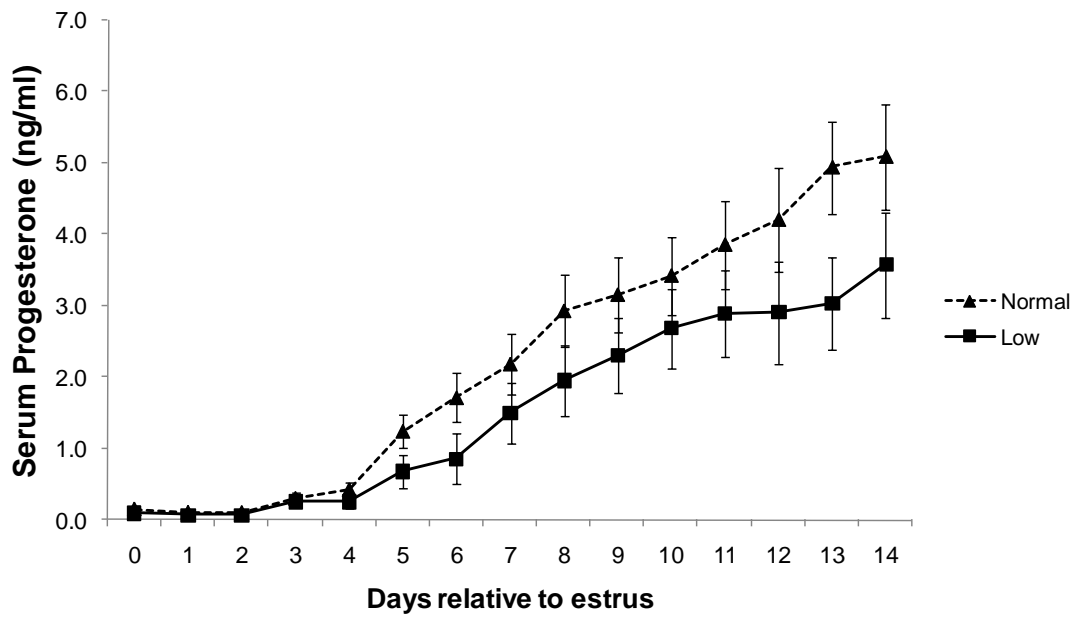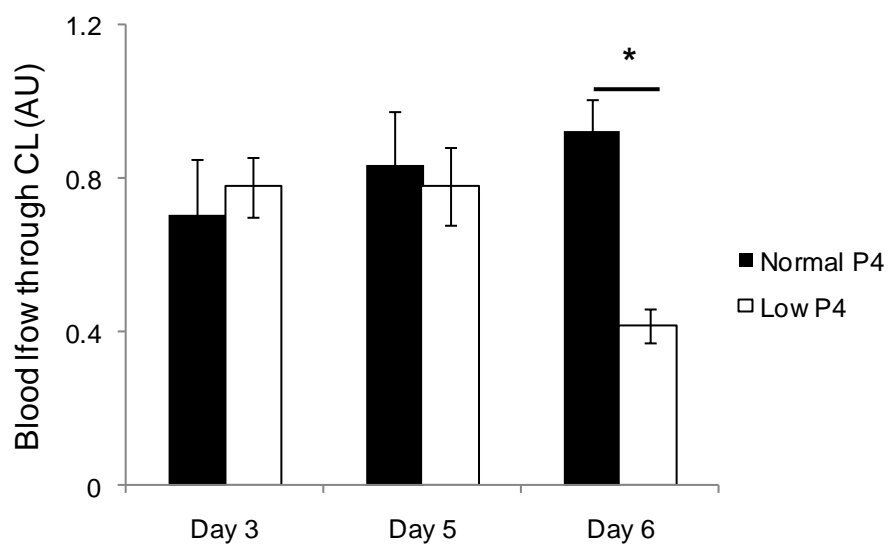
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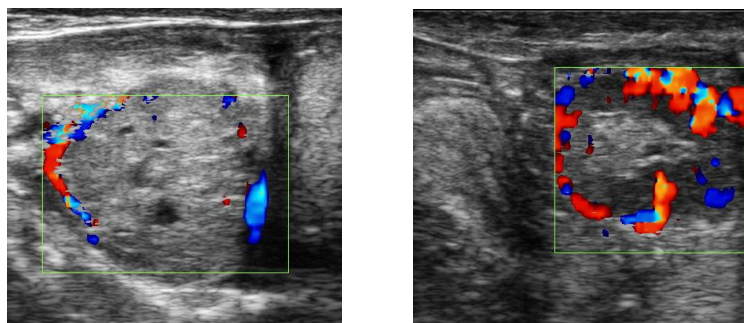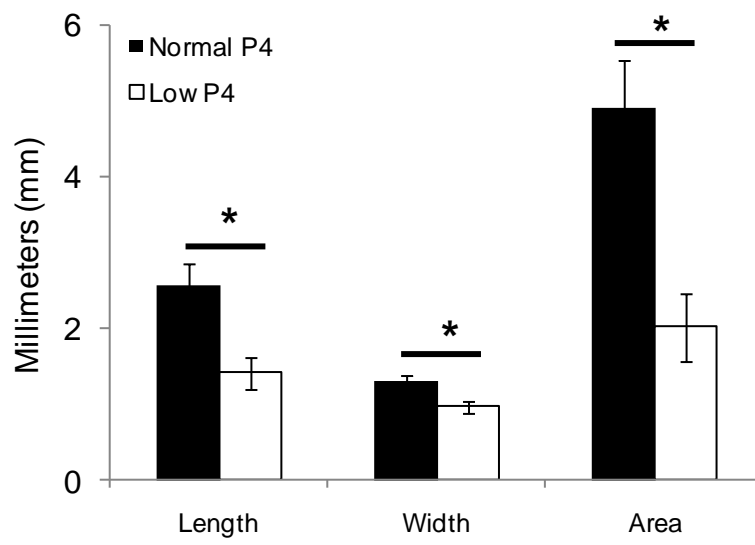

(B)

Figure 7

(A)

(B)

Figure 8

(A)

(B)

Figure 9

(A)

(B) Normal P4   Low P4