Analysis of the Uterine Lumen in Fertility-Classified Heifers: I. Glucose, Prostaglandins and Lipids

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Abstract

Survival and growth of the bovine conceptus (embryo and associated extraembryonic membranes) is dependent on endometrial secretions or histotroph found in the uterine lumen. Previously, serial embryo transfer was used to classify heifers as high fertile (HF), subfertile (SF), or infertile (IF). Here, we investigated specific histotroph components (glucose, prostaglandins (PGs) and lipids) in the uterine lumen of day 17 pregnant and open fertility-classified heifers. Concentrations of glucose in the uterine lumen were increased by pregnancy, but did not differ among fertility-classified heifers. Differences in expression of genes encoding glucose transporters and involved with glycolysis and gluconeogenesis were observed between conceptuses collected from HF and SF heifers. In the uterine lumen, PGE2 and PGF2α were increased by pregnancy, and HF heifers had higher concentrations of PGE2, PGF2α and 6-keto-PFG1α than SF heifers. Differences were found in expression of genes regulating PG signaling, arachidonic acid metabolism, and PPAR signaling among conceptuses and endometrium from fertility-classified heifers. Lipidomics was conducted exclusively in samples from HF heifers, and phosphatidylcholine was the main lipid class that increased in the uterine lumen by pregnancy. Expression of several lipid metabolism genes differed between HF and SF conceptuses, and a number of fatty acids were differentially abundant in the uterine lumen of pregnant HF and SF heifers. These results support the ideas that uterine luminal histotroph impacts conceptus survival and programs its development and is a facet of dysregulated conceptus-endometrial interactions that result in loss of the conceptus in SF cattle during the implantation period of pregnancy establishment.

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), the bovine blastocyst slowly grows into an ovoid or tubular form on days 12 to 14 and is then termed a conceptus [6]. The conceptus is about 2 mm in length on day 13, 6 mm by day 14, 60 mm (6 cm) by day 16, and 20 cm or more by day 19 [6, 7]. Peri-implantation growth of the conceptus is highly dependent on substances present in the uterine lumen. Uterine epithelia are present in the endometrium of all mammals [8], and their secretions constitute an important component of the histotroph, which is essential for preimplantation conceptus survival and development in sheep [9, 10]. Histotroph in the uterine lumen of cattle has been characterized [11-19] and is a complex mixture of amino acids, glucose, lipids, proteins, carbohydrates, vitamins, ions, cytokines, hormones, growth factors, among other substances. However, little is known about how different levels of specific constituents in the uterine lumen regulate pregnancy success in cattle.

Glucose is an essential nutrient for pre-implantation conceptus development [20, 21] and increases in the uterine lumen during conceptus elongation in sheep [21]. The capacity of the uterus to generate free-glucose through gluconeogenesis is debatable [23-25], thus glucose transporters are thought to play an important role modulating intrauterine concentrations of glucose during pregnancy [20]. Mammalian cells express three families of glucose transporters, the facilitative glucose transporter family (solute carriers SLC2A/GLUT), the sodium/glucose cotransporter family (solute carriers SLC5A/SGLT) [26], and the recently discovered SWEET (SLC50) sugar transporter [27, 28]. The uterus expresses several members of these families [29],

including SLC2A1, SLC5A1 and SLC5A11 whose expression increase in pregnant compared to cyclic endometrium in sheep [20].

Prostaglandins (PG) are lipid hormones derived from arachidonic acid [30] and essential for conceptus development. In sheep, intrauterine infusion of meloxicam, a selective inhibitor of prostaglandin-endoperoxide synthase 2 (PTGS2), inhibited conceptus elongation [31]. Concentrations of PGs in the uterine lumen increase during early pregnancy in ruminants [20, 32], because both the conceptus and endometrium produce prostaglandins, and endometrial production of PGs is further stimulated by interferon tau (IFNT) [33, 34], the signal for maternal recognition of pregnancy which is secreted predominantly by the trophectoderm of the elongating bovine conceptus after day 15 [35]. Classical signaling of PGs is through membrane-bound G protein-coupled receptors, and different types of PGs act through their specific receptor(s) and therefore regulates distinct biological processes [36, 37]. Nonetheless, select PGs can also activate nuclear receptors [38-40]. PGI2 signals through the peroxisome proliferator-activated receptor (PPAR) delta (PPARD) [41], and 15-deoxy-$\Delta^{12,14}$-PGJ2, a metabolite of PGD2, can activate PPAR gamma (PPARG) [42, 43]. PPARs are ligand-regulated transcription factors that upon heterodimerization with retinoid X receptor (RXR) and ligand binding, regulate the expression of target genes and consequently cellular function [44].

Lipids are another important component present in the uterine lumen. In addition to being a precursor of eicosanoids [45], they play many critical roles in the body, serving as a source and storage for energy, providing substrates for membrane biogenesis, and acting as signal molecules [46]. The lipid content of the uterus fluctuates throughout the estrous cycle in several species [47], and the accumulation of lipid droplets in the endometrium luminal

epithelium (LE) during the estrous cycle in sheep is attributed to progesterone from the ovarian corpus luteum [48-50]. This lipid accumulation has been hypothesized to serve as a reservoir to support increasing lipid demands of the endometrium and conceptus during early pregnancy [49, 51]. Progesterone increases the abundance of metabolites involved with membrane biogenesis in the uterine lumen during the onset of conceptus elongation in cattle [18], and the number of extracellular vesicles (EV) in the uterine luminal fluid during the period of conceptus elongation in sheep [52]. This increase in lipid availability during late diestrus is hypothesized to be important to support the growth of trophectoderm cells during conceptus elongation [53]. Fatty acids present in the uterine lumen likely participate in the crosstalk between the conceptus and endometrium during pregnancy, as saturated and unsaturated fatty acids can serve as ligands of PPAR receptors [44, 51, 54-56], which are expressed by both, the bovine conceptus and endometrium [1, 57]. Although fatty acids are essential biological components, uterine lumen fatty acid composition remains poorly understood in ruminants.

To identify changes in the uterine lumen constituents 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 embryo (Grade 1) on day 7 followed by pregnancy determination on day 28 [1, 58]. Interestingly, conceptus development and survival on day 14 (7 days post-transfer) was not different among fertility-classified heifers and only minimal differences in their endometrium transcriptome were observed on day 14 [58]. In a subsequent study, we found that on day 17 (10 days post-transfer), pregnancy rate was higher in HF (71%) and SF (90%) than IF (20%)

heifers. Day 17 conceptuses were longer in HF (mean 10.6 cm; range 1.2-32.2 cm) than SF (mean 4.7 cm; range 1.5-13.5 cm) heifers, and the endometrial and conceptus transcriptome was dysregulated in SF heifers [1]. The current study tested the hypothesis that specific histotroph constituents would be altered in the uterus of fertility-classified heifers with distinct uterine capacity to support pregnancy and be altered by pregnancy status. Because differences in endometrial transcriptome can translate into differences in the uterine lumen constituents [59], the focus of the current study was to investigate concentrations of glucose, prostaglandins (PGE2, PGF2α, and 6-keto-PFG1α, a metabolite of PGI2 [60, 61]) and lipids in the uterine lumen from fertility-classified heifers.

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. All fertility-classified heifers participating in the study were housed in the same pasture at the Beef Research and Teaching Farm of the University of Missouri and were subjected to the same management and diet.

Collection of the uterine luminal flush (ULF). Fertility-classified heifers (HF, n=21; SF, n=14; IF, n=6) were synchronized to estrus (day 0) and received two in vivo-produced embryos on day 7. All heifers were slaughtered on day 17 (10 days post-transfer) at the University of Missouri slaughter facility, and reproductive tracts were collected within ~30 minutes 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.

Glucose Analysis. Concentrations of glucose in ULF and blood plasma were measured in samples collected from all the fertility-classified heifer enrolled in the study, which includes pregnant (HF, n=15; SF, n=9; IF, n=1) and open (HF, n=6; SF, n=5; IF, n=5) heifers. Glucose levels were determined using a glucose oxidase linked assay [62], that is based on the oxidation of glucose by glucose oxidase (Pointe Scientific, MI, USA) forming gluconate and hydrogen peroxide, and H_2O_2 reacts with available phenol and 4-aminophenazone generating a quinoneimine dye that is measured at 500 nm. Briefly, each assay included a standard curve, and ULF (100 μL) and plasma (2 μL) samples were incubated at 37°C with glucose oxidase (200 μL) for 45 and 15 min, respectively. Product formation was measured on a EnVision 2104 Multilabel plate reader (Perkin Elmer, MA, USA). Intra-assay coefficient of variation was 3.0% and 6.6% for ULF and plasma assays, respectively.

Statistical analyses of glucose levels in plasma and ULF were conducted in SAS (SAS Institute Inc., Cary, NC) by analysis of variance (ANOVA) using the GLM procedure. Post-test comparisons were conducted using the LSMEANS statement with the Fisher's protected LSD option. Pearson's correlation between glucose concentrations in plasma and in the ULF, as well as the correlation between conceptus size and glucose in the ULF were determined using the

CORR procedure. In all analyses conducted, conceptus length for heifers with two conceptuses was equal to the sum of both conceptuses present in the flush.

Prior to data analysis, glucose concentration was assessed for normality using the UNIVARIATE procedure, and both glucose in plasma and ULF were determined not normally distributed and therefore were log transformed for statistical analyses. Because glucose has been shown to increase in the ULF during conceptus elongation in sheep [21], we first tested whether pregnancy affected day 17 ULF glucose concentration in our combined dataset which included samples from pregnant and open HF, SF, and IF heifers. The models used to test the effect of pregnancy on plasma and ULF glucose concentrations consisted of glucose levels as the depended variable and pregnancy status (pregnant vs open) as the independent variable.

The effect of fertility group on glucose concentration was analyzed separated for pregnant and open heifers. Among pregnant heifers, the initial models used to test the effect of fertility classification on glucose levels was composed by ULF or plasma glucose concentrations as the dependent variable, and fertility-classification, conceptus number, conceptus length, the two-way interactions of fertility-classification with conceptus number and conceptus length, and the three-way interaction of fertility-classification, conceptus number and conceptus length as independent variables. Nonsignificant ($P > 0.05$) variables were removed from the initial model by a manual backward stepwise elimination procedure. For ULF glucose, only the effect of fertility classification was retained in the final model. For plasma glucose, the variables retained in the final model were fertility classification, conceptus length, and the interaction of fertility group with conceptus length.

Among open heifers, the models used to test the effect of fertility-classification on plasma and ULF glucose concentrations consisted of glucose levels as the depended variable and fertility-classification (HF, SF and IF) as the independent variable. Statistical significance was defined as $P \leq 0.05$

Eicosanoids Analysis. The assay was conducted at the Eicosanoid Core Laboratory of Vanderbilt University on ULF samples from a subgroup of 25 heifers that were determined to be pregnant (HF, n=5; SF, n=5) or open (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 have 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 $\pm$ 7.6 cm (range: 1.2 to 32.2 cm) for HF heifers and 4.7 $\pm$ 4.2 cm (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 12.0 $\pm$ 8.1 cm (range: 1.3 to 25 cm) for HF and 6.4 $\pm$ 4.5 cm (range: 2.1 to 13.5 cm) for SF heifers.

To quantify PGs in the ULF, a total of 100 μL of each sample was placed in a microcentrifuge tube containing 25% methanol in water (500 μL) and internal standard (d_4-PGE_2 and d_4-LTB_4, 1 ng each). The sample was vortexed and then extracted on an Oasis MAX μElution plate (Waters Corp., MA, USA) as follows. Sample wells were first washed with

methanol (200 μL) followed by 25% methanol in water (200 μL). The sample was then loaded into the well and washed with 600 μL 25% methanol. Eicosanoids were eluted from the plate with 30 μL 2-propanol/acetonitrile (50/50, v/v) containing 5% formic acid into a 96-well elution plate containing 30 uL water in each well.

Samples were analyzed on a Waters Xevo TQ-S micro triple quadrupole mass spectrometer connected to a Waters Acquity I-Class UPLC (Waters Corp., MA USA). Separation of analytes was obtained using an Acquity PFP column (2.1 x 100 mm) with mobile phase A being 0.01% formic acid in water and mobile phase B acetonitrile. Eicosanoids were separated using a gradient elution beginning with 30% B going to 95% B over 8 minutes at a flow rate of 0.250 mL/min.

Statistical analyses of PG concentrations in the ULF were conducted in SAS by ANOVA using the GLM procedure. Post-test comparisons were conducted using the LSMEANS statement with the Fisher's protected LSD option. Among pregnant heifers, Pearson's correlation between PG concentrations in the ULF and conceptus length were determined using the CORR procedure.

The models used to test the effect of pregnancy on PG concentrations in the ULF consisted of PGE2, PGF2α or 6-keto-PFG1α as the depended variable and pregnancy status (pregnant vs open) as the independent variable. Among pregnant heifers, a multivariate model that accounted for conceptus number and conceptus length was used to test the effect of fertility classification on ULF PG concentrations. The initial model was composed by PGE2, PGF2α or 6-keto-PFG1α concentration as the dependent variable, and fertility-classification, conceptus number, conceptus length, the two-way interactions of fertility-classification with

conceptus number and conceptus length, and the three-way interaction of fertility-classification, conceptus number and conceptus length as independent variables. Nonsignificant ($P > 0.05$) variables were removed from the initial model by a manual backward stepwise elimination procedure. For PGE2 and 6-keto-PFG1α, only the effect of fertility group was retained in the reduced model. For PGF2α, the effects of fertility group and conceptus number were retained in the reduced model.

Among open heifers, the models used to test the effect of fertility-classification on ULF concentrations of PG consisted of PGE2, PGF2α or 6-keto-PFG1α levels as the depended variable and fertility-classification (HF, SF and IF) as the independent variable. Statistical significance was defined as $P \leq 0.05$ and statistical tendencies $0.05 < P \leq 0.10$

Fatty acids Analysis. Total fatty acid analysis was conducted by high-resolution mass spectrometry at the Southeast Center for Integrated Metabolomics (SECIM) of the University of Florida on ULF samples collected from the same subgroup of 25 heifers described in the eicosanoid analysis. Unless otherwise stated, all reagents used were of LC-MS grade and obtained from Fisher Scientific (Fairlawn, NJ). A sample of 100 mg of ULF was weighed into a conical glass tube (5 mL volume) and 1 mL of acetonitrile containing 100 mg/L of butylated hydroxy toluene (BHT) was added, followed by the addition of 0.5 mL of hydrochloric acid (37%). The sample was sonicated for 5 min and heated between 80-90°C for 2 hours in a heating block. The addition of hydrochloric acid and heating causes hydrolysis to release fatty acids from complex lipids. After cooling at room temperature, 2 mL of hexane was added, and the sample was centrifuged for 1 min at 3,260 x g. The top layer (1.5 mL) was removed,

transferred to a clean glass culture tube, and dried under a gentle stream of nitrogen. The dried sample was reconstituted in 0.5 mL of 80/20 acetonitrile/5 mM ammonium acetate, and 10 µL of the injection standard mixture (DHA-D5, EPA-D5, α-LA-D14) was added to the reconstituted sample. The final sample was transferred to an LC vial for LC-MS analysis.

Fatty acid analysis was performed on a Thermo Q-Exactive Orbitrap Mass Spectrometry with Dionex Ultimate 3000 UHPLC and autosampler. Separation was achieved on a Waters HSS T3 column (150 x 2.1 mm, 1.8 µm) at a flow rate of 0.5 mL/min and column temperature of 30°C with mobile phase A and B consisting of 1 mM ammonium acetate in water and 0.1% acetic acid in acetonitrile, respectively. Gradient elution started at 25% A and 75% B from 0 to 0.5 min with a linear increase to 90% B from 0.5 to 7 min, a further increase to 95% B from 7 to 8 min and then held constant at 95% B from 8 to 21 min. The column was returned to initial conditions in 0.5 min and equilibrated for 4.5 min. The mass spectrometry was operated in negative heated electrospray ionization (HESI) mode with a resolution setting of 70,000 at *m/z* 200, collecting *m/z* 100 to 700. Data dependent analysis was conducted on the top 5 most abundant peaks throughout the run with a resolution setting of 17,500, ion time of 75 ms and collision induced dissociation of 30, 50 and 70. The HESI settings were 3 kV, 100°C probe temperature, 300°C capillary temperature, 40 arb sheath gas, 5 arb auxiliary gas, and 1 arb sweep gas. Fatty acids were identified by accurate mass (<10 ppm accuracy), retention time and tandem mass spectrometry.

Statistical analyses of ULF fatty acid data were conducted in SAS by ANOVA using the GLM procedure. Post-test comparisons were conducted using the LSMEANS statement with the Fisher's protected LSD option. The models used to test the effect of pregnancy on ULF fatty acid

abundance consisted of fatty acid peak area (**Dataset S5**) as the depended variable and pregnancy status (pregnant vs open) as the independent variable. Among pregnant heifers, the initial models used to test the effect of fertility classification on ULF fatty acid profile was composed by fatty acid data as the dependent variable, and fertility-classification, conceptus number, conceptus length, the two-way interactions of fertility-classification with conceptus number and conceptus length, and the three-way interaction of fertility-classification, conceptus number and conceptus length as independent variables. Nonsignificant ($P > 0.05$) variables were removed from the initial model by a manual backward stepwise elimination procedure. The variables retained in the reduced models used to analyze ULF fatty acid data of pregnant heifers are described in the results section of the manuscript. Among open heifers, the models used to test the effect of fertility-classification on ULF fatty acid profile consisted of fatty acid peak area as the depended variable and fertility-classification (HF, SF and IF) as the independent variable. Statistical significance was defined as $P \leq 0.05$.

Untargeted Lipidomic Analysis. To investigate changes in ULF lipidome that are normally induced by pregnancy, global lipidomics was conducted exclusively on ULF samples of HF heifers that were open (n=5) or pregnant (n=5). The ULF samples used in this analysis are from the same HF heifers which ULF samples were analyzed for PGs and fatty acids.

The assay was conducted at the Southeast Center for Integrated Metabolomics (SECIM) of the University of Florida. Samples were extracted following a cellular extraction procedure without pre-normalization to the sample protein content. Global lipidomics profiling was performed on a Thermo Q-Exactive Orbitrap 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 Acquity BEH C18 1.7 µm, 100 x 2.1 mm column with mobile phase A as 60:40 Acetonitrile:10 mM Ammonium formate with 0.1% formic acid in water and mobile phase B as 90:8:2 2-propanol: acetonitrile: 10mM ammonium formate with 0.1% formic acid in water. The flow rate was 500 µL/min with a column temperature of 50°C with 5 µL was injected for negative ions and 3 µL for positive ions.

Data from positive and negative ion modes were separately analyzed using LipidMatch software. First, all MS2 raw files were converted to "ms2" and MS raw files to "MzXML" using MSConvert. A peak list was generated after running MzMine on all MzXML files. An input folder that included all "ms2" files and the peak list were used to run LipidMatch to identify features.

Statistical analysis of the lipidomics data was performed on MetaboAnalyst 4.0 [63-65]. A table matrix of m/z peak intensities with samples in columns and features in rows were created and imported to MetaboAnalyst 4.0, and data pre-processing consisted of autoscaling normalization [66]. Fold change (FC) analysis was conducted to identify features that increased or decreased in the ULF of HF heifers with pregnancy, and the standard fold change threshold of 2 was used. Additionally, a t-test was used to investigate if features were differently ($P < 0.05$) abundant in the ULF of HF heifers that were pregnant or open.

Integration of uterine luminal content and endometrium and conceptus transcriptome. To further investigate the biology of subfertility, we integrated the present ULF data with transcriptome data from endometrium and conceptuses which have been performed in the

same group of animals and recently published [1]. The gene expression data generated in our previous publication (available in Gene Expression Omnibus (GEO) database 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 [67], and a false discovery rate (FDR) of < 0.05 was used as the cutoff for determining the differently expressed genes (DEGs). After a list of DEG were generated for each comparison of interest, we use the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) to verify if the genes associated with glucose, prostaglandins and lipid metabolism were differently express in our transcriptome data.

Relationship of endometrium transcriptome and ULF composition. Two analyses were conducted to integrate data of ULF components and endometrium transcriptome. 1) The first one investigated the effect of pregnancy on endometrial expression of genes associated with synthesis and metabolism of glucose, PGs and lipids. This analysis was performed on endometrial transcriptome 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 analyzes. 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 open) as the independent variable. 2) The second analysis investigated endometrial expression of the genes of interest only in the subgroup of pregnant heifers (HF, n=5; SF, n=5) selected for ULF analyzes. One of the most important finding of our previous work was that the endometrial response to pregnancy was dysregulated in SF heifers [1]. Thus, this analysis aimed to explore if the diminished endometrial

response to pregnancy observed in SF heifers affected the synthesis and metabolism of the ULF components of interested, as differences might provide insights on the biological mechanisms involved with pregnancy loss in SF heifers. The model used to test the effect of fertility group on endometrial gene expression of pregnant heifers was composed by gene expression data as the dependent variable and fertility group (HF vs SF) as the independent variable.

Relationship of conceptus transcriptome and ULF composition. A similar approach was used to investigate the relationship of ULF composition and the 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 [6, 57, 58, 68, 69] and the conceptus transcriptome changes as it develops [57], 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

Glucose Concentrations in ULF. Glucose concentrations in ULF were higher (*P* = 0.05) in pregnant compared to open heifers on day 17 (**Figure 1A**). Among pregnant heifers, there was no effect (*P* = 0.88) of fertility classification on ULF glucose concentrations (**Figure 1B**). Furthermore, ULF glucose did not differ (*P* = 0.66) among open fertility-classified heifers (**Figure 1C**).

Circulating concentrations of glucose in plasma were not different (*P* = 0.20) between pregnant and open heifers (**Figure 1D**). Among pregnant heifers, plasma glucose was affected (*P* = 0.02) by the interaction of fertility group and conceptus length, as plasma glucose was positively correlated with conceptus length in HF heifers but not in SF heifers (**Figure 1E**). Among the open animals, IF heifers had higher (*P* = 0.04) plasma glucose concentrations than HF heifers (**Figure 1F**). Further, ULF and plasma glucose concentrations were not correlated (**Figure 2A;** r = 0.22; *P* = 0.17), and there was no significant correlation (r = 0.27; *P* = 0.19) between conceptus length and concentrations of glucose in the ULF (**Figure 2B**).

Transcriptome analysis of endometrium and conceptuses for glucose transport and metabolism genes. Expression of genes encoding glucose transporters that increased in pregnant compared to open endometrium (*SLC2A1, SLC5A1, SLC5A11, SLC35D2* and *SLC5A4*) were not different (FDR P $\geq$ 0.68) between pregnant HF and SF endometrium (**Table 1**). Interestingly, expression of other glucose transporters (*SLC2A10, SLC2A3* and *SLC37A4*)

decreased (FDR < 0.05) with pregnancy, but their expression was also not different (FDR P $\geq$ 0.65) in pregnant endometrium of HF and SF heifers (**Table 1**).

No differences were observed in conceptus transcripts encoding glucose transporters between short (mean length: 2.5 ± 0.4 cm) and long conceptuses (mean length: 14.5 ± 1.9 cm), and the five most abundant glucose transporters on day 17 conceptuses were *SLC2A1, SLC2A5, SLC37A1, SLC2A8* and *SLC2A13* (**Dataset S1**). Interestingly, expression of *SLC2A2* (*GLUT2*) and *SLC2A4* (*GLUT4*) was higher in SF than HF conceptuses (**Dataset S1**).

For genes involved with glycolysis and gluconeogenesis according to the KEGG database, expression of 13 genes increased and 3 decreased in pregnant compared to open endometrium (**Dataset S2 and Table 2**), and expression of one gene (*FBP1*, fructose-bisphosphatase 1) was increased in pregnant endometrium of HF compared to SF heifers (**Dataset S2 and Table 2**). None of the selected genes involved with glycolysis and gluconeogenesis were differently expressed in short vs long conceptuses (**Dataset S2**), but expression of *HK1, FBP1* and *GALM* was increased in HF conceptuses, and *ALDOB* and *ALDH3B1* was increased in SF conceptuses (**Dataset S2 and Table 3**).

Prostaglandins (PGs) in the ULF. Pregnant heifers tended (P = 0.07) and had (P<0.01) greater concentrations of PGE2 and PGF2α in the ULF than open heifers (**Figures 3A and 3D**, respectively). However, ULF concentrations of 6-keto-PFG1α was not affected (P = 0.18) by pregnancy status (**Figure 3G**). Although 6-keto-PFG1α concentrations did not differ between pregnant and open SF heifers (P = 0.61; Open = 29.7 $\pm$ 8.3 ng/mL, Pregnant = 23.5 $\pm$ 8.3 ng/mL), 6-keto-PFG1α was increased by 5.4 fold in the ULF of pregnant than open HF heifers (P < 0.01;

Open = 8.5 ± 6.4 ng/mL, Pregnant = 45.8 ± 6.4 ng/mL). Among pregnant heifers, ULF concentrations of PGF2α (**Figure 3E**) was affected by fertility group (*P* = 0.01; HF = 25.31 ± 1.91; SF = 16.36 ± 1.91) and conceptus number (*P* = 0.02; One conceptus = 16.65 ± 1.91; Two conceptuses = 25.02 ± 1.91). Additionally, concentrations of PGF2α in ULF was significant correlated with conceptus length (r = 0.66, *P* = 0.04; **Figure 4**). Furthermore, ULF of HF pregnant heifers tended to have greater concentrations of PGE2 (*P* = 0.10; **Figure 3B**) and 6-keto-PFG1α (*P* = 0.08; **Figure 3H**) than ULF of pregnant SF heifers. Nonetheless, there was no significant correlation between PGE2 (r = 0.32, *P* = 0.37) and 6-keto-PFG1α (r = 0.26, *P* = 0.46) with conceptus length (**Figure 4**). Among open heifers, there was no effect ($P \geq 0.32$) of fertility group on ULF PG concentrations (**Figures 3C, 3F and 3I**).

Transcriptome analysis of endometrium and conceptuses for PG signaling, arachidonic acid metabolism and PPAR signaling genes. Expression of selected genes involved with PG signaling by day 17 endometrium and conceptuses are summarized in **Table 4**. Expression of selected genes encoding PG synthases did not differ in the endometrium of pregnant HF vs SF heifers, or between conceptuses collected from HF or SF heifers. Expression of *PTGR1, PTGR3, PTGFR, PTGIR* and *PPARG* was very low (FPKM < 1) in the endometrium. Among conceptuses, PGF receptor (*PTGFR*) expression was higher in HF than SF conceptuses, and *PTGFR* was the overall highest PG receptor expressed by the conceptuses. Conceptus expression of *PTGER1, PTGER2, PTGER3, PTGER4, PTGIR* and *PPARA* was very low (FPKM < 1). Additionally, the overall expression of *PPAR* receptors by the endometrium or conceptuses did not differ in all comparisons. Interestingly, the expression of the PG transporter *SLCO2A1* was higher in HF than

SF conceptuses, but no differences were observed among short and long conceptuses or in the endometrium (**Table 4**).

Expression of genes involved with arachidonic acid metabolism differed between pregnant and open endometrium. Expression of seven genes were increased in open endometrium (*CYP2C18, ALOX15B, PTGDS, PTGIS, PLA2G4B, ALOX15* and *CYP2U1*), and 10 genes increased in pregnant endometrium (*CYP2J2, PLA1A, ALOX12, CBR3, PLA2G3, HPGDS, LOC506594* (prostaglandin F synthase 1-like), *PTGES3, CBR1* and *PLA2G2A-2*) (**Dataset S3**). However, none of the genes associated with arachidonic acid metabolism were differently expressed in endometrium of pregnant HF and SF heifers (**Dataset S3**). In conceptuses, expression of only one gene (*LOC506594*; prostaglandin F synthase 1-like) was increased in short compared to long conceptuses (**Dataset S3**), and expression of one gene (*PLA2G15*) increased and three (*PLA2G1B, GPX1* and *CBR1*) decreased in HF compared to SF conceptuses (**Dataset S3**).

Among genes involved with PPAR signaling, expression of 11 genes (*PCK2, PLIN2, ACSL5, FABP3, SCD, OLR1, UBC, SLC27A5, FADS2, SLC27A6* and *ACSL4*) increased and 5 decreased (*FABP7, PLTP, SCD5, RXRA* and *CPT1C*) in pregnant compared to open endometrium (**Dataset S4**). However, expression of only one gene differed between pregnant HF vs SF endometrium, as expression of *FABP3* was increased by 2-fold in the endometrium of pregnant HF heifers (**Dataset S4**).

In conceptuses, expression of one gene (*FADS2*) in the PPAR signaling pathway increased in long compared to short conceptuses (**Dataset S4**), and expression of one gene

increased (*PDPK1*) and 6 decreased (*ACADL, ACADM, APOA5, FABP3, LPL* and *SORBS1*) in HF compared to SF conceptuses (**Dataset S4**).

Fatty Acids in the ULF. A total of 37 fatty acids were identified in the ULF of the fertility-classified heifers, and their concentrations in pregnant and open ULF is presented in **Figure 5A**. Two saturated fatty acids, palmitic acid (16:0) and stearic acid (18:0), were the predominant fatty acids detected in the ULF of both open and pregnant heifers (**Figure 5A**), accounting for about 90% of the total fatty acid content detected in the ULF (**Figures 5B and 5C; Dataset S5**). The percentage of the subsequent most predominant fatty acids in the ULF relative to total fatty acid content for both open and pregnant heifers were: 3.1% octacosaheptaenoic (28:7); 1.9% octacosaoctaenoic acid (28:8); 1.2% arachidate (20:0); 0.8% Behenate (22:0) and 18:1; 0.7% myristate (14:0); and 0.6% margaric (17:0) (**Figures 5B and 5C**). The remaining 28 fatty acids together accounted for only around 1.1% of the total fatty acids detected in ULF (**Dataset S5**). A heatmap for fatty acid concentration in the ULF across samples is presented in **Figure 5D**. Surprisingly, there was no difference in fatty acid content between ULF from open and pregnant heifers. When HF and SF heifers were analyzed separately, the fatty acid content that were influenced by pregnancy was not consistent in HF and SF ULF (**Table 5**).

In the comparison of only pregnant HF and SF heifers, concentrations 11 fatty acids increased and 3 decreased in the ULF of HF compared to SF heifers (**Table 5**). Among the fatty acids that increased in the ULF of pregnant HF heifers, concentrations of 17:0, 28:7 and 28:0 were also affected by conceptus number ($P < 0.01$, $P = 0.01$, $P = 0.01$; **Figure 6A**), by the interaction between conceptus number and fertility group ($P < 0.01$, $P = 0.01$, $P = 0.01$), by the

interaction of conceptus length and fertility group (P < 0.01, *P* < 0.01, *P* < 0.01), and by the three-way interactions of conceptus number, conceptus length and fertility group (*P < 0.01*, *P = 0.01*, *P* = 0.01; **Figures 6B, 6C and 6D**).

Furthermore, among the fatty acids that increased in the ULF of pregnant SF than HF heifers, concentrations of 20:0 was affected by the interaction of conceptus number and fertility group (*P* = 0.01; **Figure 6E**), and ULF concentrations of 6:0 was affected by conceptus number (*P* < 0.01; One = $1.79x10^6 \pm 3.84x10^3$; Two = $1.66x10^6 \pm 8.15x10^3$), conceptus length (*P* < 0.01; **Figure 6F**), and by the interaction of conceptus length and fertility group (*P* < 0.01), and by the three-way interaction of conceptus number, length and fertility group (*P* < 0.01; **Figure 6G**). For all the remaining analysis, only the effect of fertility group was retained in the reduced model.

Among the open HF, SF and IF heifers, concentrations of three fatty acids were increased in the ULF of HF compared to SF heifers, and two fatty acids increased in the ULF of HF compared to IF heifers (**Table 5**).

Transcriptome analysis of endometrium and conceptuses for lipid metabolism genes. Expression of 66 genes associated with lipid metabolism differed between pregnant and open endometrium, with 41 genes increased and 25 decreased in the pregnant endometrium (**Dataset S6**). Of note, expression of genes regulating fatty acid biosynthesis (*FASN, FADS2, ELOVL1*, and *ELOVL6*) and fatty acid transport (*SLC27A5, SLC27A6, FABP3,* and *LOC100299715*) were upregulated in the endometrium by pregnancy. However, expression of only three genes differed in the endometrium of pregnant HF and SF heifers, as *FABP3*, *ST6GAL2* and *GALNT16* were increased in the endometrium of HF heifers (**Dataset S6**).

In conceptuses, expression of six genes associated with lipid metabolism differed between short and long conceptuses (**Dataset S6**). Expression of *ELOVL7* and *INPP4B* was increased in short conceptuses, and expression of *FADS1, FADS2, ELOVL5* and *B3GALT5* was increased in long conceptuses. Interestingly, expression of 24 genes differed between HF and SF conceptuses (**Dataset S6**). Expression of ten genes (*ELOVL5, FADS1, GPAM, CHKA, CHSY1, CYP11A1, PI4KB, B3GALT5, XYLT2,* and *B3GNT5*) were increased in HF conceptuses, and 14 genes (*ELOVL7, FABP3, SLC27A3, INPP4B, ASAH1, B4GALT4, GALNT5, ACAT1, CEPT1, PIGN, NDST3, ITPKA, ST6GAL2,* and *CERS1*) increased in SF conceptuses.

Untargeted Lipidomics. There was a total of 17,480 features detected from the positive mode and 2,020 features detected in the negative mode (**Dataset S7**). Only features identified based on fragmentation spectra (ddMS2) were subjected to statistical analysis, which represents 22 features detected in the positive ion mode.

Fold change analysis found that abundance of nine lipids increased and one decreased (**Figure S1**) in the uterine lumen by pregnancy, and 6 of the 9 lipids which increased by pregnancy in the FC analysis were significantly different ($P \leq 0.05$) in the t-test (**Table 6**). The differently abundant lipids in the FC analysis included phosphatidylcholines, triacylglycerides and acylcarnitines.

DISCUSSION

The present study was conducted to investigate glucose, prostaglandins and lipids in the uterine lumen of fertility-classified heifers to gain insights in the mechanisms regulating uterine

capacity for pregnancy. Conceptus elongation involves an increase in trophectoderm cell number that is driven by proliferation, therefore sufficient energy and anabolic precursors are required to support cellular replication and specialization [70]. In mammalian embryos at the cleavage stage, ATP production is based on low levels of oxidation of pyruvate, lactate, and amino acids, and by the blastocyst stage, energy demands increase dramatically with the formation of the blastocoel and due to the increase in protein synthesis required for conceptus growth [71, 72]. The blastocyst preferably uses glucose as an energy source compared to embryos in earlier stages of development, and rather than direct glucose towards oxidative phosphorylation, aerobic glycolysis seems to be the preferred pathway, which is a metabolic adaptation of rapidly proliferative cells known as Warburg effect [73-76]. This same pattern of glucose metabolism was observed during conceptus elongation in sheep [77]. Additionally, the increase in available glucose during early pregnancy in cattle may be an important regulator of the onset of conceptus elongation and mediated by the glucose-AMPK-PPARγ interplay [22]. Thus, glucose availability in the uterine lumen and the expression of glucose transporters by the elongating conceptus are likely essential for pregnancy success in ruminants.

Gluconeogenesis appears not to occur in the uterus of mice and humans, because their uteri lacks expression of rate-limiting gluconeogenic enzymes required for the synthesis of glucose from non-carbohydrate carbon substrates. For instance, phosphoenolpyruvate carboxykinase (PEPCK, also known as PCK) which converts oxaloacetate into phosphoenolpyruvate and carbon dioxide, was not detected in mouse uterus [25], and PCK and fructose-1,6-bisphosphatase (FBP1), which converts fructose-1,6-bisphosphate to fructose 6-phosphate, were also not detected in human myometrium and endometrium [24]. In the

present study, the mRNA expression of rate-limiting gluconeogenic enzymes (*G6PC, PCK2, FBP1*) increased in pregnant compared to open endometrium. Similarly, expression of those same three enzymes were found to increase in the intercaruncular endometrium from day 28 to 42 of gestation in cattle [23], indicating that perhaps the bovine uterus is able to generate glucose through gluconeogenesis, and that this gluconeogenic capacity of the uterus increases as pregnancy advances. Regardless of the gluconeogenic capacity of uterine cells, the uptake of glucose into the uterine lumen through glucose transporters expressed by the endometrium appears to be the main pathway regulating glucose in the uterine lumen of ruminants [20]. In fact, concentrations of glucose in the uterine lumen of ewes increased by 6-fold from days 10 to 15 of pregnancy, which occurred concomitant with the increase in expression of glucose transporters (*SLC2A1, SLC5A1, SLC5A11*) by the endometrium [20, 21]. Those glucose transporters are non-classical IFNT-stimulated genes [78] and were also increased by pregnancy in the endometrium in the present study, thus likely contributing to the higher glucose observed in the ULF of pregnant compared to open heifers. However, no differences in uterine lumen glucose was observed between pregnant HF and SF heifers, which was supported by the endometrium transcriptome data as only one gene (*FBP1*) involved with gluconeogenesis was increased in pregnant HF than SF endometrium. Higher circulating progesterone levels have also been associated with increased uterine luminal concentrations of glucose [16], but plasma progesterone concentrations were not different between pregnant HF and SF heifers [1]. These findings indicated that the availability of glucose in the ULF is probably not a major factor influencing subfertility in this group of fertility-classified heifers. However, circulating plasma glucose was increased in IF than HF heifers in the present study. Leane et al. [79] have recently

reported that, in lactating dairy cows, increasing circulating glucose concentrations inhibited early conceptus development on Day 14, despite not affecting ULF glucose concentrations. Thus, it is possible that higher circulating levels of glucose has systemic negative effects, and may hinder fertility without affecting ULF glucose levels. Furthermore, no differences were observed between short and long conceptuses for genes encoding glucose transporters, but expression of *SLC2A2* (*GLUT2*) and *SLC2A4* (*GLUT4*) were higher in SF than HF conceptuses, as 5 genes involved with glycolysis and gluconeogenesis (*HK1, FBP1, GALM, ALDOB, ALDH3B1*) were differently expressed between HF and SF conceptuses, which indicate dysregulated energy metabolism in SF conceptuses.

Prostaglandins are major regulators of female reproduction [80, 81] and modulate conceptus and endometrial gene expression during early pregnancy in ruminants [34]. In fact, PTGS2 was predicted as a key player regulating overall gene expression of day 17 bovine conceptuses [1]. In the present study, uterine luminal concentrations of PGE2, PGF2α, and 6-keto-PFG1α were higher in the ULF of pregnant HF than SF heifers. Although no differences in endometrium and conceptus mRNA expression of selected PG synthases (PTGS2, PTGIS, PTGES, PTGFS) were observed, the increase in uterine luminal concentrations of PGs in HF heifers is likely due to differences in conceptus-derived PGs, as HF heifers had longer conceptuses and therefore a larger number of cells producing PGs. Likewise, an earlier study observed no differences in *IFNT* mRNA 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 [35].

Prostaglandins E2, PGF2α, and PGI2 signal through their designated receptors, termed EP, FP, and IP, respectively. There are 4 subtypes of PGE2 receptors (EP, also known as PTGER1 through 4) but only one PGF2α receptor (FP, also known as PTGFR) and one PGI2 receptor (IP, also known as PTGIR) [82, 83]. Among the PTGER receptors, *PTGER2* and *PTGER4* had the highest expression in the endometrium of open and pregnant cows. These receptors are members of the seven-transmembrane G protein-coupled receptors which signal through cAMP [84], and the PGE2-cAMP pathway regulates important biological processes such as angiogenesis, vasodilatation, myometrial quiescence, and decidualization, which are essential for pregnancy establishment in several species [85]. In the present experiment, the expression of PGF synthase (*PTGFS*) by day 17 conceptuses was low, but the expression of the PGF2α receptor (*PTGFR*) was the highest among the PG receptors investigated in the conceptuses, suggesting that paracrine actions of endometrial PGF2α might be of particular importance regulating bovine conceptus elongation, and interestingly, HF conceptuses expressed higher *PTGFR* than SF conceptuses. Although the effect of PGF2α on embryonic and trophectoderm cell growth has not been investigated, PGF2α modulates endometrial cell proliferation, angiogenesis, and tissue remodeling in other systems [86, 87], which are important biological events occurring during conceptus elongation. Furthermore, higher expression of a PG transporter (*SLCO2A1*) was observed in HF than SF conceptuses, possibly indicating higher PG signaling within HF conceptuses. The solute carrier organic anion transporter family member 2A1 (*SLCO2A*; also known as PGT) [88] is able to transport PGE1, PGE2, PGD2, PGF2α and, to a lesser extent TXB2 [89]. Although newly synthesized PGs may exit the cells by diffusion due to their lipophilic nature, *SLCO2A1* is involved in the transport of PGs across membranes, and

plays an important role mediating the inactivation of the PG stimulus, which depends on the uptake of the PG molecule signaling through membrane bound receptors into the cytoplasm of the cell, where it is oxidized by 15-ketoprostaglandin dehydrogenase (15-PGDH) [90, 91]. Expression of another transmembrane PG transporter, the ATP-binding cassette, subfamily C member 4 (*ABCC4*; also known as *MRP4*) [92], which is expressed by the pig endometrium and conceptus [93] was not detected on day 17 bovine endometrium or conceptus in the present study.

Higher concentrations of 6-keto-PFG1α, a stable metabolite of PGI2, were detected in the uterine lumen of HF pregnant heifers, and PGI2 synthase was highly expressed by the endometrium in pregnant and open heifers as well as the conceptus. Although the expression of PGI2 synthase and PGI2 receptors by the endometrium and conceptus during the time of maternal recognition of pregnancy have been studied in sheep [94], the downstream effects of PGI2 signaling in the endometrium and conceptus is poorly understood. PGI2 is a strong vasodilator and a potent platelet inhibitor [95, 96]. PGI2 signaling via PPARD is essential for implantation and decidualization in mice [41]. PGI2 signaling through PPARD in sheep conceptuses does not seem to be important, as inhibition of PPARD in trophectoderm cells using morpholino antisense oligonucleotides did not affect conceptus elongation [97]. In contrast, the inhibition of PPARG using morpholino antisense oligonucleotides resulted in severely growth-retarded conceptuses [97], indicating that PPARG signaling is of great importance during conceptus elongation in ruminants. Furthermore, *PPARG* expression has been reported to increase during conceptus elongation in cattle, and its expression was correlated with the expression of several genes involved in lipid metabolism [57]. Of note,

PPARA was the highest PPAR expressed by day 17 endometrium and regulates FA catabolism in several tissues [98], suggesting it may have critical roles modulating uterine luminal lipid content during early pregnancy. Among genes involved with PPAR signaling in the endometrium, the expression of fatty acid binding protein 3 (*FABP3*) was increased in pregnant compared to open heifers and in pregnant HF compared to SF heifers. FABP coordinates cellular lipid responses and reversely binds a variety of lipids, including eicosanoids and saturated and unsaturated long-chain fatty acids [99]. Of note, FABP3 modulates cell growth and proliferation [100] and is upregulated by pregnancy in the endometrium LE on days 15 and 18 of pregnancy in cattle [101].

Regarding the differentially expressed genes involved with arachidonic acid metabolism, expression of glutathione peroxidase 1 (*GPX1*), a major antioxidant enzyme [102], was increased in SF than HF conceptuses and could indicate greater oxidative stress in SF conceptuses. Furthermore, expression of one phospholipase A2 (*PLA2*) was increased in HF conceptuses (*PLA2G15*) and another one (*PLA2G1B*) increased in SF conceptuses. The superfamily of PLA2 enzymes are characterized by their ability to hydrolyze fatty acids from the sn-2 position of glycerophospholipids producing a variety of free fatty acids and lysoglycerophospholipids [103]. PLA2G15 is localized in lysosomes and hydrolyses preferentially zwitterionic phospholipids (phosphatidylcholine - PC and phosphatidylethanolamine - PE) [103, 104], and PLA2G1B is described as a pancreatic lipase [103] expressed primarily by acinar cells, and in a much lesser extent by the lungs and kidney, and has higher affinity for anionic phospholipids (phosphatidic acid - PA, phosphatidylserine - PS, phosphatidylglycerol - PG) than PC [105, 106]. Those differences in PLA2 expression by HF and SF conceptuses perhaps

contributed to the observed differences in ULF fatty acid composition between pregnant HF and SF heifers.

Simintiras et al. [18] have recently reported changes in the uterine fluid lipidome associated with progesterone and day of the estrus cycle during the period of onset of conceptus elongation (days 12 to 14) in heifers. Surprisingly, in the current study, there was no effect of pregnancy on the fatty acid profile of day 17 ULF. Palmitic acid (16:0) and stearic acid (18:0), which are the two most abundant saturated fatty acids in mammalian cells [50, 107], accounted for around 90% of the total fatty acid detected in the ULF. The low complexity of the ULF fatty acid profile observed in the present study may have been influenced by the amount of PBS used to perform the uterine flushes (20 mL), which perhaps diluted the fatty acid containing molecules in ULF, and thus biased the fatty acid profile towards the ones with highest intensity.

Two unusual ultralong-chain fatty acids ($C \geq 26$) were the third (28:7) and fourth (28:8) most abundant fatty acids detected in the ULF of open and pregnant heifers, and their concentrations were increased in pregnant HF than SF ULF. Ultralong-chain polyunsaturated fatty acids (PUFAs) have also been found in the other mammalian tissues, such as brain, retina, skin, and testis [107, 108], and 28:5 and 30:5 are essential for sperm maturation and male fertility in the testis [109]. 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. However, the differently abundant fatty acids had overall low concentrations in the ULF, accounting together for only 1.2% of the total fatty acid detected. The source of long-chain saturated fatty acids in animals comes from the diet or from fatty acid de novo synthesis, which is mediated by FASN [110, 111]. Expression

of *FASN* by the endometrium was increased by pregnancy, but it did not differ in the endometrium of pregnant HF and SF heifers, or between HF and SF conceptuses. Fatty acid biosynthesis and catabolism involves dynamic processes in which the number of carbons and double bonds change under the influence of elongation, desaturation and b-oxidation reactions [107]. Fatty acid extension depends on elongases (ELOVL), which are enzymes that catalyzes carbon chain extension [112]. In the present study, *ELOVL1* and *ELOVL6* were upregulated in the endometrium by pregnancy. In addition, *ELOVL5* was upregulated in long and in HF conceptuses, and *ELOVL7* was upregulated in short and in SF conceptuses. Similarly, Barnwell et al. [68] also observed increased expression of *ELOVL5* in long (24.7 $\pm$ 1.9 mm) compared to short (4.2 $\pm$ 0.1 mm) day 15 bovine conceptuses. In the present study, differences were also observed in the expression of fatty acid desaturases 1 and 2 (*FADS1* and *FADS2*). Fatty acid desaturases controls the degree of fatty acid unsaturation by catalyzing the insertion of double bonds into the fatty acid chain [107]. Fatty acid desaturase 1 and 2 (FADS1 and FADS2) are essential enzymes for PUFA biosynthesis [113, 114], and expression of *FADS1* was increased in HF and long conceptuses and decreased in SF and short conceptuses. Furthermore, *FADS2* expression was increased long conceptuses and in pregnant endometrium. Moreover, a number of genes involved with lipid metabolism were differently expressed in HF and SF conceptuses, including genes involved with the transport of long-chain fatty acids (*SLC27A3*), and with the biosynthesis of glycerolipids (*GPAM*) [115], phosphatidylcholines (*CHKA*) [116], ceramides (*CERS1, ASAH1*) [117, 118], steroid hormones (*CYP11A1*) [119], and with cholesterol esterification (*ACAT1*) [120]. The observed differences in lipid metabolism among HF and SF

conceptuses likely contributed to the observed differences in fatty acid content among pregnant fertility-classified heifers.

To identify changes in ULF lipidome that are normally induced by pregnancy, global lipidomics was conducted exclusively on ULF samples of HF heifers that were pregnant or not. This analysis found that phosphatidylcholines, a major component of the cell membrane in eukaryotes [121], were the main lipid class that increased in the uterine lumen with pregnancy, which is possibly related to the secretion of extracellular vesicles by the elongating conceptus [52, 53]. phosphatidylcholines are the most abundant phospholipid in sheep endometrium on days 3, 12 and 15 of the estrus cycle and pregnancy [122], and the main lipid component of uterine epithelial cells during the peri-implantation period in mice [123]. It is likely that conceptus-derived EVs are responsible for the increased abundance of PC observed in day 17 pregnant ULF, as pregnancy appears to inhibit endometrial-derived extracellular vesicles through induction of interferon stimulated gene 15 (ISG15) by IFNT [124].

In summary, the main findings of the current study are that concentrations of glucose, PGE2, PGF2α and phosphatidylcholines were increased in the ULF by pregnancy and that ULF from pregnant HF heifers had overall higher concentrations of fatty acids, PGE2, PGF2α and 6-keto-PFG1α than pregnant SF heifers. The observed differences in the metabolism of glucose, PG, and lipids, by the conceptus and endometrium from fertility-classified heifers, further reinforces our hypothesis of dysregulated conceptus-endometrium interactions in SF heifers [1], which consequently affects uterine luminal histotroph and negatively affects conceptus survival and development after day 17 and before day 28.

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FIGURE LEGENDS

Figure 1. Glucose concentrations in ULF were higher (P = 0.05) in pregnant compared to open heifers on day 17 (**Figure 1A**). Among pregnant heifers, there was no effect (P = 0.88) of fertility classification on ULF glucose concentrations (**Figure 1B**). ULF glucose did not differ (P = 0.66) among open fertility-classified heifers (**Figure 1C**). Glucose in plasma was not different (P = 0.20) between pregnant and open heifers (**Figure 1D**). Among pregnant heifers, plasma glucose was affected by the interaction between fertility group and conceptus length (P = 0.02; **Figure 1E**). Among the open animals, IF heifers had higher (P = 0.04) plasma glucose concentrations compared to HF heifers (**Figure 1F**).

Figure 2. Pearson correlation between glucose concentrations in plasma and in the uterine luminal flush (ULF) (**A**) and between conceptus size and glucose in the ULF (**B**).

Figure 3. Concentrations of PGE2 tended (P = 0.07) to be higher in ULF of pregnant compared to open heifers (**Figure 3A**). Pregnant heifers had (P < 0.01) greater concentrations of PGF2α in the ULF compared to open heifers (**Figure 3D**). ULF concentrations of 6-keto-PFG1α was not affected (P = 0.18) by pregnancy status (**Figure 3G**). Among pregnant heifers, concentrations of PGF2α was higher (P = 0.01) in ULF of HF compared to SF heifers (**Figure 3E**). Furthermore, ULF of HF pregnant heifers tended to have greater concentrations of PGE2 (P = 0.10; **Figure 3B**) and 6-keto-PFG1α (P = 0.08; **Figure 3H**) than ULF of pregnant SF heifers. Among open heifers, there was no effect ($P \geq 0.32$) of fertility group on ULF PG concentrations (**Figures 3C, 3F and 3I**).

Figure 4. Pearson correlations between ULF PGs (PGE2, PGF2α, 6-keto-PFG1α) and conceptus length.

Figure 5. Concentrations of fatty acids identified in the uterine luminal flush (ULF) of the fertility-classified heifers (**A**). Proportion of the nine most abundant fatty acids in the ULF relative to the total fatty acid content detected for open (**B**) and pregnant (**C**) fertility-classified heifers. Heatmap (**D**) for fatty acid concentration (molar) in the uterine lumen flush (ULF) across samples.

Figure 6. Description of the significant independent variables retained in the multivariable model investigating the effect of fertility classification on ULF fatty acid profile of pregnant heifers. For fatty acids that increased in the ULF of pregnant HF compared to SF heifers, conceptus number affected ULF concentrations of 17:0 ($P < 0.01$), 28:7 ($P = 0.01$) and 28:0 ($P = 0.01$) fatty acids, as heifers with two conceptuses had greater levels of these fatty acids than heifers with a single conceptus (**Figure 6A**). Additionally, ULF concentration of 17:0, 28:7 and 28:0 fatty acids was affected by the interaction between conceptus number and fertility group ($P < 0.01$, $P = 0.01$, $P = 0.01$), by the interaction of conceptus length and fertility group ($P < 0.01$, $P < 0.01$, $P < 0.01$), and by the three-way interactions of conceptus number, length and fertility group ($P < 0.01$, $P = 0.01$, $P = 0.01$; **Figures 6B, 6C and 6D**). For fatty acids that increased in the ULF of pregnant SF than HF heifers, concentrations of 20:0 was affected by the interaction of conceptus number and fertility group ($P = 0.01$; **Figure 6E**), and ULF concentrations of 6:0 was

affected by conceptus number ($P < 0.01$; One = $1.79x10^6 \pm 3.84x10^3$; Two = $1.66x10^6 \pm 8.15x10^3$), by conceptus length ($P < 0.01$; **Figure 6F**), by the interaction of conceptus length and fertility group ($P < 0.01$), and by the three-way interaction of conceptus number, length and fertility group ($P < 0.01$; **Figure 6G**).

SUPPLEMENTAL FIGURE LEGEND

Figure S1. Fold change (FC) analysis showing the differently abundant lipids (FC > 2) in the uterine lumen of HF heifers that were pregnant or not. The open samples were set as the reference group and the data is presented as log2 of the FC. Each dot represents a feature identified in the positive mode of the lipidomic analysis based on fragmentation spectra (ddMS2; **Dataset S7**). The graph demonstrates that the abundance of 9 lipids that increased (pink dots) and 1 decreased (grey dot) in the ULF of HF heifers by pregnancy (**Table 6**).

SUPPLEMENTAL DATASETS

Dataset S1. Expression of genes encoding glucose transporters in conceptuses.

Dataset S2. Gene expression by the endometrium and conceptuses for genes involved with glycolysis and gluconeogenesis using KEGG database.

Dataset S3. Gene expression by the endometrium and conceptuses for genes involved with arachidonic acid metabolism according to the KEGG database.

Dataset S4. Gene expression by the endometrium and conceptuses for genes involved with PPAR signaling according to the KEGG database.

Dataset S5. Raw data from total fatty acid analysis.

Dataset S6. Gene expression by the endometrium and conceptuses for genes involved with lipid metabolism according to the KEGG database.

Dataset S7. Features detected by untargeted lipidomics.

TABLES

Table 1. Expression of genes in the endometrium involved with glucose transport

			Pregnant vs Open (HF and SF)			Only Pregnant HF vs SF		
	Gene name	Gene description	FPKM[1] OPEN	FPKM[1] Pregnant	FDR	FPKM[1] HF(P)	FPKM[1] SF (P)	FDR
	SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	68 ± 11	153 ± 19	0.00	174 ± 35	132 ± 13	0.68
Increased in Pregnant	*SLC5A1*	Solute carrier family 5 (sodium/glucose cotransporter), member 1	21 ± 3	35 ± 3	0.00	31 ± 5	38 ± 4	0.90
Endometrium	*SLC5A11*	Solute carrier family 5 (sodium/inositol cotransporter), member 11	0.02 ± 0.1	1 ± 0.1	0.02	1.2 ± 0.3	0.9 ± 0.1	0.94
	SLC35D2	Solute carrier family 35 (UDP-GlcNAc/UDP-glucose transporter), member D2	12 ± 1	15 ± 1	0.04	15 ± 1	15 ± 1	1.00
Decreased in Pregnant	*SLC2A10*	Solute carrier family 2 (facilitated glucose transporter), member 10	1 ± 0.1	0.5 ± 0.1	0.00	0.6 ± 0.1	0.5 ± 0.0	1.00
Endometrium	*SLC2A3*	Solute carrier family 2 (facilitated glucose transporter), member 3	8 ± 0.5	5 ± 1	0.00	5 ± 1	6 ± 1	0.65
	SLC37A4	Solute carrier family 37 (glucose-6-phosphate transporter), member 4	7 ± 0.2	6 ± 0.2	0.04	5 ± 0.3	6 ± 0.4	0.88

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

Table 2. Differently expressed genes in the endometrium by pregnancy and between fertility-classified heifers for genes involved with glycolysis and gluconeogenesis

Comparisons	Gene name	Gene description	FPKM[1]	FPKM[1]	FDR
Pregnant vs Open			Open	Preg	
	LDHB	Lactate dehydrogenase B	125 ± 5	101 ± 2	0.04
Increased in Open	*LDHA*	Lactate dehydrogenase A	21 ± 2	13 ± 1	0.00
	PFKM	Phosphofructokinase, muscle	8 ± 0.4	5 ± 0.3	0.00
	HK2	Hexokinase 2	1 ± 0.3	2 ± 0.2	0.01
	ALDOB	Aldolase, fructose-bisphosphate B	1 ± 0.1	2 ± 0.4	0.00
	ADPGK	ADP-dependent glucokinase	9 ± 0.3	11 ± 0.5	0.00
	PCK2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	2 ± 0.1	5 ± 0.4	0.00
Increased in Pregnant	*PFKP*	Phosphofructokinase, platelet	11 ± 0.4	16 ± 0.9	0.00
	FBP1	Fructose-bisphosphatase 1	4 ± 0.4	12 ± 3.7	0.02
	HKDC1	Hexokinase domain containing 1	12 ± 2	22 ± 3	0.00
	ACSS2	Acyl-CoA synthetase short-chain family member 2	12 ± 1	23 ± 2	0.00
	AKR1A1	Aldo-keto reductase family 1, member A1 (aldehyde reductase)	30 ± 1	44 ± 5	0.00
	ENO1	Enolase 1, (alpha)	95 ± 2	110 ± 5	0.01
	PGK1	Phosphoglycerate kinase 1	61 ± 2	85 ± 5	0.00
	ACSS1	Acyl-CoA synthetase short-chain family member 1	71 ± 7	106 ± 4	0.00
Only Pregnant			SF	HF	
HF vs SF	*FBP1*	Fructose-bisphosphatase 1	7 ± 4	16 ± 6	0.00

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

Table 3. Expression of select genes involved with glycolysis and gluconeogenesis in conceptuses from fertility-classified heifers

Comparisons	Gene name	Gene description	FPKM[1]	FPKM[1]	FDR
			Short conceptus	Long conceptus	
Short vs Long	-	-	-	-	-
HF vs SF Conceptuses			SF conceptus	HF conceptus	
Increased in HF	*HK1*	Hexokinase 1	9 ± 1	19 ± 3	0.01
	FBP1	Fructose-bisphosphatase 1	0.4 ± 0.1	1.4 ± 0.4	0.02
	GALM	Galactose mutarotase (aldose 1-epimerase)	69 ± 14	207 ± 51	0.05
Increased in SF	*ALDOB*	Aldolase, fructose-bisphosphate B	1.2 ± 0.4	0.4 ± 0.1	0.04
	ALDH3B1	Aldehyde dehydrogenase 3 family member B1	2 ± 0.2	1 ± 0.1	0.04

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

Table 4. Expression of selected genes involved with prostaglandins synthesis or signaling in endometrium and conceptuses

Gene name	Gene description	HF vs SF Pregnant Endometrium			HF vs SF Conceptus		
PG synthase		FPKM[1] HF (P)	FPKM[1] SF (P)	FDR	FPKM[1] HF	FPKM[1] SF	FDR
PTGS2	Prostaglandin-synthase 2	33 ± 4	40 ± 5	0.86	1071 ± 111	666 ± 95	0.13
PTGES	Prostaglandin E synthase	2 ± 0.5	2 ± 0.2	1.00	14 ± 2	18 ± 3	0.35
PTGFS	Prostaglandin F synthase	7 ± 6	2 ± 1	0.19	0.2 ± 0.1	0.5 ± 0.3	0.43
PTGIS	Prostaglandin I2 synthase	3 ± 0.4	4 ± 0.2	0.86	16 ± 1	12 ± 2	0.19
Receptors		Open vs Preg Endometrium			HF vs SF Pregnant Endometrium		
Endometrium		FPKM[1] Open	FPKM[1] Preg	FDR	FPKM[1] HF (P)	FPKM[1] SF (P)	FDR
PTGER2	Prostaglandin E Receptor 2	5 ± 0.3	5 ± 0.4	0.29	6 ± 0.6	5 ± 0.4	0.90
PTGER4	Prostaglandin E Receptor 4	3 ± 0.2	3 ± 0.2	0.06	2 ± 0.2	3 ± 0.3	0.59
PPARA	Peroxisome proliferator-activated receptor alpha	17 ± 1	15 ± 1	0.30	15 ± 1	15 ± 1	1.00
PPARD	Peroxisome proliferator-activated receptor delta	8 ± 0.2	8 ± 0.4	0.49	8 ± 0.5	8 ± 0.7	0.92
Receptors		Long vs Short Conceptus			HF vs SF Conceptus		
Conceptus		FPKM[1] Long	FPKM[1] Short	FDR	FPKM[1] HF	FPKM[1] SF	FDR
PTGFR	Prostaglandin F Receptor (Fp)	4 ± 1	2 ± 1	0.18	4 ± 0.9	1 ± 0.2	0.04
PPARD	Peroxisome proliferator-activated receptor delta	5 ± 1	6 ± 1	0.73	6 ± 0.6	4 ± 0.4	0.07
PPARG	Peroxisome proliferator-activated receptor gamma	3 ± 0.3	2 ± 0.3	0.48	3 ± 0.2	2 ± 0.3	0.10
Endometrium		Preg vs Open Endometrium			HF vs SF Pregnant Endometrium		
PG transporter		FPKM[1] Open	FPKM[1] Preg	FDR	FPKM[1] HF (P)	FPKM[1] SF (P)	FDR
SLCO2A1	Solute carrier organic anion transporter family member 2A1	3 ± 0.3	3 ± 0.3	1.00	3 ± 0.5	3 ± 0.1	0.95
Conceptus		Long vs Short Conceptus			HF vs SF Conceptus		
PG transporter		FPKM[1] Long	FPKM[1] Short	FDR	FPKM[1] HF	FPKM[1] SF	FDR
SLCO2A1	Solute carrier organic anion transporter family member 2A1	7 ± 2	5 ± 1	0.63	7 ± 1	3 ± 1	0.02

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

14 **Table 5. Fatty acid differences in the ULF of fertility-classified heifers**

Comparisons	Significantly different fatty acids in ULF	
Preg vs Open	None	-
HF Preg vs Open	226, 20:0, 20:1 n-11	Higher in Open than Preg
SF Preg vs Open	24:3	Higher in Preg than Open
Only Preg HF vs SF	15:0, 17:0, 24:2, 18:1, 22:5w6, 22:4, 22:2, 20:3, 20:2, 28:7, 28:8	Higher in HF than SF
	8:0, 6:0, 20:0	Higher in SF than HF
Only Open HF vs SF vs IF	22:1	Higher in HF than SF
	22:0, 24:1	Higher in HF than SF and IF

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Table 6. Lipids differentially abundant in the ULF of open and pregnant HF heifers

ID	Pregnant/Open Fold Change (FC)	log2(FC)	P-value
Phosphatidylcholine (34:1)	5.28	2.40	0.01
Phosphatidylcholine (34:2)	2.74	1.46	0.01
Phosphatidylcholine (36:2)	3.17	1.66	0.01
Phosphatidylcholine (34:2)	2.88	1.53	0.01
Phosphatidylcholine (36:1)	3.18	1.67	0.01
Triacylglyceride (16:0_18:1_18:1)	2.39	1.25	0.05
Triacylglyceride (18:0_18:1_18:1)	2.31	1.21	0.09
Acylcarnitine (4:0)	3.59	1.84	0.18
Acylcarnitine (2:0)	2.52	1.34	0.33
Triacylglyceride (18:1_18:1_18:1)	0.38	-1.38	0.44

Figure 1

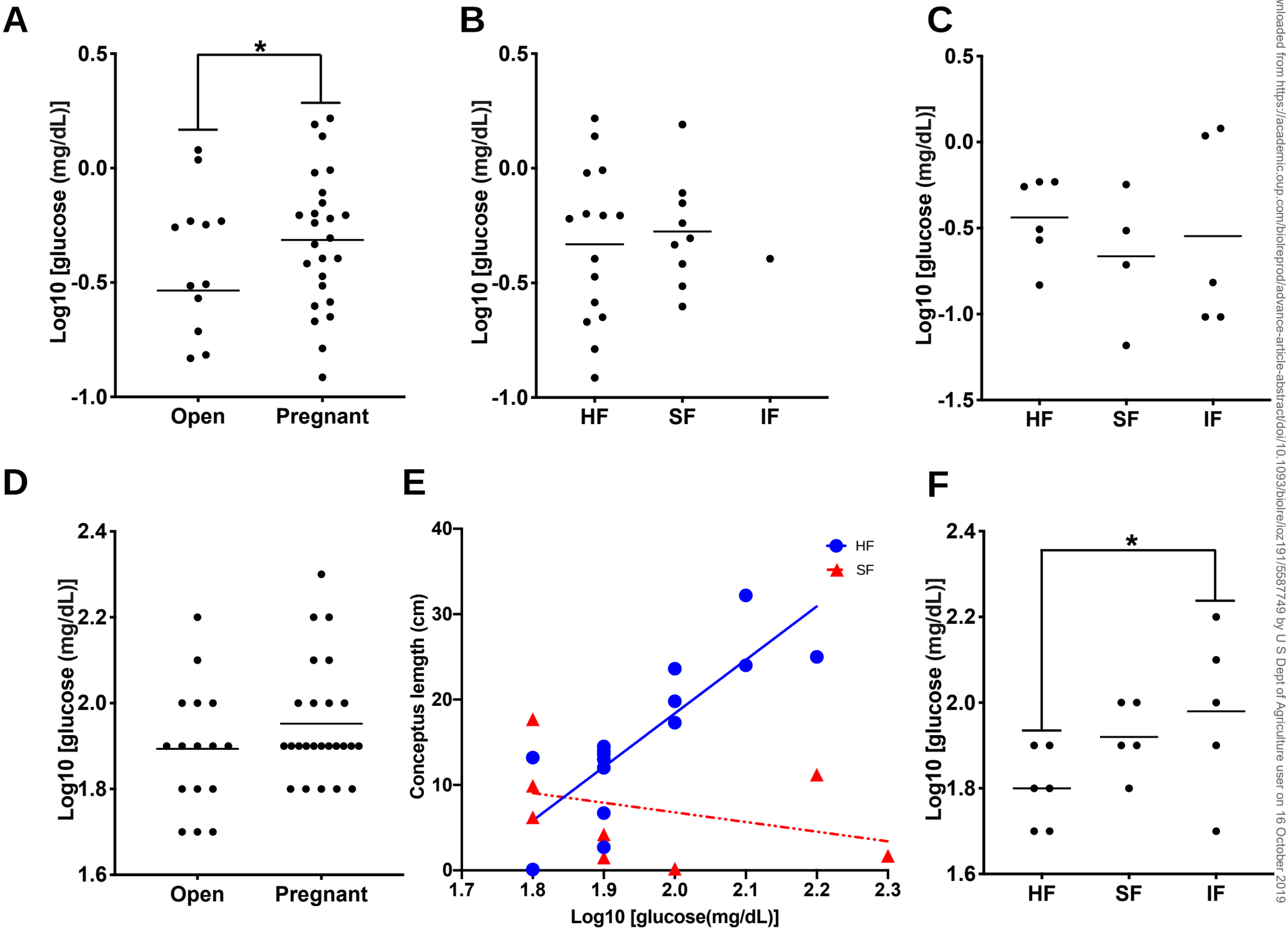


Figure 2

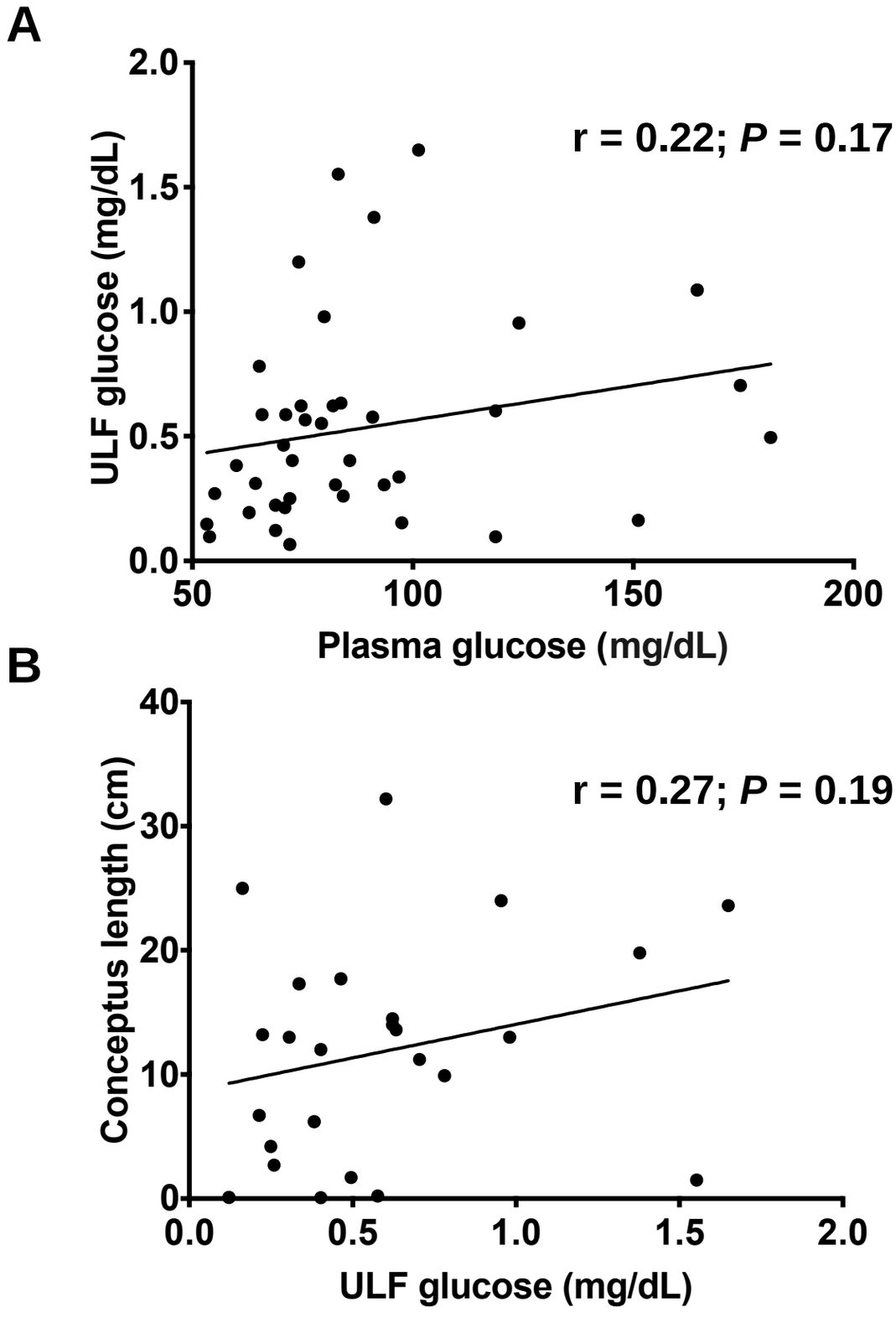


Figure 3

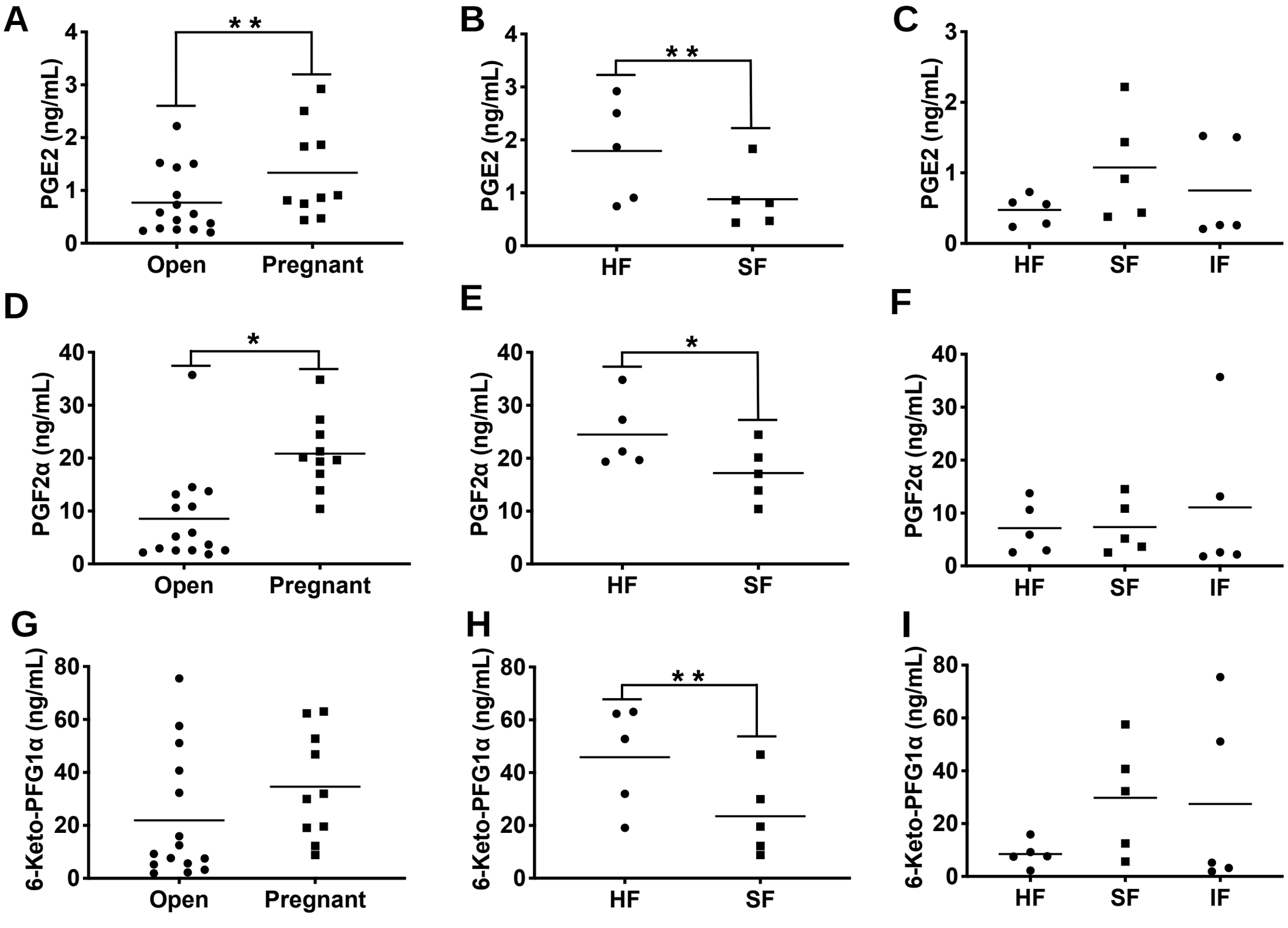


Figure 4

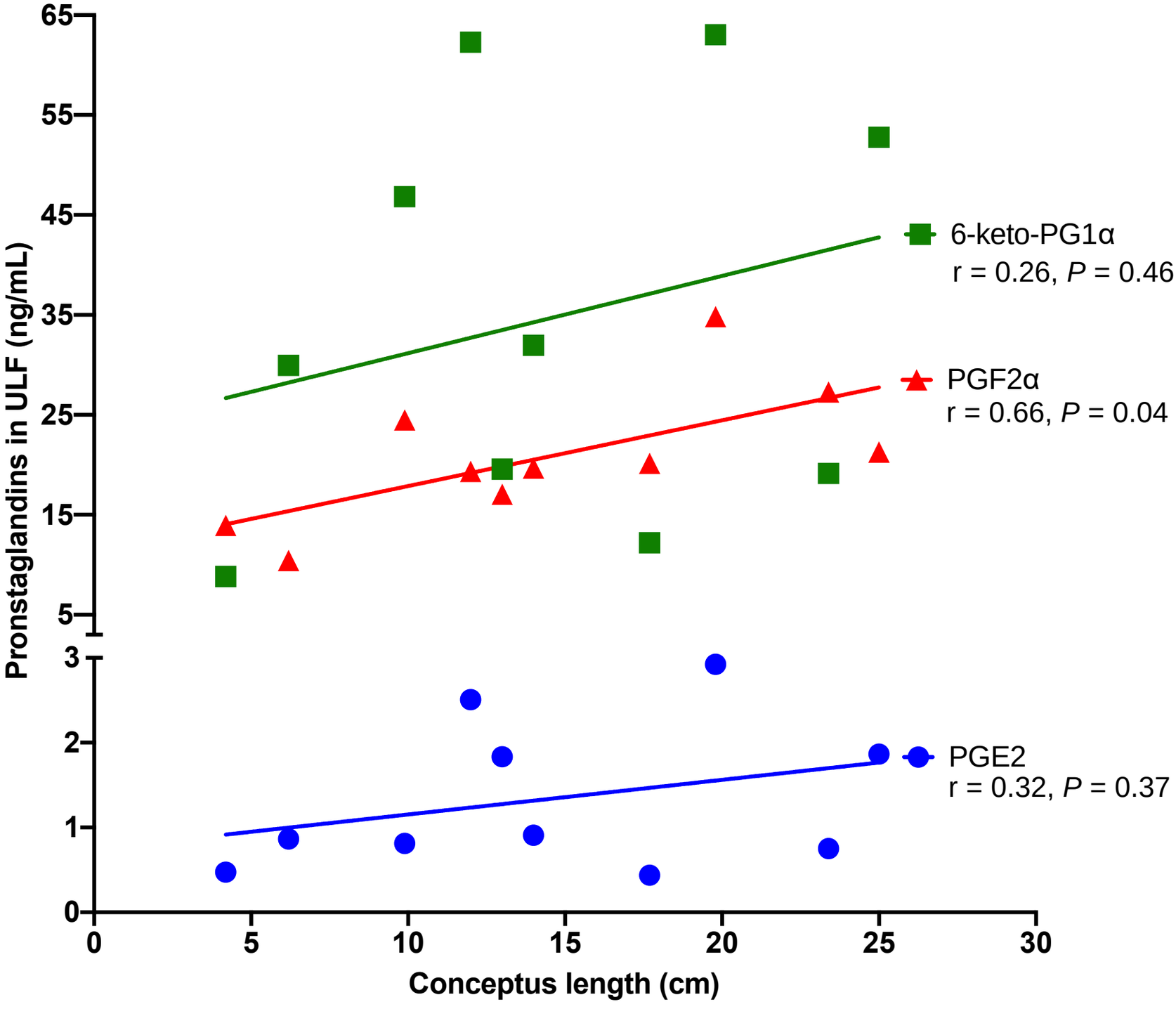
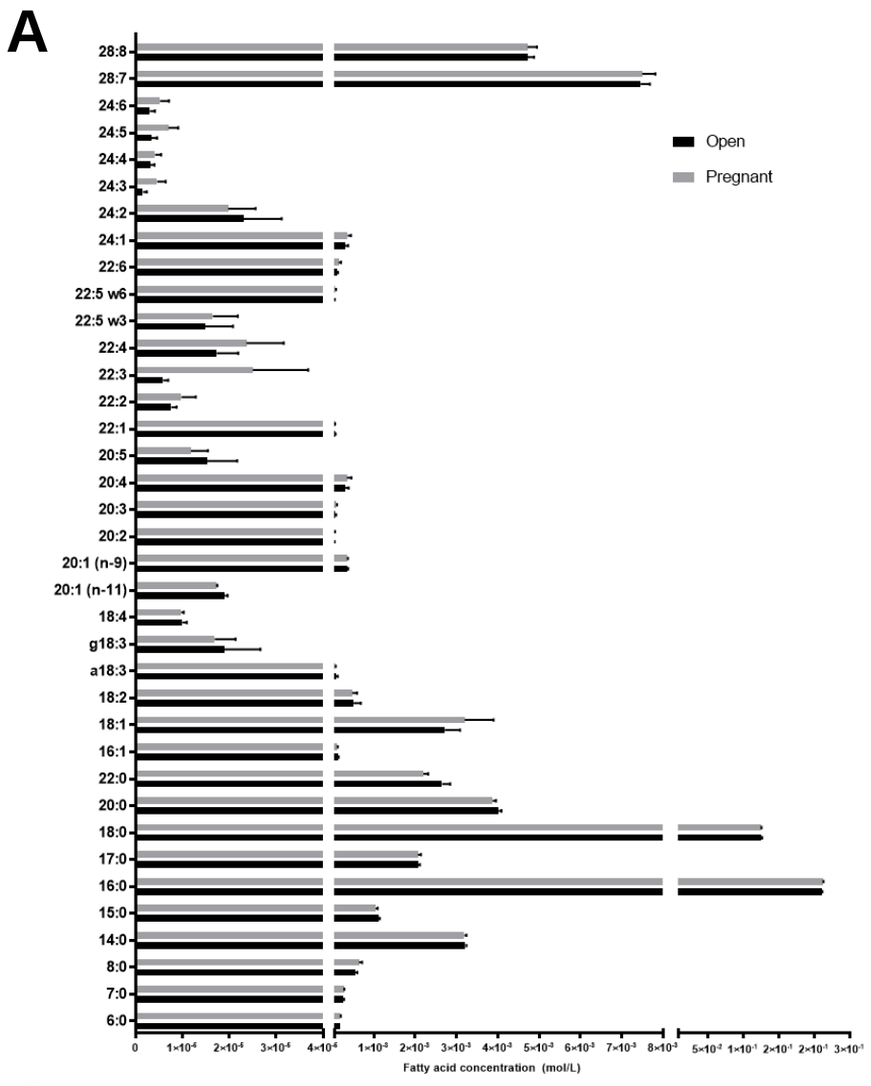
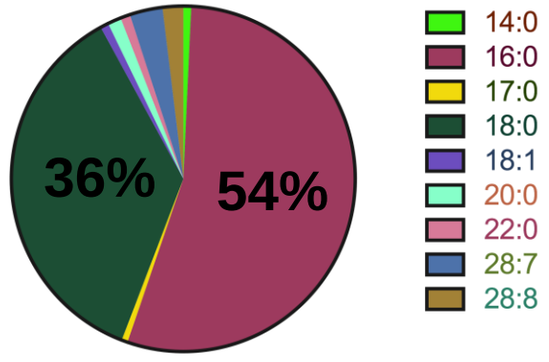
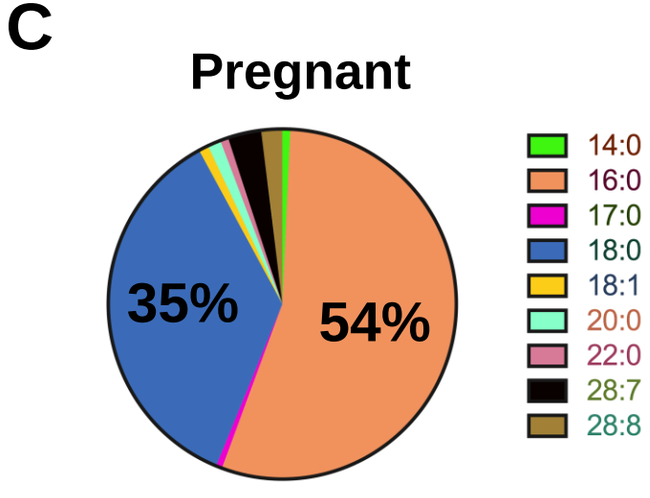


Figure 5







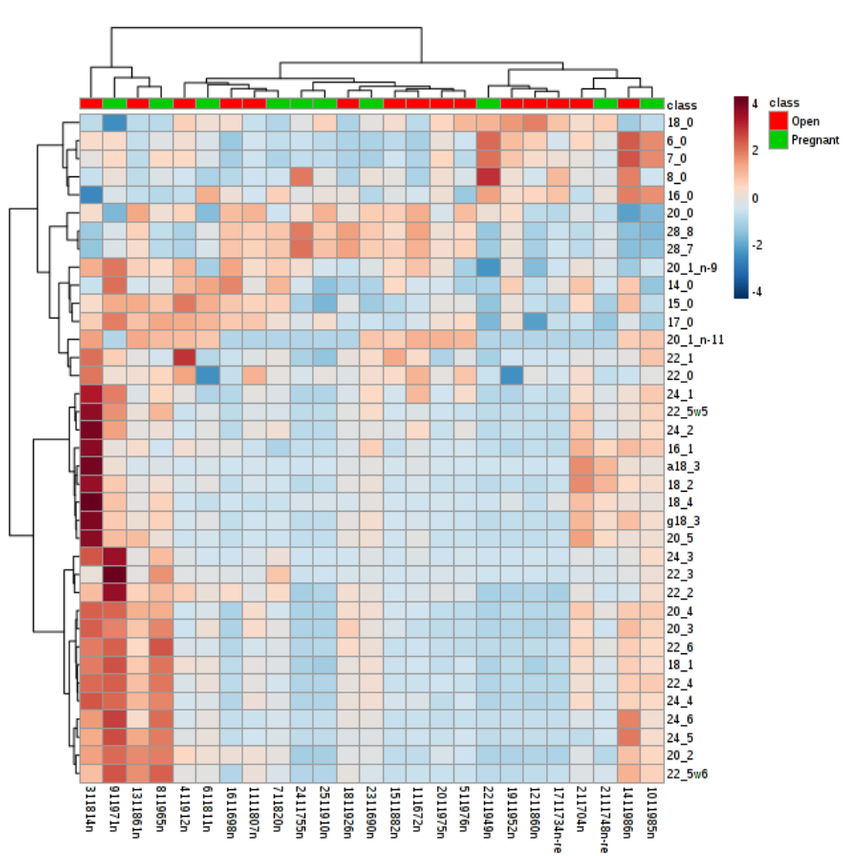


Figure 6