Analysis of the Uterine Lumen in Fertility-Classified Heifers: II. Proteins and Metabolites

Joao G.N. Moraes[1], Susanta K. Behura[1], Jeanette V. Bishop[2], Thomas R. Hansen[2], Thomas W. Geary[3], Thomas E. Spencer[1,#]

[1]Division of Animal Sciences, University of Missouri, Columbia, Missouri
[2]Animal Reproduction and Biotechnology Laboratory, Department of Biomedical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado
[3]USDA-ARS, Fort Keogh Livestock and Range Research Laboratory, Miles City, Montana

[#]To whom correspondence should be addressed: Thomas E. Spencer, Division of Animal Sciences, 158 ASRC, 920 East Campus Drive, University of Missouri, Columbia, MO, 65211, USA. Tel.: 573-882-3467; E-mail: spencerte@missouri.edu

Running title: Uterine lumen of early pregnant cattle

Summary Sentence: Pregnancy induced changes in the uterine lumen are dysregulated in subfertile heifers

Key words: Uterus, Endometrium, Conceptus, Preimplantation embryo, Fertility, Proteomics

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ABSTRACT

Survival and growth of the bovine conceptus is dependent on endometrial secretions or histotroph. Previously, serial blastocyst transfer was used to classify heifers as high fertile (HF), subfertile (SF), or infertile (IF). Here, we investigated specific histotroph components (proteins and metabolites) in the uterine lumen of day 17 fertility-classified heifers. Interferon tau (IFNT) was more abundant in uterine lumenal fluid (ULF) of pregnant HF than SF animals as the conceptus was longer in HF heifers. However, no differences in endometrial expression of selected classical and nonclassical interferon-stimulated genes (ISGs) were observed, suggesting that IFNT signaling in the endometrium of pregnant HF and SF heifers was similar. Pregnancy significantly increased the abundance of several proteins in ULF. Based on functional annotation, the abundance of a number of proteins involved in energy metabolism, oxidative stress, amino acid metabolism, and cell proliferation and differentiation were greater in the ULF of pregnant HF than SF heifers. Metabolomics analysis found that pregnancy only changed the metabolome composition of ULF from HF heifers. The majority of the metabolites that increased in the ULF of pregnant HF as compared to SF heifers were associated with energy and amino acid metabolism. The observed differences in ULF proteome and metabolome are hypothesized to influence uterine receptivity with consequences on conceptus development and survival in fertility-classified heifers.

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INTRODUCTION

The uterus clearly impacts conceptus (embryo/fetus and associated extraembryonic membranes) survival and development, thus affecting pregnancy success [1-5]. After hatching from the zona pellucida (days 9–10) [6], the bovine blastocyst slowly grows into an ovoid or tubular form on days 12 to 14 and is then termed a conceptus [7]. Peri-implantation growth of the conceptus is highly dependent on substances present in the uterine lumen [8]. Uterine epithelia are present in the endometrium of all mammals [9], and their secretions constitute an important component of the histotroph, which is essential for preimplantation conceptus survival and development in sheep [10, 11]. Based on candidate and comprehensive analyses [12-20], histotroph in the uterine lumen of cattle is a complex mixture of amino acids, glucose, lipids, proteins, carbohydrates, vitamins, ions, cytokines, hormones, growth factors, and other substances.

In cattle, the endometrium transcriptome is greatly affected by progesterone and pregnancy, e.g. the conceptus [21]. Pregnancy-induced changes in the endometrium transcriptome are hypothesized to influence uterine lumenal secretome composition, thereby affecting conceptus development and pregnancy success [17, 22-24]. For instance, pregnancy increases the availability of basic (e.g. arginine, lysine, histidine) acidic (e.g. aspartic acid/aspartate, glutamic acid/glutamate) and neutral amino acids (e.g. glutamine, isoleucine, leucine, phenylalanine, tyrosine and valine) in the uterine lumen during the preimplantation period in cattle and sheep [16, 25, 26]. Additionally, pregnancy modulates the availability of proteins in the uterine lumen during the preimplantation period in cattle [12, 22]. A series of studies found that interferon tau (IFNT) has distinct effects on the endometrial transcriptome and uterine histotroph in sheep and cattle [27-31]. IFNT is a type 1 IFN produced

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exclusively by mononuclear trophectoderm cells of the elongating conceptus in ruminants, and is secreted predominantly between days 15 to 22 in cattle [32-34]. A primary role for IFNT is to suppress development of the endometrial luteolytic mechanism [27, 35, 36]. However, the paracrine actions of IFNT also stimulate the expression of classical and non-classical interferon-stimulated genes (ISGs) in the endometrium that, along with progesterone induced effects, modulate uterine gene expression, which is essential for establishing uterine receptivity in ruminants [37-40].

It has been recently demonstrated that the metabolite composition of the uterine lumen can be altered by pregnancy as early as day 7 of gestation [17], and that progesterone plays important role regulating uterine lumenal fluid constituents around the time of onset of conceptus elongation in cattle (days 12-14) [18-20]. Alterations in the profile of uterine lumenal components during early pregnancy are a result of complex conceptus-endometrium interactions which are required to support conceptus growth and pregnancy establishment. The conceptus-endometrium crosstalk regulates conceptus and endometrium gene expression, ensures corpus luteum maintenance, provides substrates for cell proliferation and differentiation while preventing cell damage through oxidative stress, and induces the state of uterine receptivity. In order to identify changes in the uterine lumenal constituents that are associated with increased or reduced uterine capacity to support pregnancy, the current experiment utilized heifers that were previously fertility-classified as high fertile (HF; 100% pregnancy rate), subfertile (SF; 25-33% pregnancy rate), or infertile (IF; 0% pregnancy rate) using serial transfer of a single *in vitro* produced blastocyst on day 7 followed by pregnancy determination on day 28 [1, 41]. Interestingly, conceptus development and survival on day 14 (7 days post-transfer) was not different among fertility-classified heifers and only minimal differences in endometrial

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transcriptome were observed on day 14 [41]. Subsequently, it was observed that day 17 (10 days post-transfer) pregnancy rate was higher in HF (71%) and SF (90%) than IF (20%) heifers. Although no differences in conceptus recovery rate was observed between HF and SF heifers on day 17, the conceptuses from HF heifers were on average twice as long compared to SF conceptuses [1]. Additionally, the analysis of transcriptome data generated from endometrium and conceptus revealed dysregulated conceptus-endometrium interactions in SF heifers [1]. The endometrium of SF heifers was found less responsive to pregnancy on day 17, and this was hypothesized as the main cause of embryonic mortality observed in SF heifers by day 28 [1], Thus, in order to investigate the biology of subfertility, the present study tested the hypothesis that specific histotroph constituents in the uterine lumen is altered in fertility-classified heifers. Because differences in endometrial transcriptome can translate into differences in the components available in the uterine lumen [24], the focus of the current study was to investigate proteins and metabolites in the uterine lumen of pregnant and open fertility-classified heifers using targeted and untargeted approaches.

MATERIALS AND METHODS

Animals. All animal procedures were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committees of the USDA-ARS Fort Keogh Livestock and Range Research Laboratory and the University of Missouri.

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Collection of Uterine Lumenal Contents. As described previously [1], fertility-classified heifers were synchronized to estrus (day 0) and received two *in vivo*-produced embryos on day 7 (HF, n=21; SF, n=10; IF, n=5). Conceptus recovery rate was higher in HF (71%; 15/21) and SF (90%; 9/10) than IF (20%; 1/5) heifers. Additional heifers (SF, n=4; IF, n=1) were synchronized to estrus but no embryo transfer was performed. All heifers (HF, n=21; SF, n=14; IF, n=6) were slaughtered on day 17 (10 days post-transfer or 17 days post-estrus) at the University of Missouri slaughter facility, and reproductive tracts were collected within 30 min of slaughter. Immediately after collection, the reproductive tracts were transported to the laboratory, and the uterine lumen was gently flushed with 20 ml of sterile and filtered 1X PBS (pH 7.0). The conceptuses were removed, if present, the ULF clarified by centrifugation (3000 x g at 4°C for 15 min), and the supernatant was carefully removed with a pipette, mixed, divided into aliquots, frozen in liquid nitrogen, and stored at -80°C until analyzed.

IFNT Analysis. The amount of IFNT in ULF was measured in samples from pregnant (HF, n=15; SF, n=9; IF, n=1) and nonpregnant (HF, n=6; SF, n=1; IF, n=4) fertility-classified heifers that received two embryos on day 7. Bovine IFNT was generated as a glycosylated recombinant protein (rbIFNT) using bovine trophoblast protein 1 cDNA (bTP509) as template [42] and human HEK cells by Colorado State University in collaboration with a biopharma company (J.V. Bishop and T.R. Hansen, manuscript in preparation). Purified rbIFNT was used to generate a monoclonal antibody in mice (9.1.1; 16.2 μg/ml) and a polyclonal antibody in goats (5.3 μg/ml), which were used as capture and biotinylated detector antibodies, respectively, in a sandwich Enzyme Linked Immunosorbent Assay (ELISA). The range of detection for this ELISA was 100 pg to 3,000 pg and the limit of detection for this assay was 100 pg/ml. This ELISA has been

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validated to be specific for IFNT, and does not cross-react with IFNs omega, alpha/beta, or gamma. Uterine flush samples from open and pregnant heifers were analyzed in the same assay. Uterine flushings were analyzed as neat (undiluted) samples or at dilutions of 1:10 or 1:500 in order to detect IFNT in the linear range of the assay.

Proteomics. ULF proteomics analysis was conducted by the Proteomics Center of the University of Missouri in a subgroup of 25 heifers that were determined to be pregnant (HF, n=5; SF, n=5) or nonpregnant/cyclic (HF, n=5; SF, n=5; IF, n=5) at day 17 slaughter. The selected 25 heifers used here are the same subgroup of animals which we performed RNA sequencing (RNA-seq) of endometrial samples in a recent publication [1]. The selection of the pregnant heifers (HF, n=5; SF, n=5) used in these analyzes was based on data of conceptus length and number, in an effort for selecting samples that represented well the overall data collected within each fertility group. For instance, in the complete data collected [1], 38.1% of HF heifers and 40% of SF heifers had two conceptuses, and the average conceptus length was 10.6 ± 7.6 (range: 1.2 to 32.2 cm) for HF heifers and 4.7 ± 4.2 (range: 1.5 to 13.5 cm) for SF heifers. In the selected subgroup of pregnant heifers, 2 HF and 3 SF heifers had two conceptuses, and the average conceptus size was 11.97 ± 8.14 cm (range: 1.3 to 25 cm) for HF and 6.38 ± 4.5 (range: 2.1 to 13.5 cm) for SF heifers.

Two analytical procedures were used to investigate the proteomic profile of the ULF. In the first procedure, ULF proteins were precipitated, and in-solution digestion performed. To increase the number of proteins identified, a second procedure was conducted in which precipitated proteins were first loaded in 12% acrylamide SDS-PAGE for size separation, and protein digestion was performed in slices of SDS-PAGE gels.

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Description of the in-solution digestion method. Proteins in a total of 1 mL of ULF were precipitated using 4 volumes of 5% trichloroacetic acid in 100% of ice-cold acetone solution. Samples were vortexed and incubated at -22°C for 24 h for protein precipitation. Samples were then centrifuged at 16,000 x *g* for 10 min, and the supernatant discarded. Protein pellets were resuspended in a solution of urea/ammonium bicarbonate (6 M urea, 100 mM ammonium bicarbonate), digested with trypsin (www.osa.sunysb.edu/Proteomics/ProteinDigestPrep), and peptides purified by large-format, 100 μL, C18 tips (according to the manufacturer's instructions, Pierce/Thermo Scientific).

Description of the SDS-PAGE method. The amount of protein in ULF were first determined using the Qubit® Protein Assay Kit with a Qubit® 3.0 Fluorometer, and variable volumes of ULF containing 200 μg of protein were aliquoted. ULF proteins were precipitated as described in the in-solution method, and the protein pellet was resuspended in 20 μL of 1X Laemmli buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 100 mM DTT), and loaded on one of three 12% acrylamide SDS-PAGE gels. After staining with colloidal Coomassie blue, the gels were destained in water and each lane sliced into 8 pieces. Gel slices were frozen at -80°C until processing. Samples were trypsin digested according to the available protocol (http://proteomics.missouri.edu/protocols) for digestion of Coomassie-stained 1D gel bands. Peptides were then lyophilized and resuspended in 40 μL solution of 5% acetonitrile and 1% formic acid. Half of the sample (20 μL) was transferred to autosampler vials, and liquid chromatography tandem mass spectrometry (LC-MS/MS) performed using a LTQ Orbitrap XL™ mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA).

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LTQ orbitrap mass spectrometry and protein identification. A full-loop injection (18µL) of sample was loaded onto a C8 trap column (pepmap100, ThermoFisher Scientific, Waltham, MA, USA). Peptides were eluted from the trap column and separated on a 25 cm x 150 µm inner diameter pulled-needle analytical column packed with HxSIL C18 reversed phase resin (Hamilton Co.) with a step gradient of acetonitrile at 400 nL/min. The Proxeon Easy nLC HPLC system is attached to an LTQ Orbitrap XL™ mass spectrometer. Liquid chromatography gradient conditions were initially 5% B (A: 0.1% formic acid in water, B: 99.9% acetonitrile, 0.1% formic acid), followed by 2 min ramp to 10% B. Gradient of 10-20% B over 35 min, gradient of 20% B to 30% B over 40 min, gradient of 30% B to 90% B over 5 min, hold at 90% B for 22 min, ramp back to (1 min) and hold at (5 min) initial conditions.

Fourier Transform Mass Spectrometer (FTMS) data were collected (30,000 resolution, 1 microscan, 300-1800 m/z, profile, AGC 5e5) and then at each cycle (approximately 3 sec), the 9-most-abundant peptides (ignore +1 ions, ignore trypsin autolysis ions, pick peptides with > 1,000 counts) were selected for MSMS (2 m/z mass window, 35% normalized collision energy, centroid). Dynamic exclusion was applied with the following parameters: repeat count 1, repeat duration 30 sec, exclusion list max 500, exclusion duration 180 sec. Raw data was copied to the Sorcerer2 IDA (SageN Research) and peak lists prepared using ReAdW. Bovine protein entries were retrieved from NCBI using a "protein" search with the keyword bovine. A total of 126,866 *Bos taurus* entries were downloaded in FASTA format. A reversed sequence decoy database was generated using DecoyDBCreator V0.1 (http://www.p3db.org/p3db1.0/tools/DecoyDBCreator/) in which forward and reversed sequences were linked together into a single FASTA file (253,732 total sequences). Sequest searches were performed with trypsin as enzyme, two missed cleavages

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allowed, carbamidomethyl cysteine as a fixed modification, oxidized methionine as variable mod, 25 ppm mass tolerance on precursor ions, and 1Da on fragment ions.

Scaffold (version Scaffold 4.0.6.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds (XCorr >1.5) and with a mass accuracy of <10 ppm. Protein identifications were accepted if they contained at least 2 identified peptides. The minimum number of unique peptides was set at 2 in order for a protein to be identified. Peptide threshold was set at 95% peptide probability, with +2 accepted charge, and parent mass tolerance of 10 ppm. Using the parameters above, the decoy False Discovery Rate (FDR) was calculated to be 1.1% on the protein level and 0.0% on the spectrum level [43]. Total spectrum counts for proteins were used for comparisons and statistical analysis.

Untargeted Metabolomics. Global metabolomic analysis of the ULF was conducted by the Southeast Center for Integrated Metabolomics (SECIM) at the University of Florida (Gainesville, FL) in the same 25 samples selected for proteomics. All samples were extracted following a cellular extraction procedure without pre-normalization to the sample protein content. Global metabolomics profiling was performed on a Thermo Q-Exactive Oribtrap mass spectrometer with Dionex UHPLC and autosampler. All samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 35,000 at *m/z* 200 as separate injections. Separation was achieved on an ACE 18-pfp 100 x 2.1 mm, 2 μm column with mobile phase A as 0.1% formic acid in water and mobile phase B as acetonitrile. This is a polar embedded stationary phase that provides comprehensive coverage, but has some limitations for the

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coverage of very polar species. The flow rate was 350 µL/min with a column temperature of 25°C. A total of 4 µL of each sample was injected for negative ion mode analysis and 2 µL was injected for positive ion mode analysis.

Statistical analyses. Statistical analyses for measurements of IFNT in ULF was conducted using SAS (SAS Institute Inc., Cary, NC). IFNT concentrations were assessed for normality using the UNIVARIATE procedure, and because concentrations were determined not to be normally distributed, data were rank transformed. The effect of pregnancy status (pregnant vs open), conceptus number (one vs two) and fertility classification on ULF IFNT concentrations were determined by analysis of variance (ANOVA) using the GLM procedure. Post-test comparisons were conducted using the LSMEANS statement with the Fisher's protected Least Significant Difference (LSD) option. Pearson's correlation for conceptus size and ULF IFNT concentrations were determined using the CORR procedure. Of note, for heifers with two conceptuses, conceptus length was equal to the sum of both conceptuses present in the flush.

Statistical analysis of the proteomics data was performed in Scaffold (version Scaffold 4.0.6.1, Proteome Software Inc., Portland, OR). Significant proteins were identified based on Fisher's exact test for comparisons including two treatments (e.g. Pregnant vs Open), and ANOVA with Fisher's LSD post-hoc adjustment for analyses containing more than two treatments (e.g. HF vs SF vs IF). Enrichment analysis (http://www.geneontology.org) was performed to identify pathways that were over-represented among significantly different proteins identified in ULF.

Statistical analysis of the metabolomics data was performed using the web server MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) [44-49]. For this analysis, a table matrix of

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m/z peak intensities with samples in columns and features in rows were created and imported to MetaboAnalyst 4.0. Data from positive and negative ion modes were separately subjected to statistical analyses. Data filtering was performed based on the interquartile range to identify and remove low-quality data points, and then, the data was normalized by the sum method, log transformed and scaled using the auto scaling method. A t-test was used to investigate if features were differently expressed for comparisons including two treatments (e.g. Pregnant vs Open), and ANOVA with Fisher's LSD post-hoc adjustment was used when the analyses contained more than two treatments (e.g. HF vs SF vs IF). Fold change (FC) analysis was conducted to detect whether the abundance of differential metabolites increased or decreased in ULF for each comparison. The calculation of FC is based on the ratio between two group means (e.g. pregnant/open), and the fold change threshold of two was set for all analyses. Differential metabolites were further investigated using the metabolite set enrichment analysis (MSEA) module of MetaboAnalyst 4.0, to identify biologically meaningful pathways associated with the significantly altered metabolites. To account for multiple comparisons, a false discovery rate (FDR < 0.05) was applied for all analyses. Additionally, using the human database of MetaboAnalyst 4.0, joint pathway analyses that combined the uterine transcriptome data from fertility-classified heifers [1] with the differential metabolites detected in the ULF were conducted, in order to further explore biological pathways that were overrepresented among fertility-classified heifers during early pregnancy.

Integration of uterine lumenal secretome and endometrium and conceptus transcriptome. The present ULF data was integrated with transcriptome data from endometrium and conceptuses [1]. Gene expression data, available in Gene Expression Omnibus (GEO) database

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under the accession number GSE107891), was reanalyzed to address comparisons not performed in the original work. Differential gene expression analysis was conducted using edgeR-robust [50], and a false discovery rate (FDR) of ≤ 0.05 was used as the cutoff for determining the differently expressed genes (DEGs).

Because IFNT modulates endometrial gene expression, an analysis was conducted to investigate the expression of ISGs by the endometrium of fertility-classified heifers. Furthermore, we explored the expression of known genes regulating IFNT transcription in the conceptus and endometrium.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) was used to identify genes encoding amino acid transporters, and the expression of those genes by the conceptus and endometrium was investigated. Additionally, the origin of proteins that increased in the ULF by pregnancy (conceptus versus endometrium secreted) was explored using the transcriptome data from conceptus and endometrium. Furthermore, we investigated endometrial genes (FDR<0.05) and ULF proteins (P<0.01) that were similarly increased by pregnancy, to identify proteins that are robustly upregulated by the endometrium during pregnancy.

The analysis performed on endometrial transcriptome included data generated from the same subgroup of pregnant (HF, n=5; SF, n=5) and open (HF, n=5; SF, n=5) heifers selected for ULF analysis. The model used to test the effect of pregnancy on endometrial gene expression was composed by gene expression data as the dependent variable and pregnancy status (pregnant vs nonpregnant) as the independent variable. The model used to evaluate differences among only pregnant heifers (HF, n=5; SF, n=5) was comprised of gene expression data of pregnant

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endometrium as the dependent variable and fertility group (HF vs SF) as the independent variable.

A similar approach was used to investigate the relationship of ULF composition and conceptus transcriptome. Because there is natural variation in conceptus length among conceptuses collected in the same day during the period of conceptus elongation in cattle [7, 23, 41, 51, 52] and the conceptus transcriptome changes as it develops [23], we first analyzed day 17 conceptus transcriptome data from HF (n = 15) and SF heifers (n = 7) that were either short (n = 11; mean length: 2.5 ± 0.4 cm) or long (n = 11; mean length: 14.5 ± 1.9 cm) to explore differences in the transcriptome of conceptuses that were likely due to stage of development. The model used to test the effect of conceptus length on conceptus transcriptome was composed by gene expression data as the dependent variable and category of conceptus size (short vs long) as the independent variable. Then, the transcriptome of HF (n = 17) and SF (n = 10) conceptuses were compared for the same set of genes of interest, to investigate the influence of ULF composition on conceptus transcriptome, in order to explore the mechanisms associated to the retarded growth of SF conceptuses and reduced pregnancy success in SF heifers. The model used to test the effect of fertility-classification on conceptus transcriptome was composed by gene expression data as the dependent variable and fertility group (HF vs SF) as the independent variable.

RESULTS

Interferon tau (IFNT). As expected, IFNT was not detected in the ULF of open heifers (**Figure 1A**). Among pregnant heifers, conceptus number did not influence (P = 0.22) ULF IFNT

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abundance (**Figure 1B**). Consistent with differences in conceptus length [1], IFNT was higher in in the ULF of pregnant HF than SF ($P = 0.045$) and IF ($P = 0.02$) heifers (**Figure 1C**). As expected, the amount of IFNT in the ULF was correlated (r = 0.82, $P < 0.01$) with conceptus length (**Figure 1D**).

Integrating ULF IFNT and transcriptome data. There were no differences in abundance of IFNT transcripts in short compared to long conceptuses (FDR = 0.98) or between HF and SF conceptuses (FDR = 0.21). The expression of select classical and nonclassical ISGs in the endometrium were also not different in pregnant HF and SF endometrium (**Table 1**). The expression of select genes involved with the transcriptional control of IFNT by the conceptus and endometrium [53] is presented in **Table S1**. Expression of *POU5F1* in conceptuses was not different between short and long (FDR = 0.20) or between HF and SF conceptuses (FDR = 0.11). *EOMES* expression in day 17 conceptuses was very low (FPKM<1). Interestingly, conceptuses from HF heifers had higher (FDR $\leq$ 0.01) expression of the IFNT transactivators *DLX3* and *GATA3* compared to SF conceptuses, and *GATA3* and *CREBBP* tended (FDR < 0.1) to be higher in HF than SF conceptuses.

Among factors from the uterus that stimulates conceptus IFNT production *in vitro*, colony stimulating factor 1 (*CSF1*) was increased (FDR $\leq$ 0.01) in endometrium from pregnant as compared to open heifers, but no differences were observed in the endometrium of pregnant HF and SF heifers (**Table S1**). Additionally, endometrial *FGF2* expression on day 17 was not affected by pregnancy or different between pregnant HF and SF heifers. Of note, endometrial *CSF2* and *IL3* expression was very low (FPKM<1; **Table S1**).

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ULF Proteomics. There were 699 proteins identified in the ULF using the in-solution method (**Dataset S1**) and 899 proteins identified using the SDS-PAGE method (**Dataset S2**). The expected differences in sensitivity observed between the SDS-PAGE and the in-solution methods are attributed to the up-front fractionation of proteins by size in the gel approach, and due to differences in total LC-MS acquisition time between the two approaches. The gel technique was performed using 880 min of total acquisition time per sample (8 slices x 110 min acquisition each), whereas the in-solution digestion was analyzed in a single gradient of 110 min. The increase in acquisition time results in increased number of proteins being identified. The differences in nature of proteins identified by the two procedures can also be explained by methodological differences between the two approaches. The SDS-PAGE method is detergent based, and SDS solubilize membrane associated proteins with greater efficiency than the in-solution digestion buffer, which does not contain any detergent. Nevertheless, there was a significant overlap in the proteins identified between the two procedures. The majority of the proteins identified by the in-solution method (63%; 440/699) were also detected by the SDS-PAGE method, and 51% of the significantly different proteins identified among all comparisons performed in the data generated by in-solution method were also significant in the analyses conducted in the data from the SDS-PAGE method (**Table 2**).

The abundance of 167 (**Dataset S3**) and 446 (**Dataset S4**) proteins were differently present in the ULF of pregnant compared to open heifers for the in-solution and SDS-PAGE methods, respectively, and 103 of those proteins were consistently different in abundance in both procedures (**Table 2; Dataset S5**).

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Based on the 103 different proteins detected by both methods, the abundance of 62 proteins increased and 41 decreased in pregnant compared to open ULF (**Dataset S5**). Interestingly, pathways associated with the 62 proteins that increased in pregnant ULF using the Reactome database included amino acid biosynthesis and metabolism (CKMT1, ACADVL, ACAA1, ENO1, ALDH2, ACAT1, GOT2, P4HB, GOT1, ALDOC, AHCY, PGD, CAT, ACO2, IDH2, FABP3, ALDOA, PSPH, FH, ATIC, PSAT1, PGM2, ABHD14B, HSD17B10 and DDAH2), metabolism of carbohydrates (ENO1, GOT1, GOT2, ALDOC, PGD, ALDOA, PGM2), and TCA cycle (ACO2, IDH2, FH) (**Table S2**). Conversely, pathways associated with the 41 proteins that increased in open compared to pregnant ULF using the Reactome database included intraflagellar transport due to the increased abundance of three cytoskeletal proteins (TUBB2B, TUBA1A, TUBB5), and regulation of insulin secretion, due to the increased abundance of three G-protein subunits (GNAI2, GNB2, GNAQ) in open ULF (**Table S3**).

Because the SDS-PAGE method was more sensitive (**Table 2**), the description of the subsequent results was focused on the data generated by the SDS-PAGE procedure. The abundance of selected top 10 most significant proteins that increased in ULF of pregnant versus open heifers (**Table 3**) included embryonic secreted factors (TKDP1, PAG11), nuclear-envelope proteins (Lamin A/C; LMNA), mitochondrial proteins (DLD, ACAA1, ACAA2, HSPD1, HSPA9, GLUD1), and glutathione synthase (GSS). Likewise, the selected top 10 most significant proteins that increased in open than pregnant ULF (**Table 3**) included cytoskeletal proteins (EZR, 2P4N, MYO1B), G-protein subunits (GNAQ, GNAI2), other membrane-bound proteins (PAS-6/7, FAM234A, RARRES1, GPC1), and a protein of the coagulation system (Factor V).

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There were 221 different proteins in ULF of pregnant HF and SF heifers of which 142 increased and 79 decreased (**Dataset S6, Figure 2**). Panther pathways associated with the 142 proteins that increased in ULF of pregnant HF compared to SF heifers included vitamin B6 metabolism, amino acid metabolism (asparagine and aspartate biosynthesis, serine glycine biosynthesis), energy metabolism (pyruvate metabolism, pentose phosphate pathway, ATP synthesis and glycolysis), p38 MAPK pathway, and cytoskeletal regulation by Rho GTPase (**Table S4, Figure 2)**. Panther pathways associated with the 79 proteins that increased in ULF of pregnant SF compared to HF heifers were related to hemostasis and included the plasminogen activating cascade and blood coagulation pathways (**Table S5, Figure 2**).

There was a total of 48 differently abundant proteins in the ULF of open fertility-classified heifers (**Dataset S7**, **Figure 3**), but there were no pathways associated with the differently abundant proteins.

Integrating ULF proteomics with transcriptome data from the endometrium and conceptus. Among the 62 proteins commonly increased by pregnancy in both methods (**Dataset S5**), 56 were expressed by the endometrium based on our previously published RNA-seq data [1]. The remaining 6 proteins not expressed by the endometrium includes conceptus secreted factors (PAG11), a precursor for trophoblast Kunitz domain protein (TKDP1; GenInfo Identifier: 296481028) and TKDP1, a precursor of serpin A3-7-like protein (LOC784932; GenInfo Identifier: 985701132) that was neither expressed by the endometrium or conceptuses, and two proteins not well annotated (GenInfo Identifier: 89611 and 2323392). The proteins determined to be expressed exclusively by the conceptus were PAG11, TKDP1, and the TKDP1 precursor. There were also 11 proteins (ACO2, AIFM1, GDA, HSPA9, PREP, EEF2,

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HSD17B10, MTAP, PGM2, PSAT1, TPI1) that increased in the ULF by pregnancy that were determined to be encoded only by genes expressed by the endometrium and not by conceptuses.

The expression of 45% (27/60) of the genes encoding proteins commonly increased in the ULF by pregnancy were found to be increased in the endometrium of pregnant than open heifers (**Figure 4A, Dataset S8**), indicating that these proteins are upregulated in the endometrium by pregnancy. Interestingly, the abundance of 56% (15/27) of these proteins were also increased in pregnant HF than SF ULF (LAP3, PSPH, ENO1, WARS, QPRT, ATIC, MDH2, AHCY, ACAA2, CAP1, GOT1, HSPA9, ACTN4, LOC615277, GSS), but none of them were increased in pregnant SF than HF ULF (**Dataset S6**). Conversely, the expression of only 13% (5/39; FCGBP, GNAI2, SERPINF1, TUBA1A, TUBB, and two proteins not well annotated GenInfo Identifier: 7547266, 7547965) of the genes encoding proteins that increased in open ULF were found to be increased in the endometrium of open heifers (**Figure 4B, Dataset S9**), suggesting that these proteins are downregulated in the endometrium by pregnancy. Of those, the abundance of FCGBP was decreased, but TUBB and TUBA1A increased in pregnant HF than SF ULF.

Metabolomics

A total of 1,378 metabolites were detected (122 identified and 1,256 unknown) in the positive ion mode (**Dataset S10**) and 551 metabolites detected (84 identified and 467 unknown) in the negative mode (**Dataset S11**). Identified metabolites included amino acids and amino acid derivatives, ions, carbohydrates, purines, polyphenols, lipids, and other constituents. Overall, there were 315 differential metabolites (70 identified) in the ULF of pregnant versus open heifers (**Datasets S12 and S13**). A striking difference was observed in pregnancy induced changes in

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the metabolites found in the uterine lumen of HF and SF heifers (**Table 4**). In HF heifers, there were 271 differential metabolites (67 identified) comparing ULF from pregnant and open heifers (**Datasets S14 and S15**). Surprisingly, no significant differences in metabolite composition were observed in the ULF of SF heifers that were pregnant or open.

Additionally, there were 13 differential metabolites in the ULF of pregnant HF and SF heifers, but only one (L-Methionine) identified (**Datasets S16 and S17**), and its concentration increased in pregnant HF than SF ULF. Furthermore, in the comparison among only open fertility-classified heifers, there was one unidentified metabolite (m/z 109, retention time: 8.5 min) that was increased in the ULF of SF than HF and IF heifers (**Dataset S18**).

Results from the fold change (FC) analysis of pregnant and open ULF are presented on **Datasets S19** and **S20**, and **Supplementary Figures 1A** and **S1B**. The top 20 metabolites with highest FC differences are summarized in **Table 5**. Of note, the abundance of 76% (145/192) of the metabolites with FC > 2 were increased in pregnant compared to open ULF. Results of FC analysis comparing only ULF from pregnant HF and SF heifers are presented on **Datasets S21** and **S22,** and the top 20 identified metabolites with highest differences in FC are summarized in **Table 6**. The abundance of 59% (102/173) of the metabolites with FC > 2 were increased in HF compared to SF ULF, further indicating that pregnancy induced changes in uterine lumenal metabolites were greater in HF than SF heifers.

Metabolite set enrichment analysis (MSEA) identified significant pathways associated with the differential metabolites between pregnant and open ULF (**Figure 5A**). The top five pathways were urea cycle, glycine and serine metabolism, glutamate metabolism, and arginine and proline metabolism. Furthermore, the top five most significant pathways associated the metabolites uniquely increased in HF ULF by pregnancy were urea cycle, ammonia recycling,

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glycine and serine metabolism, arginine and proline metabolism, and the Warburg effect (**Figure 5B**). Additionally, joint pathways analysis identified several pathways which were overrepresented among genes (FDR < 0.05) and metabolites (FDR < 0.05) that increased by pregnancy. These pathways included key biological events during early pregnancy, including biosynthesis and metabolism of amino acids, lipids, and carbohydrates (**Dataset S23 and Figure 6A**). Interestingly, pathways associated with differently expressed genes (FDR < 0.05) and metabolites (FC > 2) between pregnant HF than SF endometrium and ULF, respectively, included amino acid biosynthesis and metabolism (e.g. phenylalanine, tyrosine, tryptophan, glutamine and arginine), energy metabolism (e.g. TCA cycle, pentose phosphate pathway), and lipid metabolism (e.g. metabolism of glycerophospholipids and steroid biosynthesis) (**Dataset S24 and Figure 6B**).

Expression of genes encoding amino acid transporters by the endometrium and conceptuses. Because the majority of the differential metabolites were associated with amino acid metabolism, we further investigated the expression of amino acid transporters by the endometrium and conceptuses. Endometrial expression of five genes encoding amino acid transporters was increased (*SLC15A3*, *SLC7A1*, *SLC15A1*, *SLC7A9*, and *SLC3A2*) and three genes decreased (*SLC7A6*, *SLC7A3* and *SLC6A14*) by pregnancy (**Dataset S25**). Additionally, among pregnant heifers, the expression of *SLC7A1*, a transporter of cationic amino acids (arginine, lysine and ornithine) [54], was upregulated in HF endometrium (**Dataset S26**).

Conceptus expression of amino acid transporters did not differ among short and long conceptuses (**Dataset S27**), but the expression of *SLC7A6* was increased in HF conceptuses, and *SLC6A19* was increased in SF conceptuses (**Dataset S28**). The top five most expressed amino

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acid transporters in day 17 conceptuses were *SLC3A2*, *SLC15A4*, *SLC43A2*, *SLC15A1* and *SLC7A8*.

DISCUSSION

In our recent work, endometrial responses to pregnancy in fertility-classified heifers was assessed by comparing the endometrium transcriptome of open with pregnant animals [1]. This analysis found 3,422 differently expressed genes (DEGs) in the endometrium of HF heifers, but only 1,095 DEGs in the endometrium of SF heifers. This diminished endometrial response to pregnancy was hypothesized as the main cause of early embryo loss by day 28 in SF heifers [1, 41]. Results of the current study further supports the theory of dysregulated conceptus-endometrium interactions in SF heifers, resulting in compromised conceptus development and failure to establish pregnancy. For instance, there were 271 differential metabolites in the ULF of HF heifers that were pregnant or open, but no significant changes in ULF metabolite profile were observed among SF heifers regardless of pregnancy status. Additionally, the majority of the metabolites differently abundant between the ULF of nonpregnant and pregnant heifers were increased by pregnancy. Sponchiado et al. (2019) reported an overall reduction in the number of metabolites detected in the ULF of pregnant than cyclic heifers on day 7 of gestation or estrous cycle. However, crosstalk between the conceptus and endometrium increases considerably during and after maternal recognition of pregnancy on day 16 [21], and the observed increase in metabolites detected in the ULF by pregnancy, as well as in pregnant HF than SF ULF, are hypothesized to influence uterine receptivity and consequently conceptus development and survival.

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In the present study, several metabolites associated with glutamine metabolism were increased in pregnant HF than SF ULF, such as 3-hydroxy-3-methylglutarate by 6.6-fold, N-methyl-L-glutamate by 4.6-fold, and glutarate by 3.5-fold. Although glutamine is a nonessential amino acid that can be synthesized from glucose, it is a key substrate for highly proliferative cells, and some cancer cells lines have been described as 'glutamine addicted' because they cannot survive without exogenous glutamine [55, 56]. In addition to providing a carbon source for the synthesis of lactic acid during glycolysis [57], glutamine is also an important donor of nitrogen for synthesis of nonessential amino acids (that are used for synthesis of new proteins) and nucleotides, and glutamine synergizes with essential amino acids to activate the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) [58]. Activation of mTORC1 promotes cell growth and metabolism [59] and also plays an important role in ovine conceptus elongation [60]. Progesterone can alter the ULF abundance of metabolites involved with glutamate metabolism (e.g. glutamate, glutamine, alpha-ketoglutaramate) during the onset of conceptus elongation in cattle [20], however circulating progesterone concentrations did not differ among the fertility-classified heifers used in the present study [1].

Glutamate and glutamine are precursors of arginine [61]. Arginine is a precursor of nitric oxide (NO) and polyamines (putrescine, spermidine, spermine and agmatine) that regulates key events during early pregnancy, such as angiogenesis, placentation and embryonic development [60, 62, 63]. The secreted phosphoprotein 1 (SPP1; also known as osteopontin), an extracellular matrix protein secreted by the uterine lumenal epithelium [11, 64], interacts with arginine and plays an important role in conceptus elongation and attachment through the activation of mTOR complexes 1 and 2 (MTORC1 and MTORC2) [60, 65]. Interestingly, in the present study, the expression of a cationic amino acid (arginine, lysine and ornithine) transporter *SLC7A1* [54] was

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increased in the endometrium by pregnancy, and also increased in the endometrium of pregnant HF than SF heifers on day 17. Furthermore, the concentrations of arginine and lysine in the ULF were significantly increased (FDR < 0.01) by pregnancy, and N-alpha-acetyl-L-Lysine, an amino acid derived from lysine, was increased by 3.4-fold in the ULF of pregnant HF than SF heifers. Lysine is another essential amino acid, and has been observed to induce cell proliferation in vitro and in vivo [66]. Lysine can be converted into carnitine [67], and two amino acid derivatives of carnitine were upregulated in pregnant HF than SF ULF; the abundance of isovalerylcarnitine increased by 7.3-fold, and acyl-carnitine (5-OH) by 2.5 fold. The primary role of carnitine is associated with the transport of long-chain fatty acids from the cytosol into the mitochondria for beta-oxidation, as acyl CoA (long chain fatty acid bound to coenzyme A) is not permeable to the inner mitochondrial membrane [68, 69]. The increase in acyl-carnitine, composed by carnitine bound to acyl CoA, may indicate increased abundance of long-chain fatty acid in the ULF of pregnant HF than SF heifers, and a number of long-chain (C11-20) and very long-chain (C21-25) fatty acids were increased in ULF of pregnant HF than SF heifers [Moraes et al., companion *Biology of Reproduction* manuscript]. Additionally, L-carnitine also possesses antioxidant [70] and anti-inflammatory [71] properties, which are important biological processes during early pregnancy [72, 73].

The importance of arginine and glutamine in porcine embryo development has been demonstrated [74-76]. Knockdown of the arginine, lysine and ornithine transporter (SLC7A1) in the ovine trophectoderm using morpholino antisense oligonucleotide (MAO) retarded conceptus development [61], indicating the importance of these amino acids during conceptus growth in ruminants. In the present experiment, the expression of SLC7A6, which mediates mediates

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arginine efflux in exchange with glutamine [77, 78] was increased in HF than SF conceptuses, supporting the idea of abnormal arginine and glutamine metabolism in SF conceptuses.

Cancer cells and perhaps preimplantation embryos metabolize glucose preferably through aerobic glycolysis rather than oxidative phosphorylation, which is a metabolic adaptation of rapidly proliferative cells known as Warburg effect [79-82]. The main explanation for the switch from oxidative phosphorylation in normal somatic cells to aerobic glycolysis in cancer cells, is that highly proliferative cells have other critical metabolic requirements that extend beyond energy production. For instance, cells undergoing mitosis have large requirements for biomass production, as one parent cell has to duplicate all its contents to form two identical daughter cells, and this alternative glucose metabolism (Warburg effect) diverges glucose to create macromolecular precursors for synthesis of fatty acids, amino acids, and nucleotides. One of the most notable characteristics of cells undergoing the Warburg effect is that these cells uptake more glucose and produce more lactic acid than noncancer cells [81, 83]. This is a result of pyruvate being diverged from the TCA cycle and metabolized to form lactic acid [79]. Although less ATP is generated through the aerobic process (2 mol of ATP per mol of glucose compared to 38 mol of ATP for oxidative phosphorylation), this alternative glucose metabolism provides sufficient energy and anabolic precursors to support cell proliferation [79]. In fact, the pentose phosphate pathway (PPP) plays a key role in this process, producing ribose sugars for nucleotide synthesis, and generating sufficient levels of nicotinamide adenine dinucleotide phosphate (NADPH) for the biosynthesis of macromolecules [84]. Interestingly, in the present study, the abundance of two key enzymes which participate in the PPP were upregulated in the ULF of pregnant HF than SF heifers; transketolase (TKT) was increased by 47-fold, and 6-phosphogluconate dehydrogenase (PGD) was increased by two-fold in the ULF of pregnant HF

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as compared to SF heifers. The Warburg effect was one of the most significant pathways associated with the differential metabolites identified between pregnant and open ULF, and with the metabolites uniquely increased in HF ULF by pregnancy. Thus, taken together, these results demonstrate increased availability of substrates required for conceptus development in pregnant HF than SF ULF, which likely contributed to the increased rate of development of HF conceptus, and consequently, with the higher pregnancy rate of HF heifers.

Histidine is an essential amino acid, and thus it cannot be synthesized *de novo* and must be provided in the diet [85]. Histidine availability in the uterine lumen during the preimplantation period has been observed to increase in cattle [16, 25] and sheep [26]. In the present experiment, histidine increased by 3.5-fold in pregnant than open ULF, and the expression of the histidine transporter (*SLC15A3*) increased by 4.6-fold in the endometrium by pregnancy. The SLC15A3 transporter is an ISG [25]. Although IFNT concentrations were higher in ULF of pregnant HF than SF, *SLC15A3* expression by the endometrium did not differ among pregnant fertility classified heifers. L-Methionine was increased by 13-fold in the ULF of pregnant than open heifers, and by 18-fold in the ULF of pregnant HF than SF heifers. Methionine is the initiating amino acid for the synthesis of proteins in eukaryotes [86, 87]. Preimplantation bovine embryos uptake methionine from its environment [88], and the absence of methionine in the culture media reduces blastocyst development rate in bovine embryos produced in vitro [89]. Methionine is also important for controlling oxidative stress, as it can be metabolized into cysteine, that along with glutamate and glycine are precursors of glutathione (GSH) [90, 91], a major antioxidant in mammalian cells [92]. Furthermore, GSH is also a key regulator of cell proliferation [93], and therefore might play an important role during the exponential growth of the ruminant conceptus during elongation. Moreover, concentrations of

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GSH in the ULF have been observed to increase during early pregnancy in ewes [26], and glutathione metabolism in the uterus appears to be influenced by progesterone and day of the cycle during the period of onset of conceptus elongation in cattle [20]. In the present study, glutathione synthetase (GSS) was increased by 19-fold in the ULF of pregnant compared to open heifers, and by 3-fold in pregnant HF than SF ULF. Additionally, glutathione disulfide (GSSG), the oxidized form of glutathione (GSH), was increased by 5-fold in the ULF of open than pregnant heifers. The increase in GSSG in ULF of open heifers may be because nonpregnant heifers lack conceptus derived glutathione reductase (GSR) and disulfide isomerases (PDI), which can converted GSSG back into GSH [92]. Under oxidative stress, glutathione peroxidases (GPX) and peroxiredoxin 6 (PRDX6) can catalyze the oxidation of GSH by hydrogen peroxide into GSSG plus water, but GSR and PDI can convert GSSG back into GSH [92].

The overall findings from the proteomics analysis further supports our theory of dysregulated endometrium response to pregnancy in SF heifers. While the pathways associated with the proteins that increased in pregnant HF than SF heifers were involved in important biological processes during early pregnancy (e.g. energy metabolism, amino acid biosynthesis, cell proliferation and differentiation), pathways associated with proteins that increased in pregnant SF than HF ULF were associated with hemostasis (plasminogen activating cascade and blood coagulation). These results strongly suggest that HF heifers have a uterine environment that is more receptive to promote conceptus growth and development than SF heifers. The present experiment also identified 27 genes and proteins that were similarly increased by pregnancy in the endometrium and uterine lumen, respectively, and therefore the expression of those genes by the endometrium is likely regulated by conceptus signaling. Interestingly, 56% of these proteins were also increased in pregnant HF than SF ULF, and are involved in crucial

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biological events during the preimplantation period, such as energy metabolism (ACAA2, ENO1, MDH2, LOC615277, QPRT) [94-98], glutathione synthesis (GSS) [99], attachment between trophectoderm and uterine lumenal epithelium (HSPA9) [100], *de novo* purine biosynthesis (ATIC) [101], amino acid metabolism (PSPH, GOT1, LAP3, WARS, AHCY) [102-106], and cytoskeletal organization and cell signaling (ACTN4, CAP1) [107, 108].

In this study, HF heifers had greater IFNT in the ULF than SF heifers, even though no differences in *IFNT* mRNA was observed between HF and SF conceptuses or between short versus long conceptuses. Longer conceptuses have a greater number of trophectoderm cells, and therefore are able to secret greater amounts of IFNT. As expected, IFNT in the uterine lumen was highly correlated with conceptus size (r = 0.82). Likewise, an earlier study reported no differences in *IFNT* mRNA levels between bovine conceptuses that were either long (>10 cm) or short (< 5 cm), but IFNT protein was substantially increased in the uterine flush from long conceptuses [32].

We further explored conceptus expression of genes that regulates IFNT transcription. The expression of IFNT transactivators *DLX3, GATA3, GATA2,* and *CREBBP* were found to increase in HF than SF conceptuses. Besides stimulating IFNT expression, these transcription factors also mediate important biological processes during preimplantation development. For instance, DLX3 and GATA2/3 play important role mediating gene expression in the placenta, regulating trophoblast differentiation, angiogenesis, and coordinating embryonic-extraembryonic signaling cross-talk [109-112], and CREBBP signaling controls vital cellular processes such as cell proliferation, differentiation and apoptosis [113-115]. Among endometrial secreted factors that stimulate IFNT transcription, *CSF1* expression was higher in the endometrium of pregnant than open heifers, but no differences were observed between pregnant HF and SF heifers. Despite the

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observed differences in ULF IFNT, IFNT signaling in the endometrium was not different in pregnant HF and SF heifers, as no differences in the endometrium transcriptome were observed for the expression of genes encoding selected classical and non-classical ISGs. This result was not unexpected, as very low amounts of IFNT and other Type I IFNs will maximally induce ISG expression based on *in vitro* experiments [116, 117], and the elongating conceptus produces and secretes a considerable amount of IFNT as it elongates [118]. Nonetheless, the transcriptome analysis in the present experiment was performed by bulk RNA-seq, and thus cell-type specific changes in ISG expression which could be of great importance for pregnancy establishment may not have been detected by this approach. Based on our findings, it is reasonable to speculate that IFNT actions in the endometrium to establish uterine receptivity [27, 40] were not substantially different between pregnant HF and SF heifers. However, the bovine conceptus can modulate endometrial gene expression independently of IFNT [119] but dependent on conceptus size [120], and HF conceptuses were on average twice as long than SF conceptuses [1]. Of note, the adequate conceptus size or concentrations of IFNT that are necessary to successfully induce pregnancy recognition and optimal uterine receptivity in cattle are still unknown and may vary among individual animals.

Studies in sheep and cattle support the idea that IFNT exits the uterus and induces the expression of ISGs in maternal tissues, such as on white blood cells (WBC), CL and liver [52, 121, 122]. Interestingly, IFNT infusions into the uterine or jugular vein in sheep during days 10-13 of the estrous cycle have been shown to protect the CL against luteolysis induced on day 11 by exogenous administration of PGF2α [117]. Similarly, intrauterine infusions of low doses of PGE1 has been reported to block the luteolytic effects induced by intrauterine infusions of PGF2α in cattle [123]. Because PGE1 and PGE2 acts through the same EP receptors, and PGE2

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was similarly increased in the ULF of pregnant HF than SF heifers [Moraes et al., *Biology of Reproduction* companion manuscript], it is possible that because SF heifers had shorter conceptuses [1], the concentrations of IFNT and PGE2 in the ULF may have been insufficient to effectively protect the CL against luteolysis, which consequently, contributed to the observed reduced reproductive efficiency in SF heifers.

In summary, the current study found substantial differences in uterine lumenal components of fertility-classified heifers. These differences are hypothesized to indicate dysregulated conceptus-endometrium interactions in SF heifers with consequences on conceptus growth and signaling, leading to pregnancy loss in SF animals.

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FIGURE LEGENDS

Fig. 1. Concentrations of interferon tau (IFNT) in the uterine lumenal fluid (ULF) were measured in samples from pregnant (HF, n=15; SF, n=9; IF, n=1) and nonpregnant (HF, n=6; SF, n=1; IF, n=4) fertility-classified heifers receiving two embryos on day 7. Concentrations of IFNT in the ULF according to pregnancy status (**A**), among pregnant heifers with one versus two conceptuses ($P = 0.22$) in the uterine lumen (**B**), among pregnant fertility-classified heifers (HF vs SF $P = 0.045$; HF vs IF $P = 0.02$; SF vs IF $P = 0.13$) (**C**), and the Pearson correlation ($r = 0.82$, $P < 0.01$) between IFNT in the ULF and conceptus size (**D**).

Fig. 2. Venn diagram showing proteins differently abundant in the uterine lumenal fluid (ULF) of pregnant HF and SF heifers according to the SDS-PAGE procedure. The blue arrows indicate the Panther pathways associated with the 142 proteins that increased or with the 79 proteins that decreased in the ULF of pregnant HF compared to SF heifers. The overrepresented proteins and enriched pathways are highlighted. Panther pathways associated with the 142 proteins that increased in ULF of pregnant HF compared to SF heifers included vitamin B6 metabolism, amino acid metabolism (asparagine and aspartate biosynthesis, serine glycine biosynthesis), energy metabolism (pyruvate metabolism, pentose phosphate pathway, ATP synthesis and glycolysis), p38 MAPK pathway, and cytoskeletal regulation by Rho GTPase. Panther pathways associated with the 79 proteins that increased in ULF of pregnant SF compared to HF heifers were related to hemostasis and included the plasminogen activating cascade and blood coagulation pathways.

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Fig. 3. Venn diagram showing the differently abundant proteins among open fertility-classified heifers.

Fig. 4. Summary of genes and proteins that were similarly increased by pregnancy in the endometrium and in the ULF, respectively **(A)**. Summary of genes and proteins that were similarly decreased by pregnancy in the endometrium and in the ULF, respectively **(B)**.

Fig. 5. Significant metabolic pathways associated with the differential metabolites between pregnant and open ULF **(A)**, and metabolic pathways associated with the metabolites uniquely increased in HF ULF by pregnancy **(B)**.

Fig. 6. Joint pathway analysis using differently expressed genes (DEG) in the endometrium and the differential metabolites detected in the uterine lumenal fluid (ULF) of open and pregnant heifers **(A)**. Joint pathway analysis for the DEG in the endometrium of pregnant HF and SF heifers, with the metabolites that were differently abundant in the ULF of pregnant HF and SF heifers **(B)**. The histograms present a summary of the joint evidence from enrichment and topological analysis performed on MetaboAnalyst 4.0 using the human database. The enrichment analysis uses the input datasets of genes and metabolites to identify pathways that appear more frequently than expected by random chance. The topology analysis evaluates the importance of metabolites and genes within a given pathway based on their position in the pathway [49].

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Figure 1

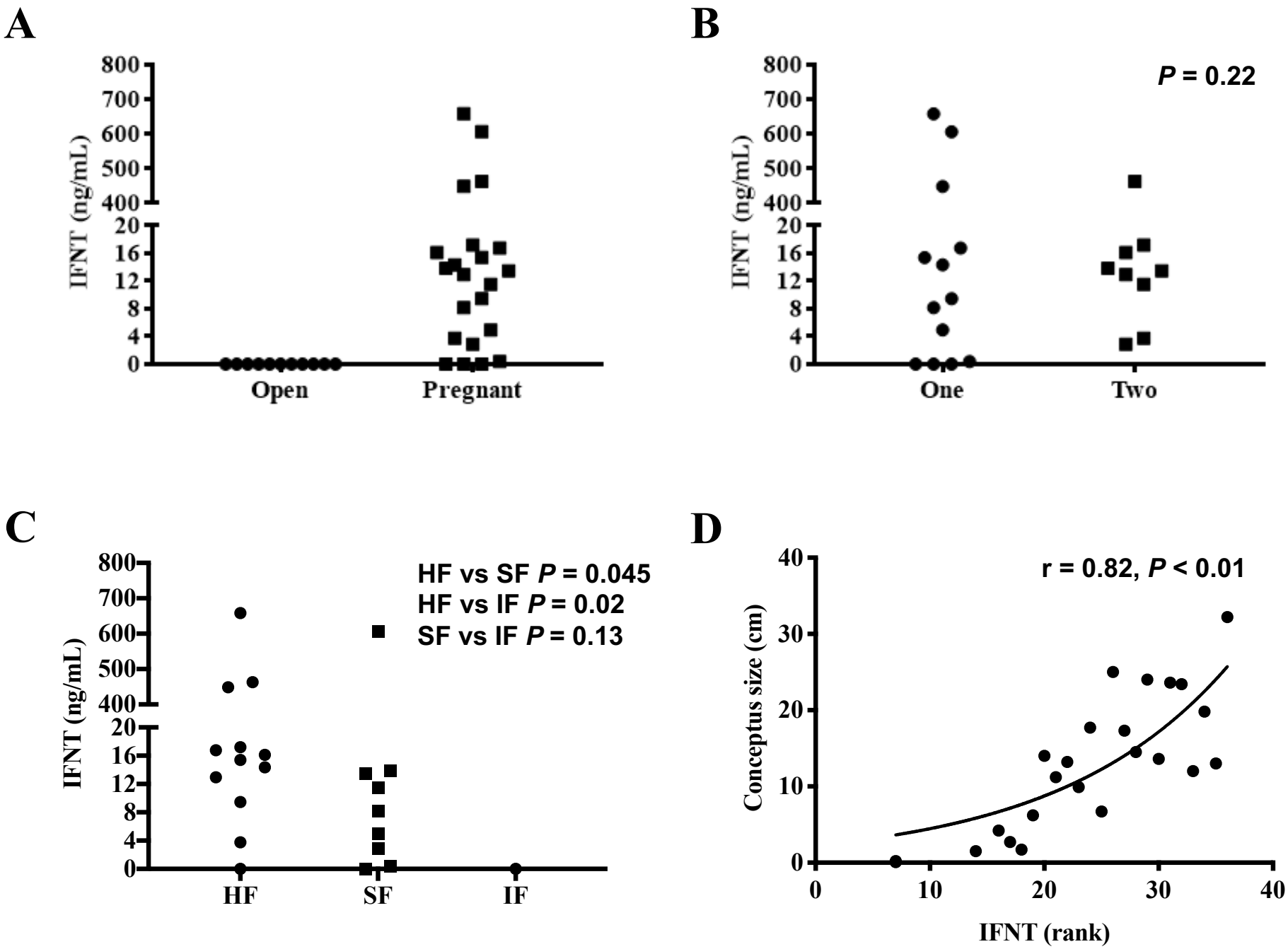

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Figure 2

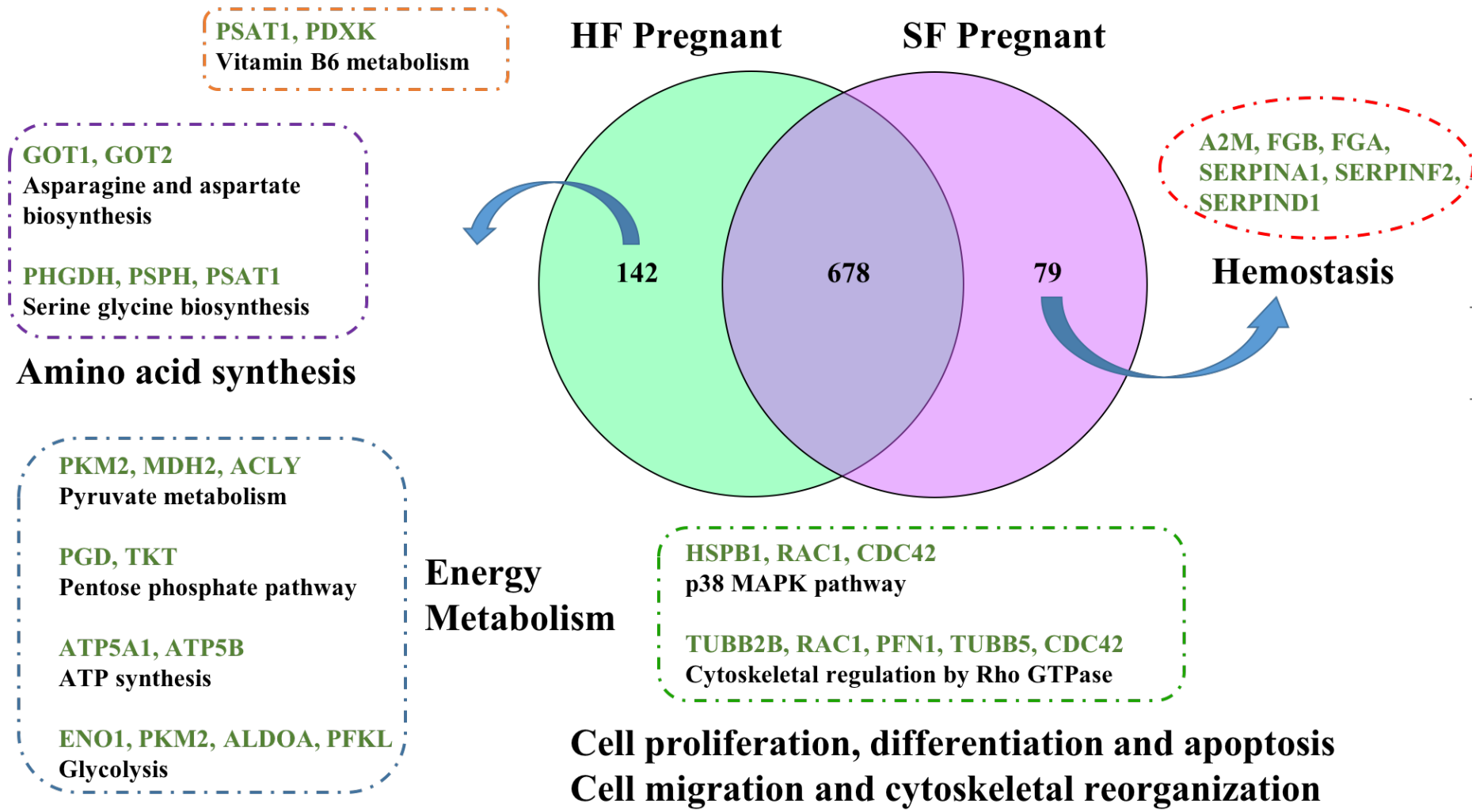

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Figure 3

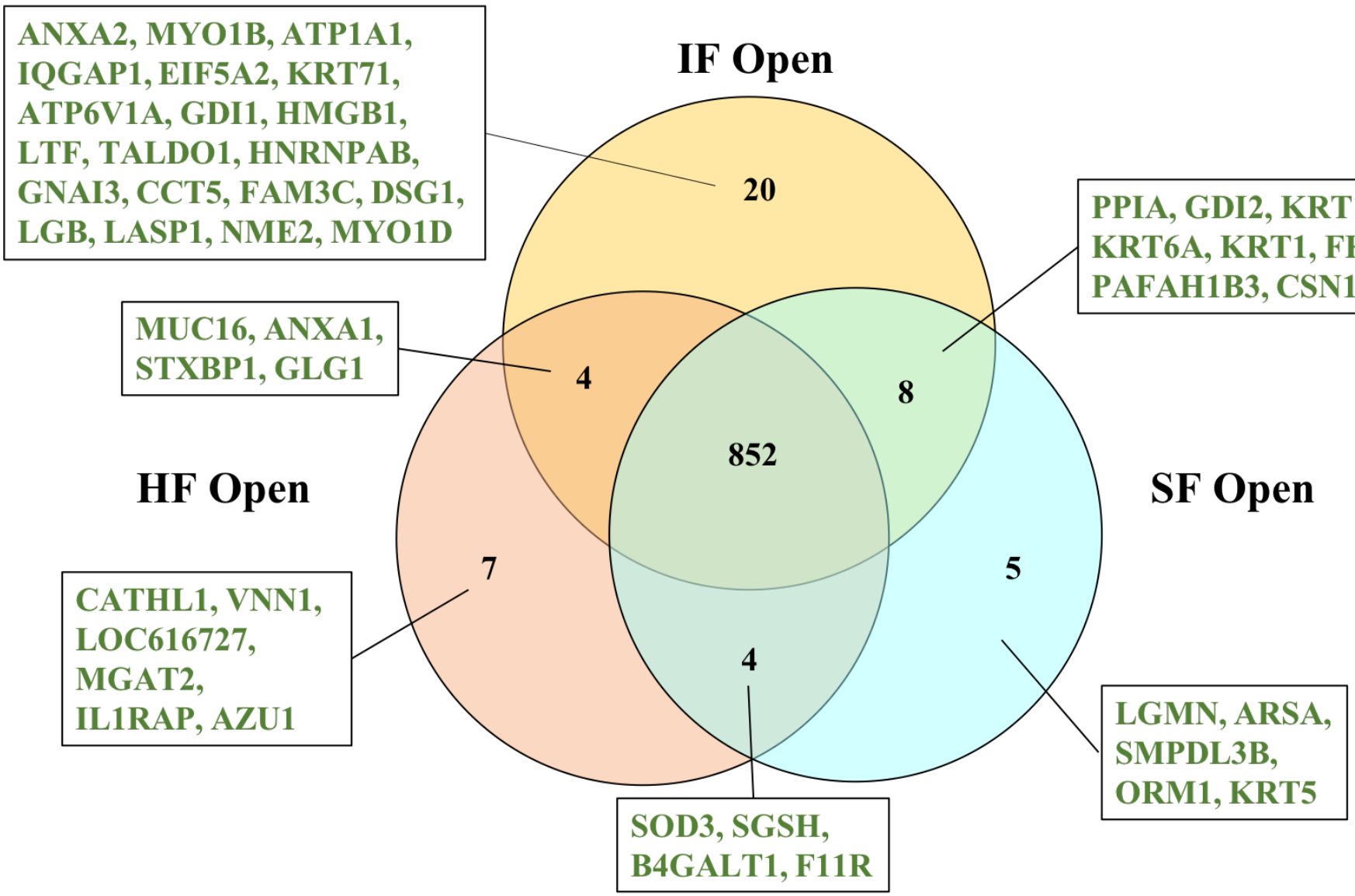

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Figure 4

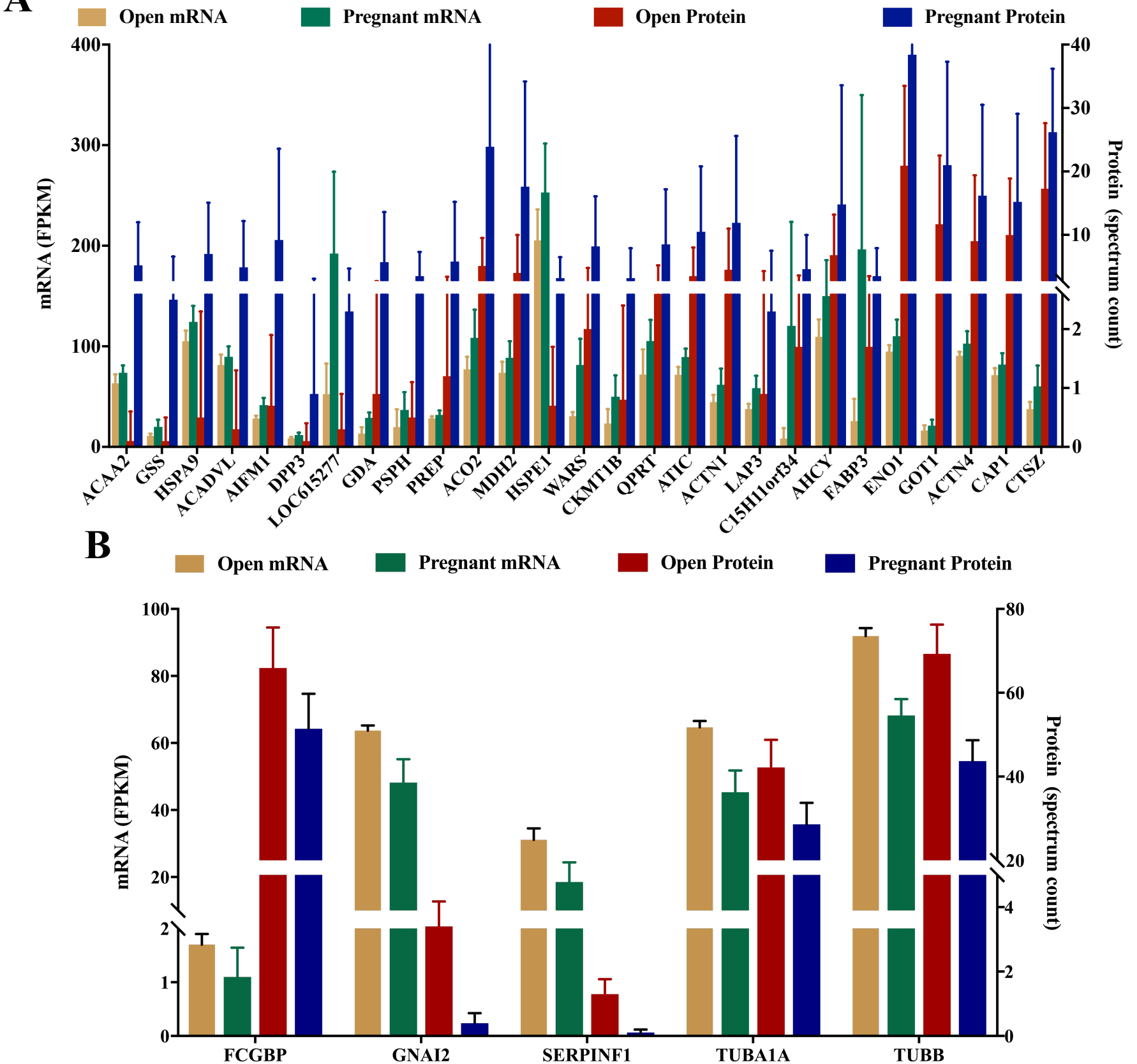

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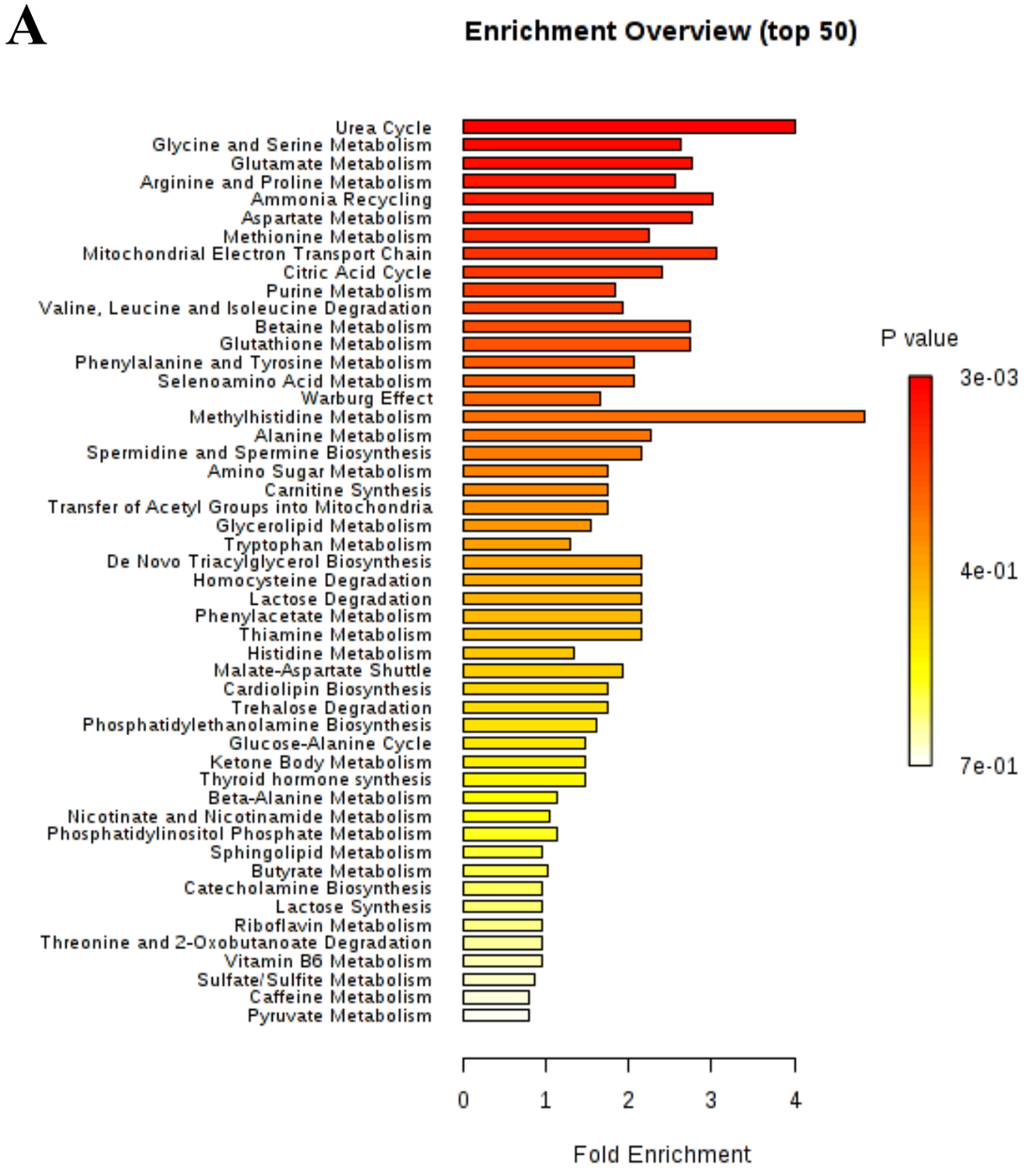
Figure 5



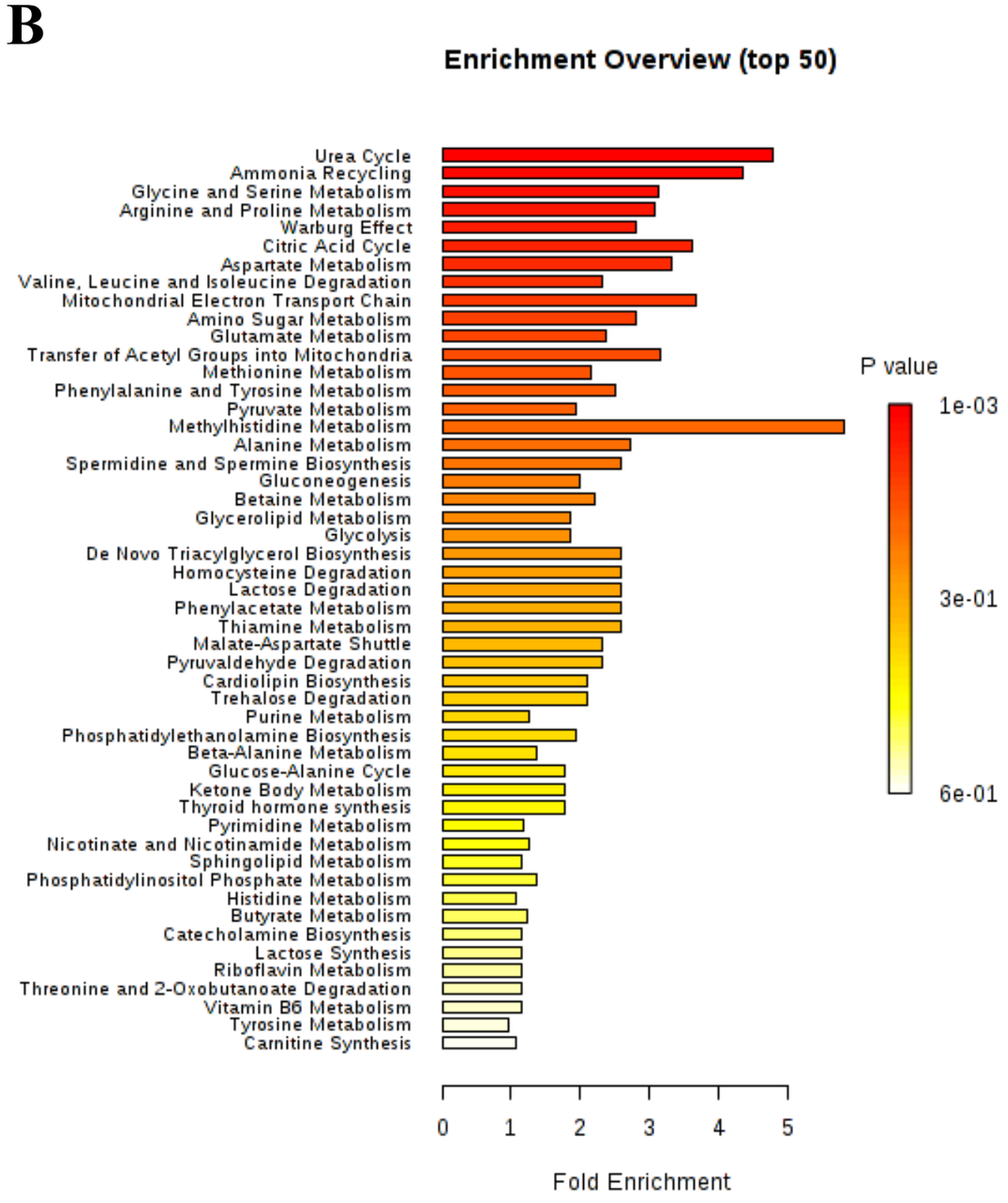


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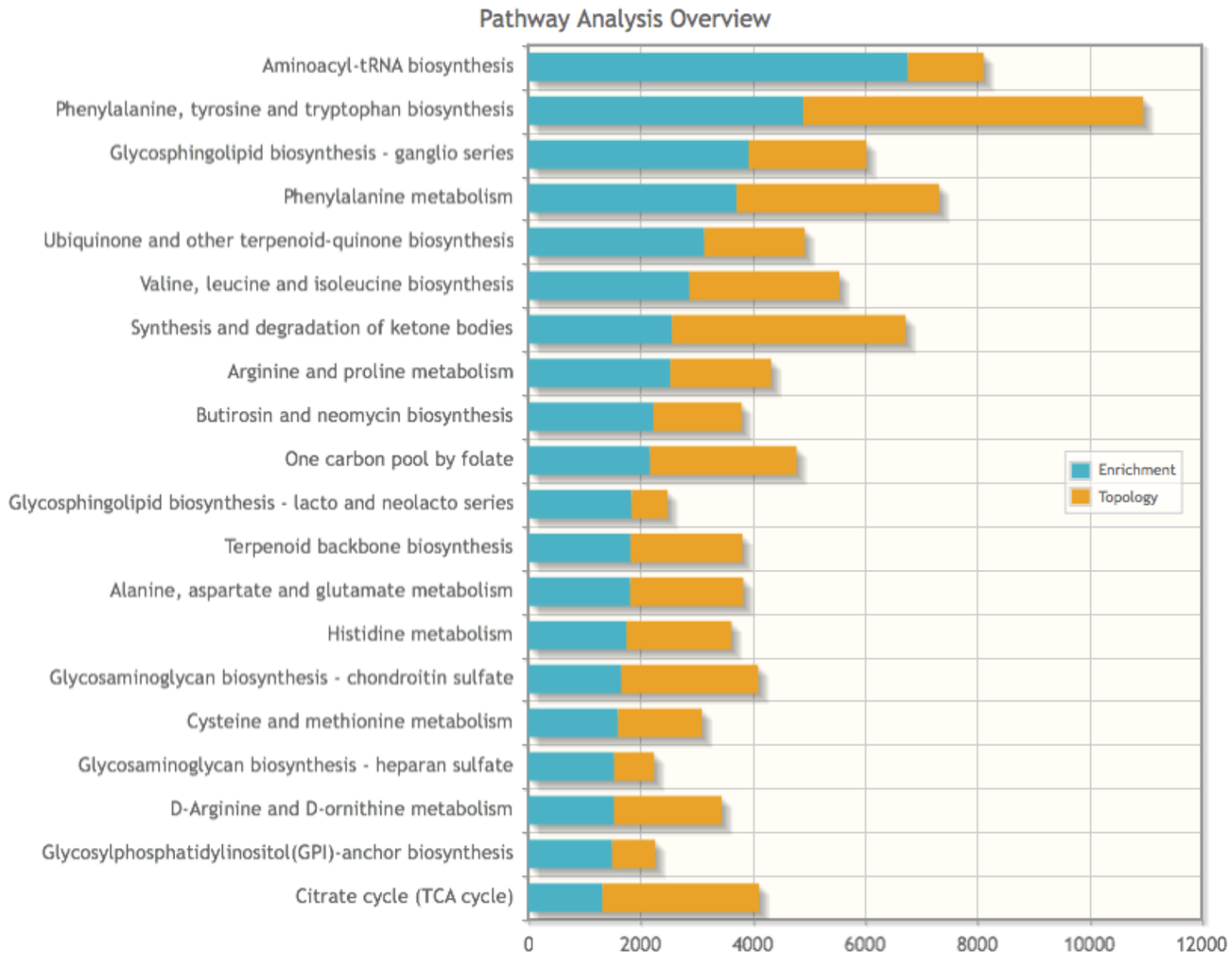
Figure 6

A



B

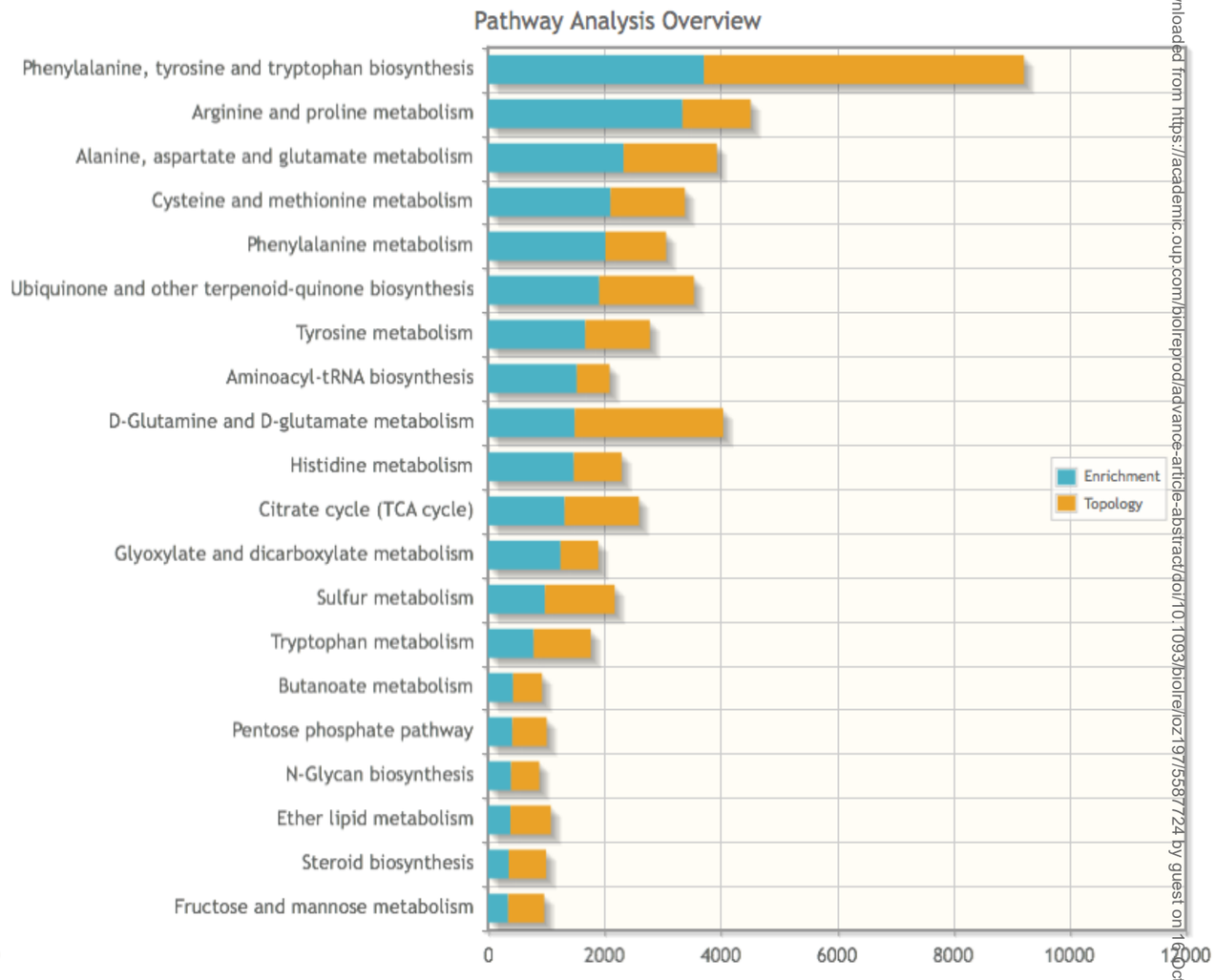


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Table 1. IFN-stimulated gene (ISG) expression in the endometrium of open and pregnant fertility-classified heifers[1]

Classical ISGs	Open FPKM[1]	Pregnant FPKM[1]	FDR	HF Pregnant FPKM[1]	SF Pregnant FPKM[1]	FDR
STAT1	50 ± 2	252 ± 18	6.61E-85	269 ± 30	236 ± 22	0.67
STAT2	16 ± 0.3	48 ± 4	8.10E-40	52 ± 6	44 ± 6	0.35
IRF9	11 ± 1	81 ± 6	2.06E-102	88 ± 8	74 ± 7	0.39
ISG15	20 ± 10	1433 ± 185	6.22E-113	1618 ± 253	1248 ± 269	0.30
B2M	721 ± 25	1956 ± 174	3.84E-31	2106 ± 209	1807 ± 285	0.40
MIC1	11 ± 2	26 ± 3	1.50E-05	26 ± 4	25 ± 6	0.90
OAS1Y	33 ± 6	576 ± 38	1.37E-106	599 ± 51	552 ± 61	0.74
RSAD2	11 ± 4	593 ± 88	2.27E-67	663 ± 116	523 ± 136	0.52
IFIH1	11 ± 1	96 ± 9	1.63E-87	101 ± 10	90 ± 15	0.67
MX1	50 ± 11	934 ± 70	7.40E-116	1007 ± 111	862 ± 83	0.56
MX2	4 ± 2	317 ± 42	9.20E-79	361 ± 60	272 ± 57	0.34
CXCL10	6 ± 1	93 ± 18	5.62E-29	110 ± 27	76 ± 25	0.35
Non-classical ISGs						
CTSL	2 ± 0.4	4 ± 1	2.43E-02	5 ± 1	3 ± 1	0.54
CST6	196 ± 21	361 ± 50	4.18E-04	408 ± 95	313 ± 35	0.93
GRP	2558 ± 518	2889 ± 171	2.98E-02	2994 ± 204	2785 ± 292	0.86
IGFBP1	79 ± 21	160 ± 27	5.71E-02	144 ± 43	177 ± 36	1.00
SLC2A1	68 ± 11	153 ± 19	1.10E-05	174 ± 35	132 ± 13	1.00
SLC5A11	0.7 ± 0.1	1 ± 0.1	2.07E-02	1 ± 0.3	1 ± 0.1	0.94

[1]Data is presented as fragments per kilobase of transcript per million mapped reads (FPKM) ± standard error of the mean (SEM).

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Table 2. Proteins differentially abundant in ULF of open and pregnant fertility-classified heifers by method

Comparisons	Number of proteins significantly ($P < 0.05$) different among comparisons		
	In-solution method (699)	SDS-PAGE method (899)	Common different proteins
Pregnant vs Open	167	446	103
HF Pregnant vs Open	97	341	50
SF Pregnant vs Open	95	212	48
Only Pregnant HF vs SF	37	221	14
Only Open HF, SF, IF	27	48	2

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Table 3. Abundance of the top 10 proteins that increased or decreased in pregnant compared to open ULF

Identified Proteins	GenInfo Identifier	Protein	*P*-value	Open[1]	Pregnant
Proteins increased in pregnant ULF					
Pregnancy-associated glycoprotein 11	28603724	PAG11	< 0.00010	0.0	5.3
Trophoblast Kunitz domain protein 1 precursor	296481028	TKDP1	< 0.00010	0.0	4.7
Mitochondrial acetyl-Coenzyme A acyltransferase 1	156254808	ACAA1	< 0.00010	0.0	4.0
Dihydrolipoamide dehydrogenase	296488519	DLD	< 0.00010	0.0	1.1
3-ketoacyl-CoA thiolase, mitochondrial	121956694	ACAA2	< 0.00010	0.1	5.2
Glutathione synthetase	296481143	GSS	< 0.00010	0.1	2.5
Bovine Glutamate Dehydrogenase	298508694	GLUD1	< 0.00010	0.6	10.3
Heat shock 70 kDa protein 9	122144079	HSPA9	< 0.00010	0.5	7.0
Lamin A/C	296489648	LMNA	< 0.00010	0.1	2.0
Heat shock protein 60 kDa, mitochondrial isoform X1	982914181	HSPD1	< 0.00010	3.0	28.1
Proteins increased in open ULF					
PAS-6 and PAS-7 proteins	1632779	PAS-6/7	< 0.00010	4.5	0.1
Guanine nucleotide-binding protein G(q) subunit alpha	158508558	GNAQ	< 0.00010	2.6	0.3
Guanine nucleotide-binding protein G(i) subunit alpha-2	198282135	GNAI2	< 0.00010	3.4	0.4
Factor V	163038	FACTORV	< 0.00010	13.9	2.2
Retinoic acid receptor responder (tazarotene induced) 1	112362395	RARRES1	< 0.00010	3.7	0.6
FAM234A; Alternative name: ITFG3	114149324	FAM234A	< 0.00010	4.2	0.7
MYO1B protein	151554811	MYO1B	< 0.00010	9.1	1.8
Bovine Tubulin (1jff)	193885177	2P4N	< 0.00010	49.1	10.0
Glypican 1	157279068	GPC1	< 0.00010	3.6	1.0
Ezrin	27806351	EZR	< 0.00010	33.3	14.0

[1]Average of total spectrum count for proteins identified in the uterine lumen flush of open and pregnant heifers.

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Table 4. Number of the differential metabolites (FDR < 0.05) identified in the uterine lumen of fertility-classified heifers for each comparison of interest

Comparisons	Identified Metabolites	Unknown Metabolites	Total Metabolites
Pregnant vs Open	70	245	315
HF Pregnant vs Open	67	204	271
SF Pregnant vs Open	0	0	0
Pregnant HF vs SF	1	12	13
Open HF, SF, IF	0	1	1

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Table 5. Top 20 identified metabolites with greatest fold change (FC) difference in the uterine lumen of pregnant and open heifers

Ionization mode[1]	m/z	Retention time	Pregnant/Open FC	log2(FC)	FDR
Positive mode					
L-Methionine	150.1	1.4	13.1	3.7	< 0.01
Hypoxanthine	137.0	6.1	0.1	-2.9	< 0.01
Glutathione Disulfide	307.1	3.9	0.2	-2.4	0.04
Isovalerylcarnitine	246.2	8.1	4.7	2.2	0.02
R-Malate	157.0	1.0	4.4	2.1	< 0.01
2-Hydroxyphenylalanine	182.1	3.4	3.9	2.0	< 0.01
L-Histidine	156.1	0.7	3.5	1.8	< 0.01
Methionine Sulfoxide	166.1	0.8	3.5	1.8	< 0.01
N-Alpha-Acetyl-L-Lysine	189.1	1.2	3.3	1.7	< 0.01
Phenylalanine	166.1	6.3	3.2	1.7	< 0.01
Negative mode					
N-Methyl-L-Glutamate	160.1	0.9	29.4	4.9	<0.01
3-Hydroxy-3-Methylglutarate	161.0	2.9	6.3	2.7	<0.01
Inosine	267.1	6.0	0.2	-2.6	0.02
6-Keto Prostaglandin G1	369.2	9.8	6.1	2.6	<0.01
N-Acetylneuraminate	308.1	0.8	4.8	2.3	0.03
Fumaric Acid	115.0	2.0	4.5	2.2	<0.01
Malate	133.0	1.0	4.4	2.1	<0.01
L-Tyrosine	180.1	3.4	4.3	2.1	<0.01
Threonine/Homoserine	118.1	0.7	3.6	1.8	<0.01
Glutarate	131.0	4.6	3.5	1.8	<0.01

[1] Global metabolomics was conducted by ultra-high-performance liquid chromatography (UHPLC)-tandem mass spectrometric (LC-MS/MS). Electrospray ionization was used to produce gas phase ions, and all samples were analyzed in the positive and negative ionization modes.

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