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Association between progesterone concentration and endometrial gene expression in dairy cows

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ABSTRACT

The objective of this study was to evaluate the association between progesterone concentration on Days 4 and 9 of the estrus cycle and endometrial transcriptome at Day 9 in lactating grazing dairy cows. Blood samples were obtained on Days 0, 4, and 9 for progesterone measurement by chemiluminescence. Cows were assigned to one of the following groups (n = 3 per group): cows with low physiological progesterone on Day 4, cows in anestrous, cows with high physiological progesterone on Day 4, and superovulated cows. Endometrial biopsy samples were obtained on Day 9 for RNA sequencing. Quality control and determination of differentially expressed genes (false discovery rate <0.05) were determined using the edgeR package for R software. We identified 3,042 differentially expressed genes among the 4 groups. Cows having high physiological progesterone and superovulated cows showed high similarities and clustered apart from those in anestrus or having low physiological progesterone. Functional analysis using Database for Annotation, Visualization, and Integrated Discovery revealed that endometrial genes upregulated by low progesterone concentration are enriched genes involved in the immune system and inflammatory response. Conversely, cows with high physiological progesterone concentration presented an endometrial transcriptome with similarities to cows with good genetic merit for fertility, showing upregulation of genes related to uterine relaxation–contraction, focal adhesion, GnRH signaling pathway, and epidermal growth factor–like related terms, suggesting a favorable embryo environment. In conclusion, our results support the concept that there is a threshold of progesterone concentration at the beginning of the luteal phase associated with endometrial expression of critical genes involved in the preparation of the uterine environment for embryo implantation.

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1. Introduction

Achieving good fertility in dairy cows is fundamental for modern dairy farming. Unfortunately, fertility has declined during the last decades, although the fertilization rate is still greater than 70% in high- and medium-producing dairy cows [1], suggesting that there are other causes for the observed declining fertility. Embryo losses mostly occur before 16 d postinsemination [2], and they seem to be related to reduced progesterone (P_4) levels. A lower systemic P_4 concentration has been associated with a smaller size of the corpus luteum (CL) [3] but also with a strong clearance of steroid hormones by the liver as a result of an increase in milk production [4,5]. Progesterone stimulates and sustains endometrial functions essential for embryonic survival, implantation, and growth [6]. Independently of the embryo presence, P_4 induces the expression of key genes in the endometrial epithelia that are then further stimulated by factors from the conceptus such as interferon tau and prostaglandins [7–9].

Cows with superior genetic merit for fertility presented a more rapid postovulatory P_4 increase and reached a higher concentration of P_4 during the estrus cycle [3]. In this sense, a quick rise in P_4 concentration at the beginning of the diestrus is more critical for the embryo survival [10] and production of the antiluteolytic protein, interferon tau [11], than the peak P_4 level reached during the whole diestrus [12]. Previous studies that aimed to characterize the endometrial transcriptomic profile according to steroid hormone levels in the cattle have reported differences in late estrus or diestrus [13], after treatment with estradiol, P_4, or both hormones in ovariectomized animals [14], or in early diestrus in cows ovulating large or small follicles [15].

Despite these studies, there is still a lack of knowledge about to what extent the physiological differences in P_4 levels in early diestrus influence the transcriptome of the endometrium during mid-diestrus when it should be able to nourish and embryo. An experimental design that can potentially shed light on this matter is to compare groups of lactating dairy cows with high and low physiological P_4 (LLP4) concentrations. In addition, anestrous (ANE) cows that lack a functional CL and superovulated (SO) cows, which have several functional CL, can also be assessed as endometrial transcriptomes of reference of a very low and very high concentration of P_4, respectively.

We hypothesized that physiological differences in P_4 concentrations at the beginning and in the middle of the luteal phase determine the endometrial transcriptomic profile during the middle diestrus. The goal of this study was to evaluate the association between P_4 concentration at the beginning and in the middle of diestrus (Days 4 and 9 of the estrus cycle) and the endometrial global gene expression at middle diestrus at the time of blastocyst's hatching (Day 9) in lactating grazing Holstein dairy cows.

2. Materials and methods

2.1. Animals and case definition

This study was conducted on a commercial dairy farm located in Buenos Aires province, Argentina. A total of 30 healthy grazing Holstein dairy cows (n = 25 cyclic and 5 ANE), with approximately 30 to 40 d in milk (DIM), with BCS ≥2.50, and with a 305 DIM accumulated milk yield (MILK305), from 6,000 to 12,000 kg were included in the study.

A subgroup of cyclic cows (n = 17 of 25) was estrus synchronized with a Presynch (SYN; 500 μg IM of cloprostenol [Ciclase DL, Syntex, Argentina] on Days −33 and −21) and an Ovsynch protocol (100 μg IM of Gonadorelin [Gonasyn, Syntex, Argentina] on Day −9), 500 μg IM of cloprostenol on Day −2, and 100 μg of Gonadorelin on Day 0, SYN cows). Another subgroup of cyclic cows (n = 8 of 25) was randomly selected to receive the same previously described Presynch and Ovsynch protocols with the addition of a superovulation protocol (2,500 UI of eCG IM [Novormon, Syntex, Argentina] on Day −7, SO cows). Moreover, ANE cows (n = 5, follicles <8 mm, without CL, ANE cows) that did not receive any treatment were also included in this study.

The SYN cows that responded to protocol (n = 12 of 17) were separated into quartiles of P_4 concentration on Day 4 of the estrus cycle, and top and bottom quartiles were selected and named as high physiological P_4 cows (HPP4 [mean ± SD = 3.03 ± 0.44 ng/mL]) and LPP4 cows (0.95 ± 0.39 ng/mL P_4), respectively. In addition, a group of SO cows (very high P_4 reference > 20 ng/mL P_4) and a group of ANE cows (very low P_4 reference <0.25 ng/mL P_4) were included.

2.2. Blood sampling

Blood samples were obtained from the tail vein on Days 0, 4, and 9 of the estrus cycle from all the enrolled cyclic cows. In the case of ANE cows, blood samples were obtained on random days. Samples were placed in glass tubes without anticoagulant and were centrifugated within 4 h. Sera obtained was stored at −20°C until P_4 measurement by chemiluminescence (Immunoanalyzer Elecsys and Cobas e, Roche, Mannheim, Germany).

2.3. Endometrial sampling

Endometrial cells were sampled on Day 0 by cytobrush technique [16] to discard cows with subclinical endometritis. The cutoff point used was 5% of polymorphonuclear cells [17]. Biopsy samples for global gene expression analysis were obtained on Day 9 (diestrus), except for ANE cows that were collected on random days, by using a biopsy instrument (10366 LL single action jaws for esophagoscopy, Karl Storz GMBH & Co, Germany). Endometrial tissue was immediately transferred into cryotubes and snap freeze in liquid nitrogen. After that, samples were stored at −80°C until processing.

Animal use in this project was approved by the Graduate School and the Laboratory Animal Care and Use Committees of the Faculty of Veterinary Sciences at the National University of La Plata (IACUC Code #:40-5-14P).

2.4. RNA extraction and preparation

RNA was extracted from the endometrial tissue samples using TRIzol (Invitrogen, Carlsbad, USA) following the manufacturer's direction. An aliquot of RNA per sample

(2 μg of total RNA) was DNase treated by using the Turbo RNase-free DNase kit (Ambion, Foster City). The RNA concentration was determined with a NanoDrop spectrophotometer (ND-1000; Thermo Fisher, Wilmington, NC), and the integrity of RNA (RIN number) was measured by using an Agilent Bioanalyzer 2100 model. For RNA sequencing, 12 samples were selected (3 samples per group), which is the minimal number of biological replicates required for any inferential analysis [18].

2.5. RNA sequencing analysis

For library construction, mRNA was purified through hybridization with oligo-dT and chemically fragmented. Double-strand cDNA was synthesized, followed by end repair, adaptor ligation, and enrichment (amplification) of the libraries according to the TruSeq Stranded mRNA protocol (Illumina Inc, CA). The quality of each cDNA library was assessed on a 2100 Bioanalyzer (Agilent Technologies) by using the DNA 1000 Kit. Libraries were quantified by quantitative PCR (Light Cycler 480 Roche) by using the Qiagen Library Quantification Kit, according to the manufacturer's protocol. Sequencing was performed on a Hiseq 1500 system (Illumina Inc, CA), generating paired-end 2 × 150 bp reads. The 92.4% of the reads had a Q score >Q30. The % reads identified varied from 82% to 92.8%. The quality of the reads in the resulting FASTQC files was checked with FastQC (version 0.11.2) [19]. Adapters and quality trimmer and filtering were performed using the Trimmomatic software (Bjorn Usadel Lab., Aarchen, Germany) [20]. The remaining reads were processed with the Rsubread package for the R software (R Foundation for Statistical Computing, Vienna, Austria) [21]. The sequence reads were aligned to the bovine reference genome (*Bos taurus* UMD3.1).

All RNA-Seq data have been deposited in National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO) under accession number GSE20168088.

2.6. Statistical analysis

To assess the dynamics of P4 concentration in time from estrus up to the middle diestrus, a linear model was run with PROC MIXED (SAS/STAT version 9.4; SAS Institute Inc, Cary, NC). The model included the random effect of the cow and the fixed effect of time (0 vs 4 vs 9 d), group (HPP4 vs LPP4), and their interaction. The covariance structure having the smallest Akaike information criterion and Schwarz's Bayesian criterion was used [22]. A polynomial contrast was used to test the linear and quadratic effect of time. Finally, a similar model without the effect of time was used to analyze MILK305.

On an unsupervised analysis, multidimensional scaling (MDS) plot was performed with the Glimma package [23]. Genes with low expression counts (<1 CPM) in 3 or more samples were filtered out before normalization. Thus, 2,930 transcripts were filtered out, retaining 11,000 for further analysis. The normalization method applied was the weighted trimmed mean of M values. Normalization factors for all samples had a mean of 1, with a minimum of 0.75 and a maximum of 1.12.

For the statistical analysis, a robust estimate of the negative binomial dispersion parameter was applied to each gene by using observation weights. These observation weights were used later for estimating regression parameters. Then, a negative binomial generalized log-linear model was fit to read counts for each gene and conduct genewise statistical tests for the coefficient contrast [24].

The matrix of contrast was built based on the comparisons between the groups with high P_4 levels on Day 4 (HPP4 and SO) and the groups with low P_4 levels on Day 4 (LPP4 and ANE). Thus, the pairwise comparisons were HPP4 vs LPP4, SO vs LPP4, HPP4 vs ANE, and SO vs ANE. Finally, the differentially expressed genes (DEGs) were determined through one-way ANOVA, defining DEG as those with a false discovery rate (FDR) <0.05. All these procedures were performed with the edgeR package for the R software [25].

2.7. Functional analysis

Upregulated or downregulated DEG on each comparison was visualized with Venn diagrams to determine the genes with the highest overlap. These common DEGs were interrogated for functional terms by using the Functional Annotation Clustering tool of the Database for Annotation, Visualization, and Integrated Discovery [26]. In addition, the expressions of the DEG were used to determine samples' similarities, according to their profile, through hierarchical clustering and a heat map of the sample to the sample distances. Distances between samples and genes were measured using centered correlation as similarity metric, and samples and genes were clustered according to the complete linkage algorithm, using the software Cluster 3.0 (Michiel de Hoon, Human Genome Center, University of Tokyo, Tokyo, Japan) [27].

2.8. Comparison with a selected external data set

As a complement of our study, a publicly available RNA-seq gene expression data set was used to compare with our results. The data set was downloaded from the NCBI GEO repository, accession number #GSE52438 [28]. In that study, endometrial biopsy samples from lactating dairy cows on Day 7 of the estrus cycle (nonpregnant) with similar genetic merit for milk production traits but with very good genetic merit for fertility (H-Fert, n = 7) or very poor genetic merit for fertility (L-Fert, n = 6) were analyzed by RNA-seq technology [29]. The matrix of gene counts was analyzed with the edgeR package for R following the same steps as detailed previously for our data set. For the sake of comparisons and to cover a wider number of genes, DEGs were defined as those with *P* value <0.05. The upregulated or downregulated DEG were compared with the upregulated or downregulated DEG (FDR <0.1) in HPP4 vs LPP4 and visualized through Venn diagrams. The test of independence (Pearson's chi-square test) was used to determine the relatedness between them. The Entrez ID of all the corresponding DEGs were used for the comparisons between ours and the external data set. The purpose of these comparisons was to determine if similar genes would be

stimulated or inhibited in H-Fert and HPP4, when compared with L-Fert and LPP4, respectively.

3. Results

The analysis showed that the groups (HPP4 and LPP4) had no effect on MILK305 ($P = 0.187$). The MDS plot shows that samples from cows having high P_4 on Day 4 (HPP4 and SO groups) are grouped apart from samples belonging to groups of cows having low P_4 (LPP4 and ANE groups; Fig. 1). Reinforcing the results from the MDS, the analysis of similarities between the expression levels of the DEG, also showed that samples in the HPP4 and SO groups clustered apart from samples in the LPP4 and ANE groups. Genes clustered into 2 main groups, upregulated in HPP4 and SO and downregulated in LPP4 and ANE, and vice versa (Supplementary Fig. 1). The total number of DEG between groups was HPP4 vs LPP4: 453; HPP4 vs ANE: 623; SO vs LPP4: 603; SO vs ANE: 1,102; LPP4 vs ANE: 261; and SO vs HPP4: 0. The number of DEG upregulated and downregulated for each comparison is shown in Figure 2. The lists of genes with the corresponding *P* values and FDR for each comparison are shown in Supplementary Table 1. The overlaps between upregulated or downregulated DEG identified for each comparison are shown in Figure 3.

The results from the functional analysis showed that clusters significantly enriched ($P < 0.05$) with DEG upregulated in HPP4 and SO, when compared with LPP4 and ANE, contain terms related to cyclic guanosine-3′,5′-monophosphate–dependent protein kinase G (cGMP-PKG), cyclic adenosine monophosphate, GnRH and oxytocin signaling pathways, vascular smooth muscle contraction, and focal adhesion. When compared only with ANE, the clusters contained terms related to glycoprotein, extracellular matrix, Wnt signaling pathway, and epidermal growth factor (EGF)–related terms. For the downregulated DEG in HPP4 and SO, when compared with LPP4, significantly enriched clusters contain terms related mainly with the immune system, such as beta-defensin type, antigen processing, and presentation of peptide antigen via major histocompatibility complex class I and innate immunity. Genes that were downregulated by SO treatment (ie, SO vs LPP4 and SO vs ANE) were strongly related to cell cycle and mitosis. The complete list of significantly enriched functional annotation clusters is presented in Supplementary Tables 2 and 3

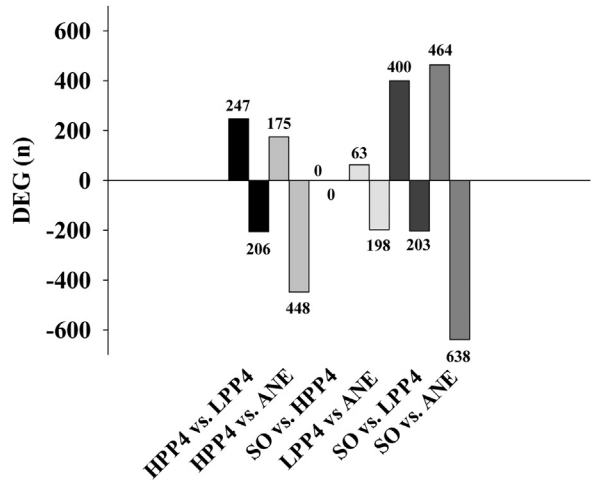

Fig. 2. Differentially expressed genes (DEGs) upregulated (above 0) and downregulated (below 0) on Day 9 of the estrus cycle for every comparison between groups of cows included in the study: low physiological levels of progesterone (LPP4), high physiological levels of progesterone (LPP4), superovulated (SO), and anestrous (ANE) cows.

The results from the comparison of upregulated and downregulated DEG between HPP4 vs LPP4 and the external data set (H-Fert vs L-Fert) are shown in Figure 4. There was a significant overlap of DEG for both comparisons, indicating similarities between genes differentially expressed in H-Fert and HPP4 cows. The most enriched cluster with the shared upregulated 30 DEG contains terms related to the cGMP-PKG signaling pathway, vascular smooth muscle contraction, cAMP signaling pathway ($P <$

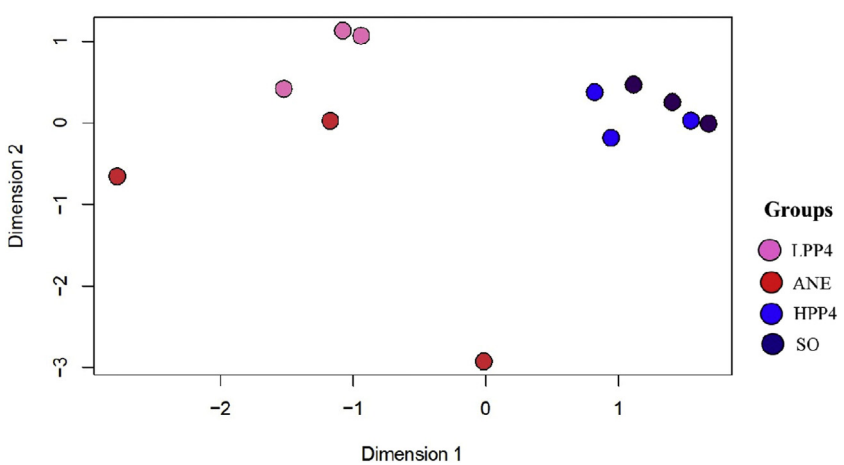

Fig. 1. Multidimensional scaling analysis for transcriptomes of uterine biopsy samples from individual cows. Blue dots represent cows with high physiological levels progesterone (HPP4), pink dots represent cows with low physiological levels progesterone (LPP4), red dots represent anestrous cows (ANE), and black dots represent superovulated cows (SO) on Day 9 of the estrus cycle. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

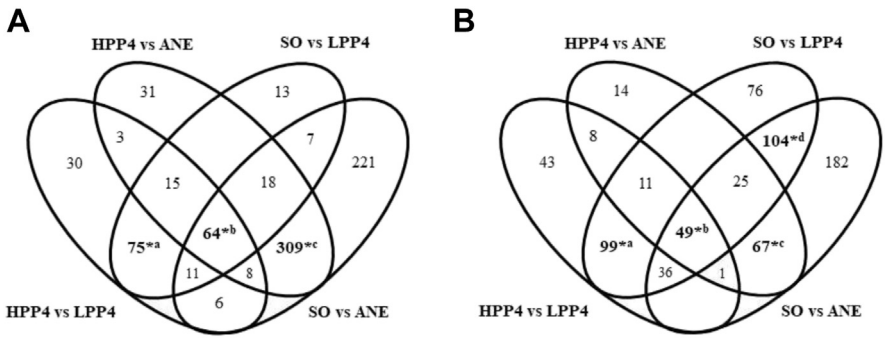

Fig. 3. Venn diagrams depicting the overlap between upregulated (A) or downregulated (B) differentially expressed genes (FDR <0.05) identified for each comparison: high physiological progesterone cows (HPP4) vs low physiological progesterone cows (LPP4), superovulated cows (SO) vs LPP4, HPP4 vs anestrous cows (ANE) and SO vs ANE. *Overlapping genes (in bold) were subjected to functional annotation clustering analysis. Superscript letters correspond with tabs in Supplementary Tables 2 and 3 (for panels A and B, respectively), containing the results from the functional analysis.

0.01), and oxytocin signaling pathway ($P < 0.05$). For the downregulated genes, there were 10 genes shared between H-Fert vs L-Fert and HPP4 vs LPP4, but they were not enriched for functional terms. On the contrary, there was no significant overlap for the DEG when H-Fert vs L-Fert were compared with LPP4 vs HPP4 cows.

4. Discussion

There is a consensus that early embryonic development until implantation is the most critical period for a successful pregnancy, and it is well known the strong positive association between circulating P_4 concentration and embryo development in cattle [6]. Gathered evidence suggests that P_4 has no direct effect on the embryo previous to the blastocyst stage. Instead, it plays a vital role during early pregnancy by inducing genes that coordinate changes in the uterine environment, leading to the establishment of uterine receptivity for posterior embryo implantation [6,30,31]. These major endometrial genes are mainly related to the immune system, adhesion molecules, and development [32]. Therefore, it is reasonable to propose that low levels of P_4 can affect the modulation of crucial genes, potentially leading to a suboptimal environment for the development and nutrition of the blastocyst [9]. These results support our hypothesis that low P_4 concentration at the beginning and in the middle luteal phase alters the endometrial gene expression during the middle diestrus in lactating dairy cows. That is in line with previous studies that demonstrate that the bovine uterus is highly sensitive to changes in P_4 concentrations during the first few days after estrus [9,33], although when the P_4 variation is as little as 0.5 ng/mL on Day 4 after ovulation [34]. Whereas P_4 concentrations (nanogram/milliliter) were similar for HPP4 and LPP4 cows on Day 0 (0.63 vs 0.29 ng/mL), in HPP4 cows, we observed a P_4 concentration 3 times higher on Day 4 (3.03 vs 0.95 ng/mL) and 2 times higher on Day 9 (8.54 vs and 4.50 ng/mL) than in LPP4 cows. So, it is feasible that the observed difference in P_4 could be associated with the DEG found and the clustering of both groups in the MDS analysis.

In our study, HPP4 cows showed a faster rise in P_4 concentration from Day 0 to 9 than in LPP4 cows. We believe that a critical level of P_4 should be reached at the beginning of the diestrus (ie, before embryo's hatching) to stimulate the expression of essential endometrial genes that ensure an ideal uterine environment supporting embryo implantation. In this sense, Kenyon et al [12] found that P_4 fold changes $\geq$2.71 from Days 0 to 7 and $\geq$1.48 from Days 7 to 14 are associated with the establishment of pregnancy in dairy cows. These results are in agreement with the fact that a quick rise in P_4 concentration at the beginning of the diestrus seems to be more critical for the healthy embryo development than the level of P_4 reached later during full diestrus [35]. That could be the reason why the administration of exogenous P_4 after the postovulatory P_4 rise (Day 7) failed to improve pregnancy rates [35].

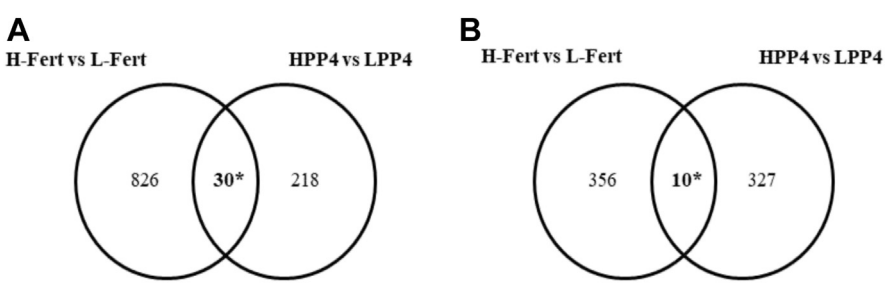

Fig. 4. Venn diagrams depicting the overlap between upregulated (A) or downregulated (B) differentially expressed genes identified for each comparison: high genetic merit for fertility cows (H-Fert) vs low genetic merit for fertility cows (L-Fert) and high physiological progesterone cows (HPP4) vs low physiological progesterone cows (LPP4). *Overlapping genes (in bold) were subjected to functional annotation clustering analysis.

A limitation of this study is that we cannot know precisely the day in which the endometrial transcriptome begins to differ between LPP4 and HPP4 cows. However, Forde et al [36] found that beef heifers at Day 5, receiving a P_4-release intravaginal device from Day 3, already presented DEG in the endometrium compared with the control animals. Besides, another study showed that there are already DEG in the endometria of high-receptive cows on Day 3 compared with those cows that were nonreceptive to an embryo [37]. On the other hand, Salilew-Wondim et al [38] found vast differences in the endometrial gene expression pattern on Day 7 between heifers whose pregnancy resulted in calf delivery compared with those resulting in no pregnancy, but those differences gradually decline to be minimal at Day 14. Our results from the comparison with the external dataset analyzed suggested that HPP4 cows had a more fertile phenotype than LPP4 cows. Therefore, we can speculate that HPP4 and LPP4 presented differences in the endometrial transcriptome already on Day 4, leading to 2 distinctive endometrial signatures on Day 9.

Surprisingly, the HPP4 and the SO cows cluster together in the MDS analysis despite that SO cows have P_4 concentration greater than 6 times higher (3.03 vs >20 ng/mL) than the HPP4 cows at Day 4. Furthermore, the hierarchical clustering and heat map (Supplementary Fig. 1) and the functional analysis of the DEG confirmed the similarities in endometrial transcriptomic profile on Day 9 of the estrus cycle between HPP4 and SO cows and between LPP4 and ANE cows. According to Forde et al [33], endometrial genes affected by low P_4 are different from those affected by high P_4, which could explain the similarities in DEG observed between cows with higher (ie, SO and HPP4) or with lower P_4 concentration (ie, ANE and LPP4). Interestingly, and although we predicted changes in endometrial gene expression according to the concentration of P_4 (eg, HPP4 vs LPP4 DEG, n = 453; SO vs ANE DEG, n = 1,102), the high level of similarities found between SO and HPP4 groups (SO vs HPP4 DEG, n = 0) was unexpected (Fig. 2). Although there are reports that described that early exogenous supplementation of P_4 in cows induces changes in the expression of a large number of endometrial genes during the luteal phase [9,14], to our knowledge, there is no other study that compares those findings with the endometrial transcriptome of SO cows.

It is necessary to take into account that some variations in expression profile between ANE samples were observed (Fig. 1). Although ANE cows were in healthy conditions and presented indeed very low levels of P_4, they may not be considered the best model to study the effect of low P_4 concentration on the endometrium. Probably ovariectomized cows that were treated with the same synchronization protocol than SYN cows would be a better choice. Unfortunately, that kind of "negative control" was not possible to work with because all cows belonged to a commercial dairy farm and were under reproductive management. Therefore, we chose to use ANE cows as low P_4 reference cows for our study. However, it was interesting to find that the transcriptomic profile of LPP4 was similar to these ANE cows, which have very low physiological levels of P_4.

In agreement with the results from the MDS (Fig. 1) and the hierarchical clustering (Supplementary Fig. 1), there was a high number of overlapping DEG for the pairwise comparisons between the groups with high (HPP4 and SO) vs low (LPP4 and ANE) concentrations of P_4. The overlap of 309 DEG (Fig. 3A) is composed of upregulated genes in HPP4 vs ANE and in SO vs ANE comparisons, which means that these genes had high expression in the endometria of cows with high P_4 concentration and very low expression in the ANE cows. Some of these DEG were involved in cellular processes associated with the catalytic activity such as redox process, which was also found to be upregulated in the 7 d endometrium of Nellore cows ovulating large follicles [15]. Other genes were related to the extracellular matrix, glycoproteins, and cell-to-cell adhesion and communication. As a part of that, the Wnt signaling pathway, which is a critical regulator of cell-to-cell communication during embryo development in mammals and modulated by sexual steroids, was also upregulated [39,40]. In this sense, Davoodi et al [32] found an increased expression of genes involved in the Wnt signaling pathway and adhesion molecules in cows with a high P_4 concentration on Day 7 of the estrus cycle. In a coincidence, Salilew-Wondim et al [38] also found an increase in the expression of adhesion molecules in the endometrium of receptive cows in comparison with nonreceptive cows on Day 7. Another functional cluster upregulated in cows with high P_4 concentration was related to EGF and EGF-like conserved site. The EGF has been pointed out as a critical factor involved in the uterine function and early embryonic development in bovine [41–43]. In addition, a diminished endometrial EGF expression profile during diestrus was studied as a cause of subfertility, and its normalization has been suggested as one of the prerequisite factors to restore fertility in dairy cows [42].

The overlap of 64 DEG upregulated in the 4 comparisons (Fig. 3A) contained genes that had high expression in cows with high P_4 concentration (HPP4 and SO) but very low expressed genes in ANE and LPP4 cows. These genes were enriched for the GnRH signaling pathway, signaling pathways related to smooth muscle relaxation/contraction (cAMP, cGMP-PKG, and oxytocin) and vascular smooth muscle contraction. Accordingly, the results from the external data set analyzed [28] from cows with high genetic merit for fertility also showed upregulation of genes related to gonadotropin gene expression and secretion and to smooth muscle relaxation/contraction. These findings could indicate that the regulation of the uterine contraction around Days 7 to 9 could be crucial for fertility. In this way, studies in women have shown that an adequate regulation of the uterine contraction is necessary for a successful transport and implantation of the embryo [44]. Cows with high genetic merit for fertility also showed an HPP4 and upregulation of genes related to estrogen signaling pathway and EGF-like related terms. Accordingly, the comparison of our results with the external data set analyzed showed a significant association for the overlapping DEG between H-Fert vs L-Fert and HPP4 vs LPP4 (Fig. 4), but not when H-Fert vs L-Fert were compared with LPP4 vs HPP4 cows. Therefore, HPP4 cows probably have more genetic merit for fertility than LPP4.

One remarkable finding of our study is that the endometria of LPP4 cows compared with HPP4 and SO cows (99

DEG; Fig. 3B) showed strong stimulation of endometrial genes related to the immune and inflammatory response on Day 9 such as those involved in antigen processing and presentation and cell adhesion molecules. Similarly, cows with inferior genetic merit for fertility, which showed low P_4 concentration during early diestrus but similar milk production than cows with superior merit for fertility, had been described as presenting an upregulation of endometrial genes related to the immune response at Day 7 [3,29], indicating that low physiological levels of P_4 during early diestrus could be related to low genetic merit for fertility and strong expression of endometrial genes involved in immunity and innate immunity. In addition, other studies also showed upregulation of several genes related to the immune system and the inflammatory responses in the endometrium of cows and heifers with low fertility [38,45]. Conversely, Cerri et al [46] described on Day 17 of the estrus cycle, several endometrial genes related to an increase in B and T cell activities that they pointed as upregulated by lactation, implying that a high milk production together with low P_4 levels would alter endometrial function affecting its immunologic balance. All those findings could be associated with a slow postpartum uterine involution, an altered immunologic balance, and an endometrial function incompatible with pregnancy establishment. Reduced fertility in lactating dairy cows could be due, at least in part, to changes in the endometrial immune response, but the exact mechanism by which lactation affects the uterine immune response is still unclear [46].

5. Conclusion

The results from the present study support the association between the concentration of P_4 at the beginning and mid-luteal phase and the endometrial expression of critical genes involved in the preparation of the uterine environment for a new pregnancy. The HPP4 cows, which presented HPP4 around the early and middle luteal phase, showed strong similarities in their endometrial transcriptional profile with SO and H-Fert cows, suggesting a favorable embryo environment. Genes related to modulation of uterine contraction–relaxation, focal adhesion, GnRH signaling pathway, and EGF-like related terms were upregulated in the endometrium of these cows. Conversely, LPP4 and ANE cows expressed endometrial genes related to the immune system and inflammatory response, which imply adverse conditions for embryo development. Our results are promising and provide useful insights into this topic. The endometrial transcriptome of SO and ANE cows, as references for very high and very low P_4 concentration, has not been reported so far. Interestingly, this study provides evidence that fair differences in P4 levels at Days 4 and 9 could lead to strong differences in the endometrial transcriptome profile at Day 9 to the extent that they become similar in the corresponding reference in each extreme.

CRediT authorship contribution statement

L.V. Madoz: Conceptualization, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing - original draft. **M.B. Rabaglino:** Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **A.L. Migliorisi:** Investigation, Methodology. **M. Jaureguiberry:** Methodology. **S. Perez Wallace:** Investigation, Methodology. **N. Lorenti:** Investigation, Methodology. **G. Domínguez:** Investigation, Methodology. **M.J. Giuliodori:** Writing - original draft. **R.L. de la Sota:** Conceptualization, Funding acquisition, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.domaniend.2020.106481.

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