

UTERINE REGULATION OF PREIMPLANTATION EMBRYO DEVELOPMENT IN FERTILITY- CLASSIFIED HEIFERS

A dissertation submitted in partial fulfillment of
the requirements for the degree of

DOCTOR OF PHILOSOPHY IN ANIMAL SCIENCES

UNIVERSITY OF MISSOURI- COLUMBIA
Division of Animal Science

By

JOAO GABRIEL NASCIMENTO MORAES

Dr. Thomas Spencer, Dissertation Supervisor
December 2018

The undersigned have examined the dissertation entitled,

**UTERINE REGULATION OF PREIMPLANTATION EMBRYO
DEVELOPMENT IN FERTILITY-CLASSIFIED HEIFERS**

presented by Joao Gabriel Nascimento Moraes,

a candidate for the degree of doctor of philosophy, and hereby certify that, in their
opinion, it is worthy of acceptance.

Chair, Dr. Thomas Spencer

Dr. Thomas W Geary

Dr. Michael F. Smith

Dr. Matthew C. Lucy

Dr. Scott Poock

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my adviser, Dr. Thomas Spencer, for his guidance, encouragement and advice throughout my doctoral program. Thank you for supporting my research and for allowing me to grow as a scientist. I feel very lucky for having had the opportunity of joining the ‘Team Uterus’ and work alongside great people on outstanding projects. I also would like to thank the current and past members from Dr. Spencer’s lab for all their help and friendship during the last four years: Sofia Ortega, Andrew Kelleher, Greg Burns, Kelsey Brooks, Eleanore O’Neil, Carolyn Allen, Pramod Dhakal, Wang Peng, Brenda Jesernig, Jessica Foster, Harriet Fitzgerald, and Linda Rowland.

My sincere thanks must go also to the members of my dissertation committee, Dr. Thomas Geary, Dr. Michael Smith, Dr. Matthew Lucy and Dr. Scott Poock. I am grateful for having joined Mizzou during the second year of my PhD program and proud to have this great group of scientists in my committee. Thank you for offering me several learning opportunities, for making me a part of your projects, and for your support to my future career as an independent scientist.

I also would like to acknowledge the groups of Dr. Holly Neibergs at Washington State University, Dr. Peter Hansen at the University of Florida, and Dr. Thomas Hansen at Colorado State University for their collaboration on these projects. Also, I want to express my appreciation to the students, faculty members and staff of University of Missouri and Washington State University for their assistance during my doctoral program. I am especially indebted to Dr. Susanta Behura for all his support with bioinformatics, to Dr. William R. Lamberson for teaching me so much about statistics, to Kenneth Ladyman and David Todd for their help at the beef farm, and to Rick Disselhorst and the meats lab crew for their assistance with animal slaughter.

Last but not least, I would like to express my deepest gratitude to my family and friends for their love and support during every step of the way.

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ABSTRACT

Infertility and subfertility represent pervasive problems in domestic animals and humans, and embryonic mortality is a major factor influencing reproductive efficiency. In cattle, the majority of embryonic loss occurs during the first month of gestation that involves the period of blastocyst formation, conceptus elongation, maternal recognition of pregnancy, implantation and beginning of placentation. Pregnancy success and embryonic mortality are affected by paternal, maternal, embryonic, and environmental factors, and the establishment and maintenance of pregnancy are a result of complex conceptus-endometrium interactions that results in adequate conceptuses (embryo/fetus and associated extraembryonic membranes) development, implantation and placentation. Our central hypothesis is that the uterus directly influences embryonic and conceptus development, and we proposed that heifers with consistently high or low fertility have distinct uterine capacity to support pregnancy. To test this hypothesis, serial embryo transfer (3-4 rounds) was used to classify heifers based on pregnancy success on day 28 as high fertile (HF; 100%), subfertile (SF; 25%), or infertile (IF; 0%). Next, a series of experiments were conducted using the fertility-classified heifers to investigate conceptus development and uterine biology in two time points: (1) day 14, to investigate conceptus development prior to the period when pregnancy induce changes are detected in the endometrium transcriptome; (2) at day 17, to evaluate conceptus-endometrial cross talk during the period of maternal recognition of pregnancy. Results from the studies conducted on day 14 supports the idea that: (1) circulating progesterone concentrations are not different among fertility-classified heifers; (2) conceptus development and survival by day 14 is not affected by fertility classification; (3) only minimal differences in endometrium transcriptome are detected among pregnant fertility-classified heifers. Collectively, these

results indicated that the biological mechanisms underlying subfertility and infertility manifests between days 14 and 28, when pregnancy recognition signaling and conceptus elongation and implantation must occur for the establishment of pregnancy. Moreover, results from the subsequent experiments conducted at day 17 indicated that: (1) the mechanism of pregnancy loss in fertility-classified heifers start to manifest around the time of maternal recognition of pregnancy; (2) conceptus survival by day 17 is compromised in IF heifers; (3) conceptus development is advanced in HF than SF heifers; (4) conceptus transcriptome is directly influenced by the uterine environment; (5) dysregulated conceptus-endometrial interactions in SF heifers seems to be the major cause of pregnancy loss. Analysis of the uterine luminal fluid (ULF) from fertility classified heifers on day 17 established that: (1) ULF composition is affected by conceptus-endometrium interactions; (2) glucose concentrations in ULF are not different among fertility-classified heifers; (3) pregnancy induced changes in the metabolites found in ULF was diminished in SF heifers, and the majority of the metabolites that increased in the ULF of pregnant HF than SF heifers were associated with energy and amino acid metabolism; (4) increased abundance of proteins involved with energy metabolism, oxidative stress, amino acid metabolism, and cell proliferation and differentiation were detected in ULF of pregnant HF than SF heifers; (5) The lipid content of the ULF is altered by pregnancy and fertility classification; (6) overall concentrations prostaglandins and interferon tau were increased in the uterine lumen of pregnant HF than SF heifers, likely due to differences in conceptus size. Collectively, results from these studies supports the idea that the dysregulated conceptus-endometrium interactions in SF heifers affects the uterine luminal contents and impairs conceptus survival and elongation. Furthermore, knowledge gained from these studies enhances our understanding of the mechanisms regulating pregnancy loss in cattle and

provides new information on uterine and conceptus biology during early pregnancy in ruminants.

SECTION I

LITERATURE REVIEW

Overview of pregnancy loss in cattle

Subfertility is a prevailing problem in cattle and humans, and early pregnancy loss is a major cause of reduced reproductive efficiency in these species [1, 2]. Pregnancy success and embryonic mortality are affected by paternal, maternal, embryonic, and environmental factors [2–4], and the establishment and maintenance of pregnancy are a result of complex conceptus-endometrium interactions, that results in adequate conceptus (embryo/fetus and associated extraembryonic membranes) development, implantation and placentation [3, 4].

Understanding the timing of pregnancy loss is important to develop strategies to reduce such losses. Reliable pregnancy diagnosis in cattle can be currently performed as early as day 26 with the use of ultrasonography or by measuring placenta-derived pregnancy associated glycoproteins (PAGs) [5, 6]. However, accurate monitoring of preimplantation embryonic development from fertilization (day 0) until implantation (~day 20) is still not possible. Historically, studies used to investigate early pregnancy events in cattle have been based on animal breeding/mating and subsequent animal slaughter or utilized surgical and non-surgical embryo collection approaches to estimate the incidences of fertilization failure and early embryonic mortality.

Fertilization rates have been estimated to be around 90% or higher in heifers, beef cows, and moderate-producing dairy cows [7-12], ~80% in high-yielding dairy cows under thermoneutral conditions, and ~60% in high-producing dairy cattle under heat stress [2, 13-15] . Thus, in thermoneutral conditions, the majority of the pregnancy losses occurs

after fertilization, which encompasses the period of blastocyst formation, conceptus elongation, maternal recognition of pregnancy, implantation and placentation.

Fertilization is the fusion of the male and female gametes, and thus is influenced by maternal and paternal factors [16, 17]. The maternal factors associated with reduced fertilization rates includes ovulation failure, oviduct obstruction [18], uterine inflammation [14, 19], high circulating progesterone concentrations near breeding [2, 20], and reduced oocyte quality [12, 15, 21, 22]. Paternal factors implicated with reduced fertilization rates includes low sperm concentration [23, 24], semen containing sperm with abnormal morphology [25, 26], use of sexed sorted semen [27, 28], and with the use of semen of low sire conception rate (SCR) [29].

Available information concerning embryonic mortality in heifers indicates that ~75 % of beef and dairy heifers have a viable embryo 1 week following breeding/mating (Figure 1). Roche et al. [30] recovered 81% (42/52) and 84% (46/55) of viable embryos from beef heifers on days 3 and 8 following insemination. Sartori et al. [12] recovered 72% (23/32) of viable embryos from dairy heifers on day 6 days after breeding. Diskin et al. [7] recovered 77% (34/44) of viable embryos from beef heifers on day 8 following artificial insemination (AI). Maurer et al. [9] recovered 67% (44/66) of viable embryos from beef heifers between days 2-6 post-mating. Embryonic mortality during the first week after fertilization has been associated with reduced oocyte quality (e.g. caused by heat stress [31], persistent follicles [11, 32], low progesterone concentrations during ovulatory follicle development [33], and negative energy balance [17]), defects in embryonic genome activation [2], and influenced by dysregulated progesterone actions in the oviduct and uterus [2, 15].

Studies investigating embryonic viability in the second week of gestation in heifers supports the idea that a low rate (~ 5%) of embryonic mortality occurs between the first and second week after fertilization. Roche et al. [30] reported that 75% (49/65) of inseminated beef heifers had a viable conceptus on day 14 after breeding. Diskin et al. [7] and Maurer et al. [9] recovered 59% (23/39) and 70% (21/30) of viable conceptuses from beef heifers on day 16 days after breeding/mating. Dunne et al. [34] and Christenson et al. [35] reported 68% (32/47) and 64% (30/47) of viable conceptuses been recovered from slaughtered dairy and beef heifers on days 14 and 12-15, respectively. Berg et al. [36] reported a higher rate of embryonic mortality (~24%) between days 7 and 16 in heifers and nonlactating dairy cows upon transfer of multiple embryos produced in vitro (IVP). The increased embryonic mortality by day 16 observed in Berg's experiment may be attributed with the reduced capacity of IVP embryos to establish pregnancy as compared to embryos produced in vivo through AI or natural service.

More recent data have estimated pregnancy loss around time of maternal recognition of pregnancy, implantation, and placentation in heifers. Ortega et al. [29] estimated pregnancy status in dairy heifers on day 19 following AI based on the expression of interferon stimulated genes (ISGs) by white blood cells (WBC), and an overall pregnancy loss of 14.8% was suggested to have occurred between days 19 and 33 among heifers bred with high and low SCR semen. Additionally, Reese et al. observed that ~14% of dairy heifers (8/57) that were diagnosed nonpregnant on day 31 had increased PAG concentrations on day 24, indicating that embryonic mortality in these animals occurred after the beginning of placental development [37].

The rate of embryonic mortality in heifers is greatly reduced after the first month of gestation, as only about 5% of fetal losses are estimated to occur between days 30 and

90 in beef and dairy heifers [38, 39], and the rate of pregnancy loss significantly reduces as pregnancy advances past day 60 (Figure 1).

Pregnancy failure in lactating dairy cows are generally higher than in nonlactating cows and heifers, and a possible antagonism between high milk production and reproductive performance has long been suggested [17, 39-41]. High producing dairy cows experience a state of negative energy balance (NEB) during early lactation, as the nutrient demands required for supporting increasing milk production exceeds the dietary intake [42]. Negative energy balance is deleterious to fertility [43], and cows experiencing NEB are at higher risk of developing postpartum diseases [44-46]. Uterine postpartum diseases (e.g. retained fetal membranes, metritis, endometritis) are particularly detrimental to fertility, and higher rates of pregnancy loss are observed in animals that developed such conditions [14].

Pregnancy loss in lactating dairy cows during the first week after breeding have been recently reviewed [2], and is summarized in Table 1. The reported losses are related to the first 6-7 days following AI in estrus or timed-AI (TAI), and thus includes fertilization failure and embryonic mortality. Pregnancy failure in the first week averaged 49% under thermoneutral conditions and 72% under heat stress (Table 1) [13].

Heat stress greatly increases pregnancy failure in the first week following AI, which is at least in part associated with the negative effects of heat shock on oocyte quality and early embryonic development [31]. Heat stress negatively affects ovarian follicle development [47, 48], impairs oocyte maturation [49, 50], oocyte fertilization [12], and reduces blastocyst rate due to oocyte damage [21, 22, 51]. Besides reducing oocyte quality, 1-cell and 2-cell embryos are very susceptible to heat shock [52-54], but at the morula (16-cell, ~ day 4) stage, embryos are significantly more resistant to heat stress [52-55]. The

increased resistance of 16-cell embryos to the increase in body temperature confers embryo transfer (ET) as a viable alternative to AI during the summer months, as ET bypasses some of the negative effects of heat shock on oocyte quality and early embryo development [31, 56].

Embryonic losses during time of maternal recognition of pregnancy, implantation and placentation in lactating cows have also been estimated. Reese et al. [37] reported that ~14% (22/162) of lactating dairy cows diagnosed nonpregnant on day 31 after breeding had increased PAG concentrations on day 24. Additionally, Wijma et al. [57] estimated that 35% (14/40) of lactating cows diagnosed nonpregnant at day 42 had experienced early embryonic mortality, which 50% (7/14) of the losses were estimated to occur before day 24, and 50% (7/14) between days 24 and 42, based on combined data from ISG expression by WBC and circulating PAG concentrations.

The timing of pregnancy loss in high producing dairy cows from fertilization to term have been reviewed [2, 15], and up to 60% of embryonic/fetal mortality was estimated to occur from fertilization to term, with 75% of those losses occurring by day 28. Pregnancy loss after the first month of gestation is increased in high-producing lactating dairy cows than in heifers, and ~12% of fetal losses are estimated to occur between days 30 and 90, and ~4% between day 90 and calving in modern high-producing lactating dairy cows [15].

Collectively, the available data indicates that fertilization failure and early embryonic mortality during the first month of gestation are the major causes of reproductive wastage in heifers and lactating dairy cows. Hence, understanding the maternal factors mediating embryonic development and pregnancy establishment is highly desirable, in order to develop strategies to improve reproductive efficiency and profitability in the beef and dairy industries.

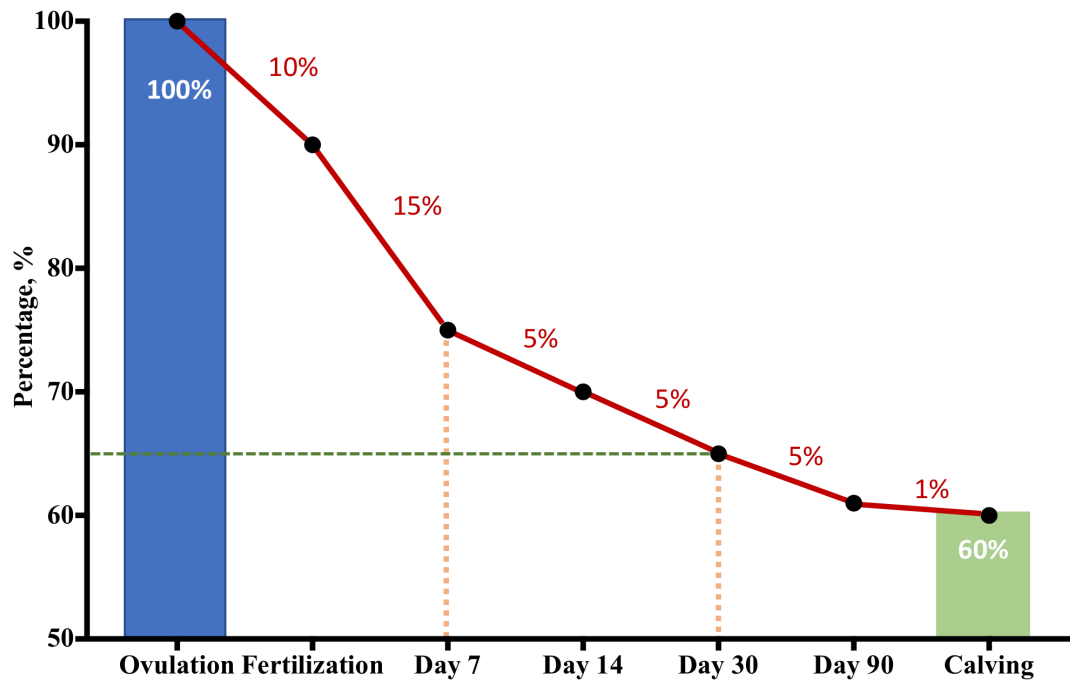


Figure 1. Proposed rate of pregnancy loss from ovulation through calving in beef heifers.

The data used to generate this graph suggests that fertilization failure and embryonic mortality accounts for ~41% of pregnancy failure in heifers. Around 61% of the losses are estimated to occur within the first week following breeding, ~12% after blastocyst formation and before maternal recognition of pregnancy, ~12% during period of maternal recognition of pregnancy, implantation and beginning of placentation, ~12% between days 30-90 of gestation, and ~2.4% from day 90 to term.

Table 1. Pregnancy loss in lactating dairy cows during the first week following artificial insemination.

The data is presented as percentage of viable embryos (VE) per total structures (embryos or oocytes; OE) collected (VE/VO), and per total embryos collected (VE/TE) (Table adapted from [13]).

Reference	AI after estrus				TAI	
	Heat stress		Thermoneutral		Thermoneutral	
	VE/OE	VE/TE	VE/OE	VE/TE	VE/OE	VE/TE
Wiebold (1988) ^a [58]			48.0 (12/25)	48.0 (12/25)		
Ryan et al. (1993) ^b [59]	47.1 (24/51)	58.5 (24/41)	41.0 (16/39)	51.6 (16/31)		
Sartori et al. (2002) ^c [12]	18.4 (7/38)	33.3 (7/21)	46.3 (19/41)	52.8 (19/36)		
Cerri et al. (2009) ^d [60]					50.0 (42/84)	62.7 (42/67)
Cerri et al. (2009) ^e [61]			61.0 (25/41)	71.4 (25/35)	59.8 (76/127)	69.1 (76/110)
Cerri et al. (2009) ^f [62]					51.8 (44/85)	65.7 (44/67)
Hackbart et al. (2010) ^a [63]	20.0 (4/20)	57.1 (4/7)	42.2 (27/64)	50.0 (27/54)		

^a 7-day-old embryos.

^b 6- or 7-day-old embryos.

^c 6-day-old embryos.

^d 5-day-old embryos of cows supplemented with calcium salt of palm oil or calcium salt of linoleic and trans-octadecenoic acids.

^e 6-day-old embryos.

^f 6-day-old embryos of cows supplemented with different sources of selenium.

Early pregnancy development in ruminants

Uterus

Major components of the female reproductive tract include the uterus, ovaries, Fallopian tubes (oviducts), cervix, vagina and external genitalia [64]. The uterus is the focus of this section, and its main functions include sperm transport and capacitation [65], control of estrus cycle [66], histotroph secretion [67], and maternal contribution to the placenta [68] and parturition [69].

During embryonic development, the uterus originates from the Mullerian ducts [70], and is still not fully differentiated at birth in domestic animals and humans. The main morphogenetic events that occurs postnatally includes the development of uterine glands (adenogenesis), organization and stratification of the endometrial stroma, and the differentiation and growth of the myometrium [71, 72].

The mammalian uterine wall is composed by three distinct layers, the endometrium, the myometrium and the perimetrium (Figure 2). The endometrium is the inner layer of the uterus consisting of luminal (LE) and glandular epithelium (GE), as well as underlying stroma. The myometrium is composed by an inner circular and an outer longitudinal layer of smooth muscle. The perimetrium is the serosal layer of the uterus, and corresponds to the part of the peritoneum that surrounds the uterus, and is composed of a membrane of simple squamous epithelium on top of a thin layer of loose connective tissue [70, 71].

The endometrium of an adult cow or ewe contains caruncular and intercaruncular areas. The caruncular endometrium is formed from proliferation of the subepithelial connective tissues, and plays important role on superficial implantation and placentation in ruminants. The synepitheliochorial placenta is formed by uterine caruncles fusing with

placental cotyledons to form the placentomes, which are responsible for maternal-fetal exchanges (e.g. gaseous, nutrients and metabolic waste) [73]. The caruncular endometrium lacks uterine glands, but a profuse amount of glands are present in the intercaruncular endometrium of cows and sheep [71].

Uterine gland development involves budding of GE from the LE [74], and although the precise mechanism regulating the differentiation of LE cells into GE cells have not been elucidated, uterine adenogenesis is regulated by complex intrinsic (e.g. transcription factors, growth factors) and extrinsic stimulus (e.g. ovary, pituitary, mammary gland) [75]. Gland secreted factors plays important role on preimplantation embryonic development in sheep and mice, as conceptus elongation does not occur in uterine gland knockout sheep (UGKO) [76, 77], and glandless mice display implantation and decidualization issues [78, 79]. Uterine luminal constituents, collectively known as histotroph, is composed of molecules secreted or transported into the uterine lumen [80]. The histotroph from cattle has been characterized [81-85], and is a complex mixture of amino acids, glucose, lipids, proteins, vitamins, ions, cytokines, hormones, growth factors, among other substances. Because the attachment of the bovine conceptus with the endometrial luminal epithelium occurs around day 20 [86], and microvillar interdigitation between trophoblast cells and the caruncular epithelium which marks the beginning of placentation only begins around day 24 [87], the development of preimplantation embryo/conceptus prior attachment and placentation is highly depended on histotrophic nutrition.

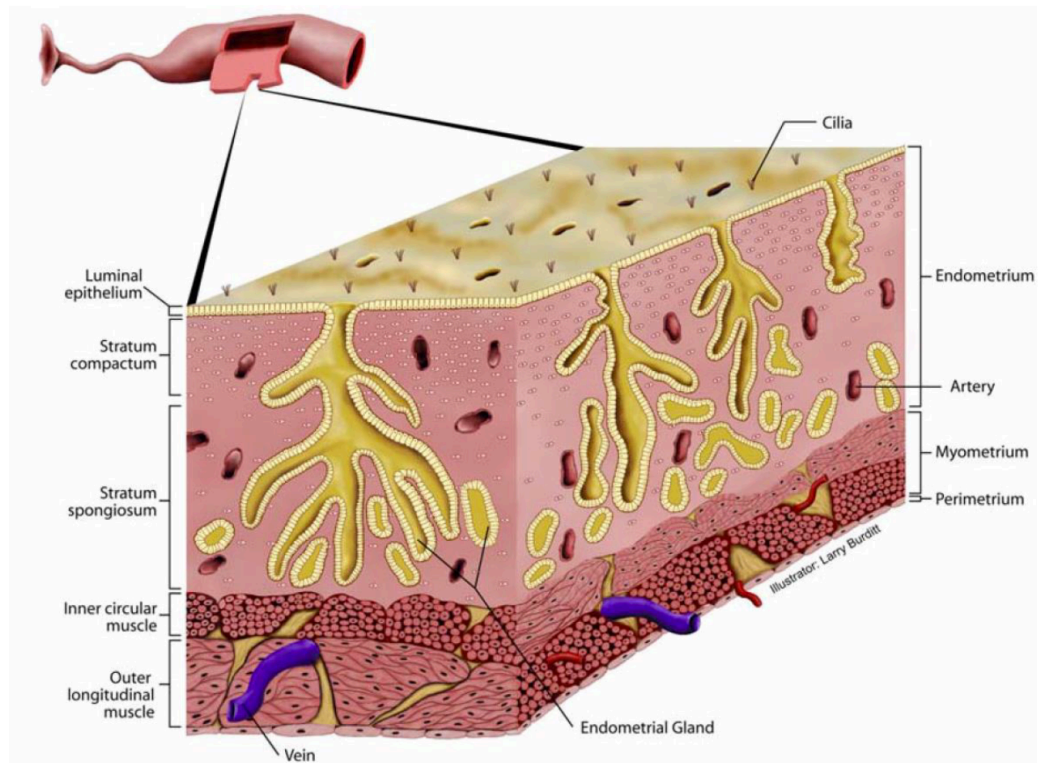


Figure 2. Uterine histoarchitecture.

The uterine wall is composed by three distinct layers, the endometrium, the myometrium and the perimetrium. The endometrium is the inner layer, and is consisted of luminal epithelium (LE), glandular epithelium (GE), and stroma. The myometrium is composed by an inner circular and outer longitudinal layer of smooth muscle. The perimetrium is the serosal layer of the uterus, which is consisted of superficial mesothelium and loose connective tissue. (The figure is courtesy of Rodney Geisert and Larry Burdett, Oklahoma State University, Stillwater, USA)

Preimplantation embryonic development

During mammalian embryonic development, the oocyte is fertilized in the ampulla of the oviduct forming a zygote [88], which undergo several mitotic divisions to form a morula [89] that enters the uterus between days 4 to 6 post-mating in cattle [90]. The morula then develops into a blastocyst that contains an inner cell mass (ICM) and a blastocoele or central cavity surrounded by a monolayer of trophectoderm (TE) cells [91]. The cells from the ICM are pluripotent and form all three embryonic germ layers (endoderm, mesoderm and ectoderm), and the differentiation of TE cells gives rise to the extraembryonic structures, including the placenta [92].

The bovine blastocyst is a 150 to 190 μm sphere on day 7 [93], and undergoes tremendous morphological changes (Figure 3) in the uterus including hatching from the zona pellucida (days 9–10), transitioning from its initial spherical appearance, into an ovoid (0.5-4mm), tubular (5–19 mm), and then filamentous (20–60 mm) form by day 15 of pregnancy [36, 94, 95]. The process of conceptus elongation is uterine dependent, as hatched blastocysts and trophoblastic vesicles do not elongate in vitro, but elongation is possible upon their transfer into the uterus of cows and sheep [96, 97].

Adequate conceptus development is critical for successful maternal recognition of pregnancy and implantation [98]. According to Guillomot [99], the implantation process in domestic ruminants includes 5 phases: (1) blastocyst shedding of the zona pellucida; (2) pre-contact and blastocyst orientation; (3) apposition; (4) adhesion; and (5) endometrial invasion (Figure 4). Apposition of the conceptus TE and endometrial LE begins around day 16 in cattle, attachment is achieved around day 20, and the microvillar interdigitation between trophoblast cells and the caruncular epithelium is observed around day 24 [87].

During attachment of mononuclear TE cells with endometrium LE (~day 20), trophoblast giant binucleate cells (BNC) begin to differentiate within the TE [86], through successive nuclear divisions without cytokinesis [100, 101]. The recently formed BNC then undergoes differentiation, in a process marked by migration of BNC away from the basement layer and tight junctions of the trophoblast. Their nuclei becomes polyploid, and their Golgi apparatus start to produce a large number of granules containing pregnancy associated glycoproteins (PAGs) and placental lactogen (also known as chorionic somatomammotropin hormone 1 or CSH1). Once fully granulated, BNC move towards the maternal side, and fuse with endometrial LE cells, thereby forming the trinucleated fetomaternal hybrid cell [102]. In sheep, the continued fusion of BNC and trinucleated cells results in the formation of syncytial plaques, that contains 20-25 nuclei, and are linked by tight junctions [100, 101]. The formation of syncytial plaques results in expansion of fetal villi and fetomaternal interface, and participates in the synthesis and delivery of placental secreted factors [101] (Figure 4). In cattle, however, areas of syncytium appears only to be formed at time of implantation [86]. Although the function of PAGs remains unknown, CSH1 binds to prolactin receptors expressed exclusively by the endometrial GE, stimulating uterine gland morphogenesis and function [103], thus affecting histotroph composition and conceptus development during early pregnancy.

Histotrophic nutrition is particularly important prior to placentome development in ruminants. In cattle, the allantois begins to form around day 23, and its vascularization is well visible on day 26. The vascularization of the allantois is a critical step in placental development, as vessels from the allantois vascularize the chorion, and cotyledons originates from the outside of the chorion (~ day 30). Around day 50, placental cotyledons fuses with uterine caruncles forming placentomes, which well established by day 70 of

pregnancy [104]. Upon placentome formation, there is a switch between histotrophic to hematomatrophic nutrition. Importantly, histotrophic nutrition of fetus continues during pregnancy, through specialized areas of the placenta named areolae [105].

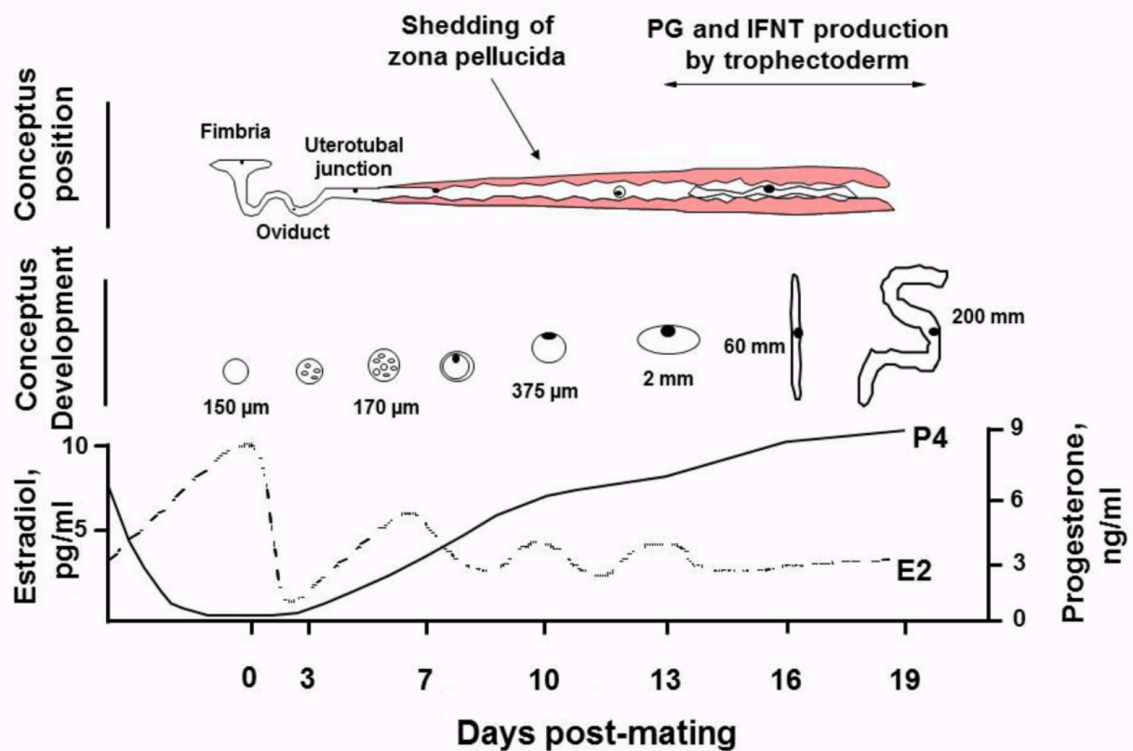


Figure 3. Early embryonic development in cattle from ovulation to attachment.

Summary of events occurring during the migration of oocytes and developing embryo/conceptus in the female reproductive tract, and the circulating concentrations of estradiol (E2) and progesterone (P4) in the maternal blood during this period. The oocyte is fertilized in the ampulla of the oviduct forming a zygote, and a morula enters the uterus between days 4 to 6 post-mating. The blastocyst is formed on day 7, and it hatches from the zona pellucida around days 9–10. At day 12 the conceptus is ovoid, and it becomes tubular by days 13–14, and mostly filamentous at or after day 15. The elongating conceptus secretes interferon tau (IFNT) and prostaglandins (PG), which are essential for maternal recognition of pregnancy and to modulate uterine gene expression for successful implantation and placentation. Figure from [106]

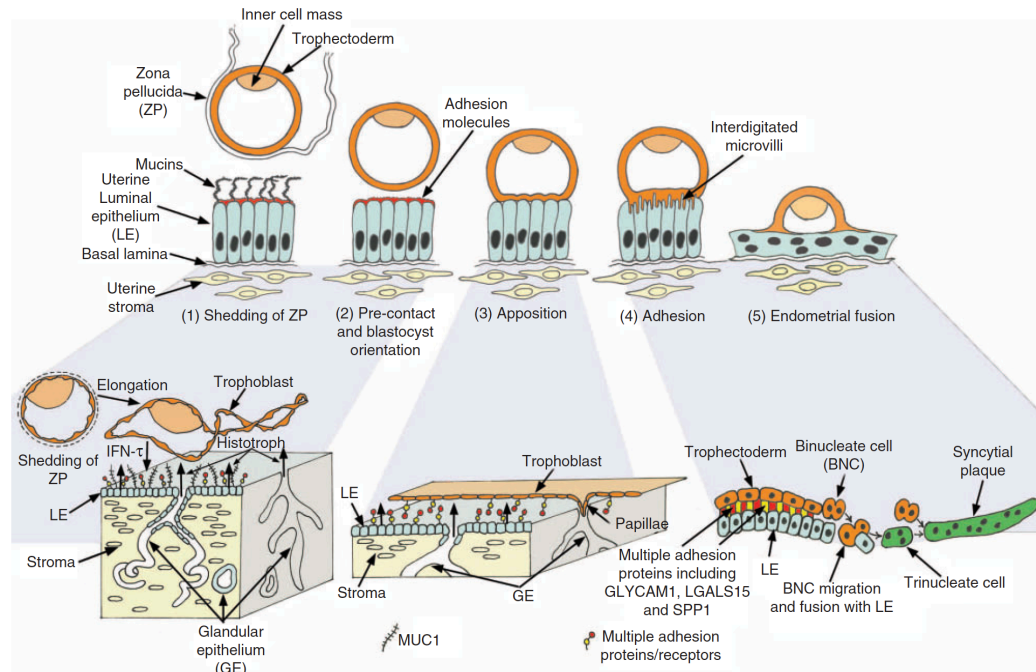


Figure 4. Phases of implantation in sheep.

The bovine blastocyst hatches from the zona pellucida around days 9-10, exposing the trophectoderm (TE) to be in direct contact with the uterine luminal epithelium (LE) (Phase 1). However, at this stage, anti-adhesive mucin 1 (MUC1) are present on the surface of the endometrium LE, limiting the contact of conceptus TE and adhesive receptors (e.g. integrins) expressed by the LE. Thus, during this period, the developing conceptus rely heavily in histotrophic nutrition. Phase 2 consists of pre-contact and blastocyst orientation. Apposition of the conceptus TE and endometrial LE begins around day 16 (Phase 3). At this stage, expression of MUC1 by the endometrium LE declines, exposing LE integrins which facilitates conceptus attachment. Attachment of conceptus TE and endometrium LE is achieved around day 20, and the microvillar interdigitation observed around day 24 (Phase 4). Several uterine secreted molecules, such as glycosylated cell adhesion molecule (GLYCAM1) 1, galectin 15 (LGALS15) and secreted phosphoprotein 1 (SPP1 or osteopontin), interact with receptors (integrins and glycoconjugates) on the apical surface

of trophoctoderm and LE to facilitate adhesion. Phase 5 involves the fusion of trophoblast giant binucleate cells (BNC) and uterine LE forming the trinucleated cells. The continued fusion of BNC and trinucleated cells results in the formation of syncytial plaques. Figure from [98].

Maternal recognition of pregnancy

The regulatory mechanisms controlling the estrus cycle and pregnancy recognition are substantially different among mammalian species. Primates for instance, have uterine independent menstrual cycle, as the luteolytic prostaglandin $F2\alpha$ ($PGF2\alpha$) is derived from intra-ovarian sources, and conceptus-derived chorionic gonadotropin (CG) plays a key role ensuring corpus luteum (CL) maintenance and sustained progesterone production [107]. Ruminants on the other hand, have uterine dependent estrus cycle, as the luteolytic pulses of $PGF2\alpha$ are rather secreted by endometrium cells, and interferon tau (IFNT) is the major conceptus secreted factor responsible for maternal recognition of pregnancy [108].

IFNT is a type 1 interferon (IFN) produced exclusively by mononuclear trophoblast cells of elongating conceptuses in ruminants, and in cattle it is secreted predominantly between days 15-24 [109-111]. IFNT prevents luteolysis by suppressing the expression of oxytocin receptor (OXTR) in the uterine luminal (LE) and superficial glandular epithelia (sGE), which abrogates the uterine luteolytic mechanism [108, 112, 113] (Figure 5). All type 1 interferons, including IFNT, signals through a common receptor (IFNAR)[114, 115], which is a heterodimeric receptor composed of IFNAR1 and IFNAR2 subunits [116]. All endometrial cells express IFNAR, but IFNT regulation of endometrial gene expression occurs in a cell type-specific manner. Expression IRF2, a potent and stable transcription repressor, increases in the endometrial LE and sGE during early pregnancy. The presence of IRF2 inhibits the expression of classical interferon stimulated genes (ISGs) in the LE/sGE, an event mediated by the direct binding of IRF2 to IFN-stimulated response elements (ISRE) and IFN response factor binding element (IRFE) that are present in the promoter region of many classical ISG, thus suppressing gene transcription. However, IRF2 is not upregulated in the endometrial stromal (ST) or deep glandular epithelium

(dGE) by pregnancy, which allows classical ISGs to be upregulated in these cell types upon IFNT signaling [117].

Differences exist in the mechanisms by which IFNT regulates luteolysis in cattle and sheep, as the bovine OXTR gene lacks a classical palindromic estrogen response element [118], and no changes in ESR1 expression are observed in uterine epithelia of pregnant and nonpregnant cows. Thus, in cattle, IFNT and pregnancy can suppress OTRX expression independently of ESR1. Nonetheless, IRF2 have also been shown to suppress OXTR expression in the bovine LE [118], indicating a common role of IRF2 mediating the antiluteolytic actions of IFNT in both, the bovine and ovine endometria [112].

IFNT signaling in the endometrium induces the expression of classical and non-classical interferon stimulated genes (ISGs) [117], which are hypothesized to play essential role establishing uterine receptivity in ruminants [98, 117, 119, 120]. The classical signaling of IFN occurs through the JAK/STAT (janus kinase/signal transduce and activator of transcription) pathway, and non-classical signaling cascades includes PI3K and p38 MAP kinase pathways [121].

A variety of IFNT genes exist in most ruminant species studied (Figure 2), and the transcriptional regulation of IFNT expression in these species appears to be somewhat conserved [122]. The conceptus expresses several transcription factors that have been implicated with the regulation of IFNT transcription. Transcription factor such as POU5F1 (also known as Oct-4) [123] and T-box protein eomesodermin (EOMES) [124] have been reported to suppress IFNT expression, while others such as ETS2 [125-127], distal-less 3 (DLX3) [128], CDX2 [124, 129] and GATA2/3 [130, 131] have been implicated with the upregulation of IFNT transcription. Additionally, a transcriptional co-activator expressed

by ovine conceptus, the cAMP-response element-binding protein-binding protein (CREBBP), have been shown to upregulate of IFNT transcription [132] (Figures 7).

Uterine derived factors are also thought to mediate conceptus expression of IFNT (Figure 7). For instance, colony stimulated factor 1 (CSF1), interleukin 3 (IL3), and fibroblast growth factor-2 (FGF2) are expressed by the endometrium LE and GE in sheep, and have been shown to induce conceptus expression of IFNT in vitro [133-135].

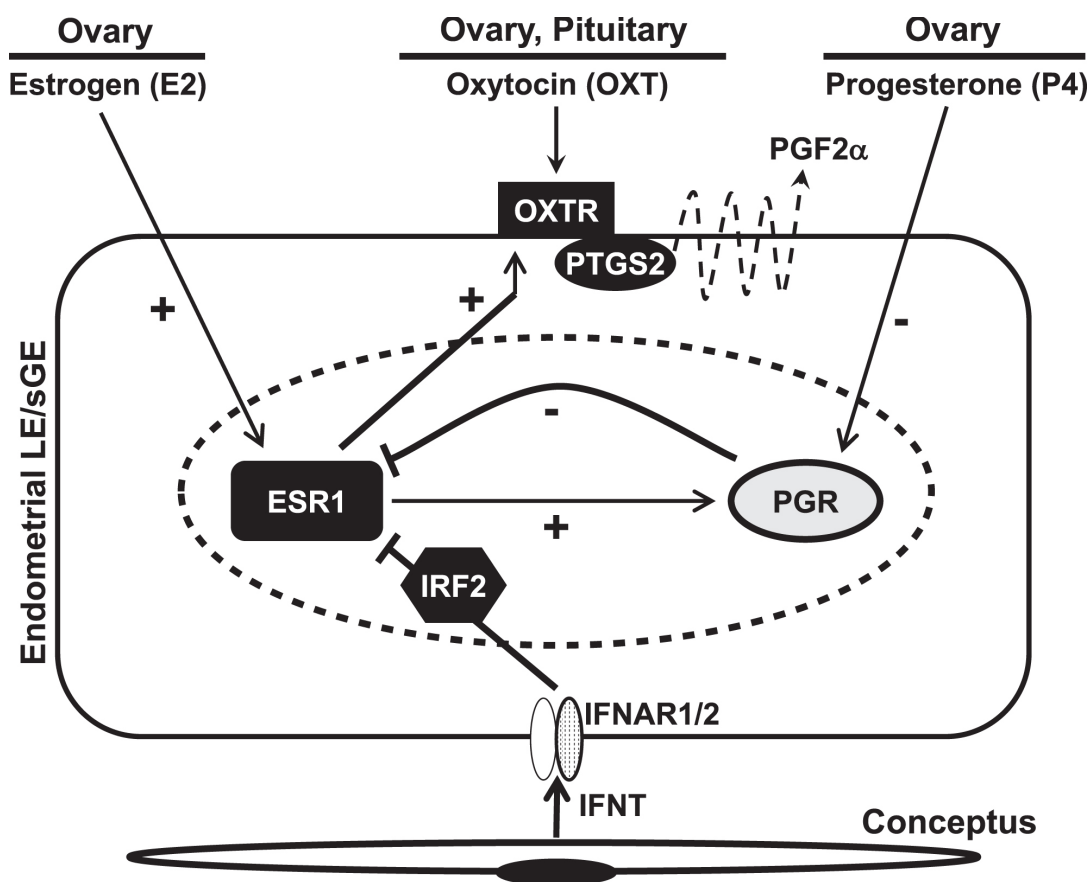


Figure 5. Regulation of the estrus cycle in pregnant and nonpregnant ewes.

During estrus and metestrus, estradiol produced by growing follicles stimulates the increase in expression of estrogen receptor alpha (ESR1) in the endometrial luminal (LE) and superficial glandular epithelium (sGE), which in turn increases the expression of progesterone receptor (PGR) and oxytocin receptor (OXTR) in these cells. Although PGR is expressed by endometrial LE/sGE during estrus, ESR1 is not downregulated by PGR signaling at this stage, as low progesterone concentrations are detected at estrus. Thus, ESR1 and OXTR are upregulated during estrus, but the luteolytic pulses of prostaglandin F2α (PGF2α) does not occur at this stage due to the absence of corpus luteum (CL) derived oxytocin. The increase in systemic progesterone in early diestrus results in downregulation of ESR1 and OXTR expression via PGR signaling, resulting in low

expression of ESR1 and OXTR during the first 8 to 10 days of the estrus cycle. Nonetheless, continuous exposure of endometrial LE/sGE cells to progesterone induces downregulation of PGR in these cell types around days 11 to 12 of the cycle. Consequently, with the absence of PGR signaling blocking ESR1 and OXTR expression, ESR1 becomes upregulated in LE/sGE cells around days 11 and 12, and upregulation of OXTR is observed on days 13 to 14. Increasing estradiol produced by growing follicles during this period further stimulates ESR1 signaling. The release of oxytocin by the CL begins around day 9. In nonpregnant ewes, oxytocin released by the CL and neurohypophysis bind to its receptor and induce the luteolytic pulses of $\text{PGF2}\alpha$, resulting in CL regression and reinitiation of the estrus cycle. In pregnant ewes, interferon tau (IFNT) is secreted by the elongating conceptus and binds to its receptor (IFNAR) expressed by all endometrial cell types. IFNAR is a heterodimeric receptor composed of IFNAR1 and IFNAR2 subunits, and its signaling induces the expression of IRF2 in the endometrial LE and sGE. IRF2 is a potent transcriptional repressor that inhibits the expression of classical interferon stimulated genes (ISGs), including ESR1. ESR1 transcriptional repression prevents OXTR upregulation and therefore inhibits the uterine luteolytic mechanism ewes. Figure from [108]

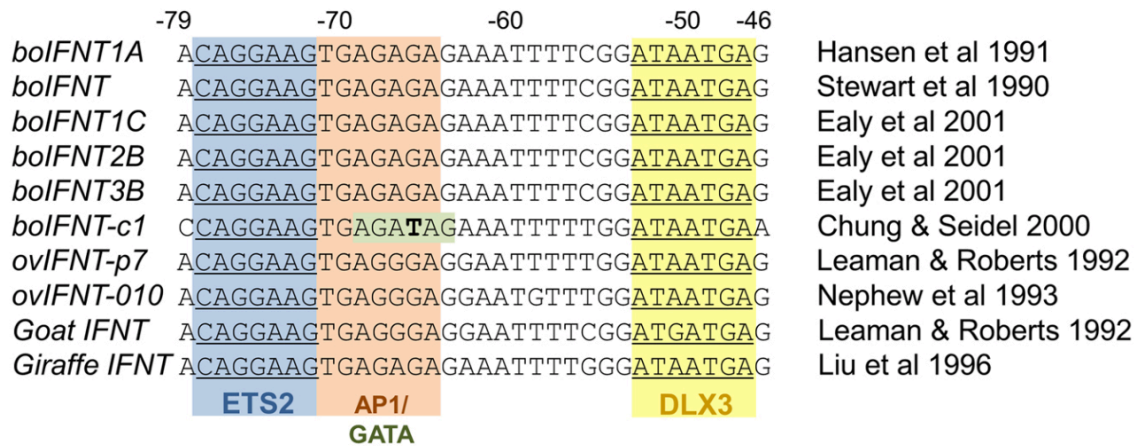


Figure 6. Conserved transcription factor binding sites on a variety of interferon tau (IFNT) genes in ruminants.

The transcriptional regulation of IFNT appears to be somewhat conserved across species, as different IFNT genes have been shown to contain a conserved ETS-2 binding site (blue), putative AP1-binding site (orange) or GATA-binding site (green), and DLX3-binding site (yellow). Figure adapted from [122]

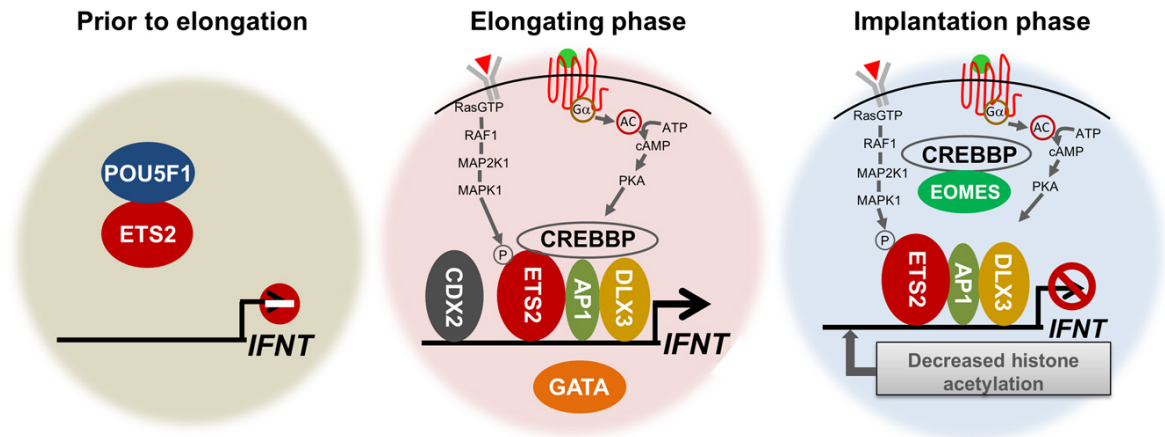


Figure 7. Suggested model of transcriptional control of interferon tau (IFNT) expression during preimplantation embryonic development.

Ezashi and Imakawa [122]. Prior to conceptus elongation, POU5F1 is thought to inhibit IFNT expression by squelching ETS2, an important IFNT transactivator. As conceptus development proceeds, the expression of POU5F1 is reduced, allowing ETS2 to increase IFNT transcription. Additionally, as the conceptus elongate, the expression of other IFNT transactivators (DLX3, CDX2, GATA2/3) increase in the trophectoderm, and growth factors released from the maternal endometrium are also thought to further stimulate IFNT transcription through the activation of MAPK and PKA signaling pathways. Conceptus expression of IFNT reduces around time of implantation, but the precise mechanisms mediating this event remains unclear. Trophectoderm expression of EOMES increases following conceptus attachment, and because EOMES has been reported to bind to CREBBP reducing CREBBP-mediated transcriptional co-activation of IFNT, it has been hypothesized to participate in the mechanism resulting in IFNT silencing during implantation. Epigenetic mechanisms are also thought to suppress IFNT transcription during the implantation phase, as reduction in histone acetylation in the upstream region of

the IFNT gene has been observed in ovine conceptuses around time of implantation (day 20) as compared to preimplantation conceptuses (days 14 and 16).

Histotroph components

Glucose

Glucose is a major energy source for mammalian cells, and its catabolism generates key substrates for protein and lipid biosynthesis [136], which are particularly important to support growth of rapidly proliferating cells such as cancer cells and preimplantation embryos [137-140].

Concentration of glucose in the uterine lumen have been reported to rise around time of conceptus elongation in sheep [139] and cattle [141], and three possible mechanisms may regulate uterine luminal concentrations of glucose in ruminants: 1) *de novo* synthesis of glucose (gluconeogenesis) by uterine cells; 2) glucose transport from blood into the uterine lumen by glucose transporters; or 3) combination of both, uterine gluconeogenesis and glucose transport.

Gluconeogenesis does not appear to occur in the uterus of mice and humans, because their uteri lacks expression of rate-limiting gluconeogenic enzymes [142, 143]. For instance, phosphoenolpyruvate carboxykinase (PEPCK, also known as PCK), which converts oxaloacetate into phosphoenolpyruvate and carbon dioxide, was not detected in mouse uterus [144], 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 [145]. However, Moore et al. [146] observed increased mRNA levels of gluconeogenic enzymes (PCK2, FBP1 and G6PC3) in bovine caruncular endometrium from days 28 to 42 of gestation, indicating that gluconeogenesis may occur in the bovine uterus during pregnancy. Although the gluconeogenic activity of the uterus remains poorly investigated in ruminants, the expression of glucose transporters by endometrial cells are thought to play important role modulating uterine luminal

concentrations of glucose [138]. Because glucose is hydrophilic, it cannot diffuse through the lipid bilayer of plasma membranes, and thus the expression of carrier proteins by uterine cells are necessary to allow glucose uptake into the uterine lumen [147, 148]. Mammalian cells express three families of glucose transporters, the facilitative glucose transporter family (solute carriers SLC2A/GLUT), the Na⁺/glucose cotransporter family (solute carriers SLC5A/ SGLT) [136], and the recently discovered SWEET (SLC50) sugar transporter [149, 150]. Member of the glucose transporter families GLUTs and SWEET are classified as uniporters, which facilitates sugar transport based on sugar gradient. Members of the SGLT (SLC5A) family are classified as symporters, as they mediate the simultaneous transport of glucose and sodium (Figure 8). Several members of GLUT and SGLT families have been identified in uterus of human, mice [151], sheep [138], and cattle [152], and the expression of selected glucose transporters (SLC2A1, SLC5A1 and SLC5A11) have been shown to increase in pregnant compared to cyclic endometrium in sheep [138].

Glucose is an essential nutrient during embryonic development, as glucose metabolism provides energy and anabolic precursors that supports cellular replication and specialization [153], and may be of particular importance during the rapid proliferation of trophectoderm cells during conceptus elongation in ruminants. Most somatic cells, in the presence of oxygen, preferentially oxidize glucose through oxidative phosphorylation, as this pathway favors ATP production [154]. Oxygen serves as electron acceptor in the electron transport chain (ETC), and therefore is required for oxidative phosphorylation [155]. Thus, under anaerobic conditions, most differentiated cells diverge pyruvate away from the TCA cycle and converts it into lactate, a reaction known as anaerobic glycolysis [154]. Otto Warburg made the important observation that cancer cells, even under aerobic

conditions, metabolize glucose preferentially into lactate, a phenomenon that became known as aerobic glycolysis or Warburg effect [156, 157]. Similar to cancer cells, embryonic cells are highly proliferative, and thus the Warburg effect has been hypothesized to occur during the growth of preimplantation embryos [140].

Oxidative phosphorylation is a more efficient pathway for generating ATP from glucose than aerobic glycolysis, as 1 molecule of glucose generates up to 36 molecules of ATP through oxidative phosphorylation but only 2 molecules of ATP via aerobic glycolysis. Thus, even though aerobic glycolysis is an inefficient way to generate ATP, it is still the preferred pathway for the metabolism of glucose in most cancer cells. 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 metabolism (Warburg effect) diverges glucose to create macromolecular precursors for synthesis of fatty acids, amino acids, and nucleotides (Figures 9 and 10) [140]. For instance, oxidation of glucose in the pentose phosphate pathway (PPP) generates nicotinamide adenine dinucleotide phosphate (NADPH) and pentoses (Figure 9), such as ribose-5-phosphate (R5P) and erythrose 4-phosphate (E4P), which are important for the synthesis of nucleotide, nucleic acids and aromatic amino acids (e.g. phenylalanine, tyrosine, tryptophan, and histidine). Additionally, de novo biosynthesis of serine and glycine, which plays important role in biomass production, occur through a glycolysis-diverting pathway, as the glycolytic intermediate 3-phosphoglycerate can be converted to serine, and serine can be converted into glycine (Figure 9).

The amino acid glutamine plays a key role in cells undergoing the Warburg effect, as glutamine metabolism provides an important carbon source for the synthesis of lactate from pyruvate [158]. 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 [159, 160]. Glutamine is also involved with energy production, and with the synthesis of nucleic acids, amino acids, and lipids (Figure 10). Additionally, glutamine participates in the biosynthesis of glutathione (GSH), a major antioxidant in mammalian cells [161] (Figure 11). Glutathione is a tripeptide consisted of glutamine-derived glutamate, glycine and cysteine. Glutamate-cysteine ligase (GCL), a heterodimer composed of a modifier (GCLM) and a catalytic (GCLC) subunit, catalyzes the ligation of glutamate and cysteine to form γ -glutamylcysteine, the first step in GSH synthesis. The second step in GSH synthesis is the coupling of γ -glutamylcysteine and glycine, a reaction catalyzed by glutathione synthetase (GSS) [162]. The antioxidant effects of GSH have been associated with the actions of glutathione peroxidase (GPX) and peroxiredoxin 6 (Prdx6), which can convert hydrogen peroxide (H_2O_2) to water (H_2O), forming glutathione disulfide (GSSG), the oxidized form of GSH [161, 163]. GSSG can be converted back to GSH, in a reaction catalyzed by glutathione reductase (GSR), through the conversion of NADPH to $NADP^+$ [163] (Figure 11). The ratio of reduced (GSH) and oxidized (GSSG) glutathione (GSH/GSSG) indicates the magnitude of oxidative stress, and while a resting cell may have a GSH/GSSG ratio that exceeds 100:1, cells experiencing oxidative stress have been shown to possess lower GSH/GSSG ratio, of 10:1 or even 1:1 [164, 165].

Glutamine metabolism is also thought to play a major role stimulating trophectoderm cell proliferation during conceptus elongation, as glutamine and glutamine derivatives synergize with other amino acids to regulate the activity of the mechanistic target of rapamycin complex 1 (mTORC1), a master regulator of cellular growth and proliferation [163, 166]. For instance, α -ketoglutarate and leucine have been shown to promote mTORC1 activation through different mechanisms (Figure 12).

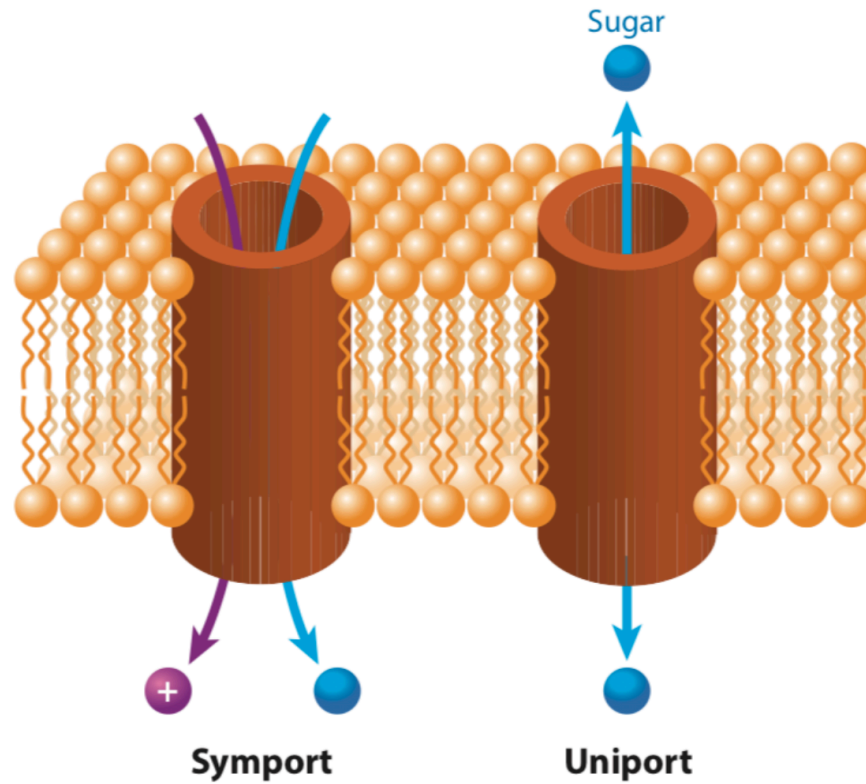


Figure 8. Transport of glucose through glucose transporters.

The glucose transporter families GLUTs (SLC2A) and SWEET (SLC50) are classified as uniporters, which facilitates sugar transport based on sugar gradient. Members of the SGLT (SLC5A) glucose transporter family are classified as symporters, as they mediate the simultaneous transport of glucose and sodium. Figure adapted from [167].

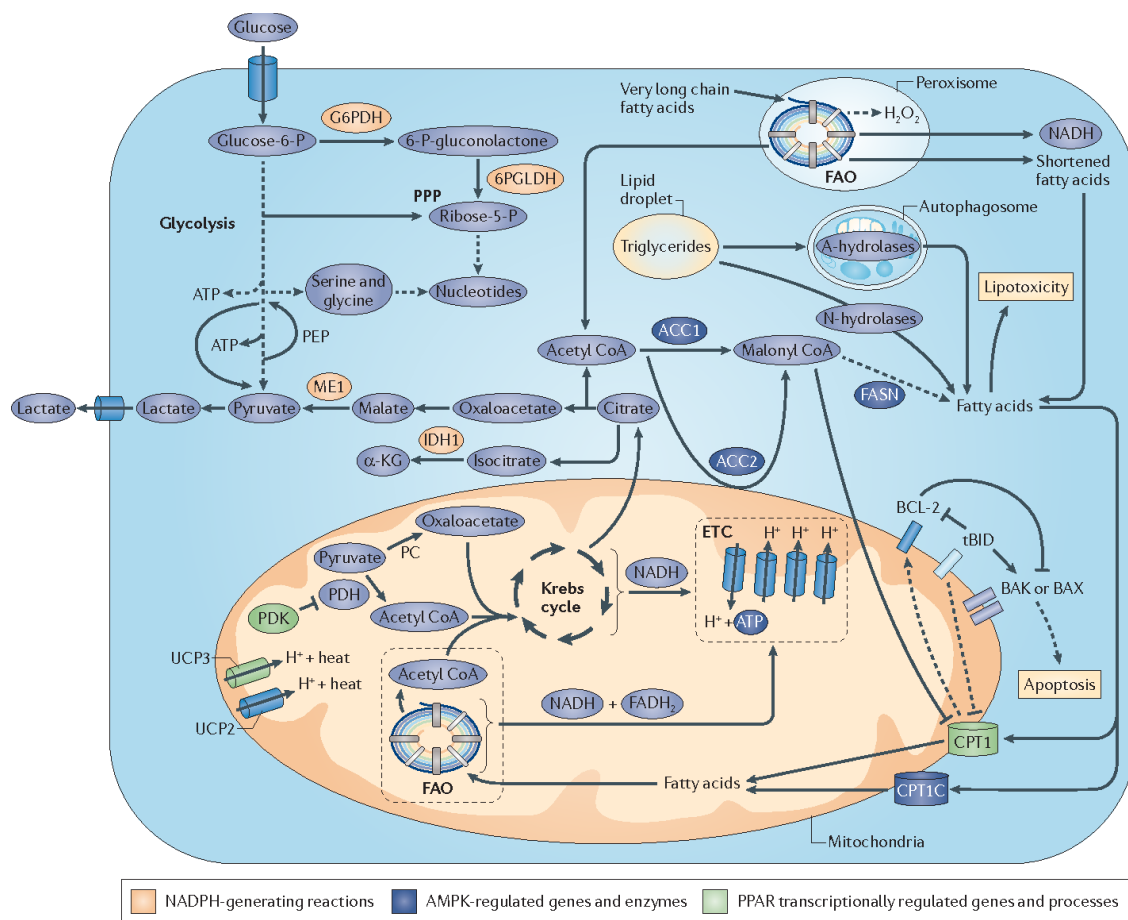


Figure 9. Glucose and fatty acid metabolism in cancer cells.

Glucose metabolism in cancer cells appears to be similar to that of in preimplantation embryos. The metabolic pathways represented include fatty acid metabolism, aerobic glycolysis, oxidative phosphorylation, serine and glycine biosynthesis, and pentose phosphate pathways (PPP). Figure from [168]

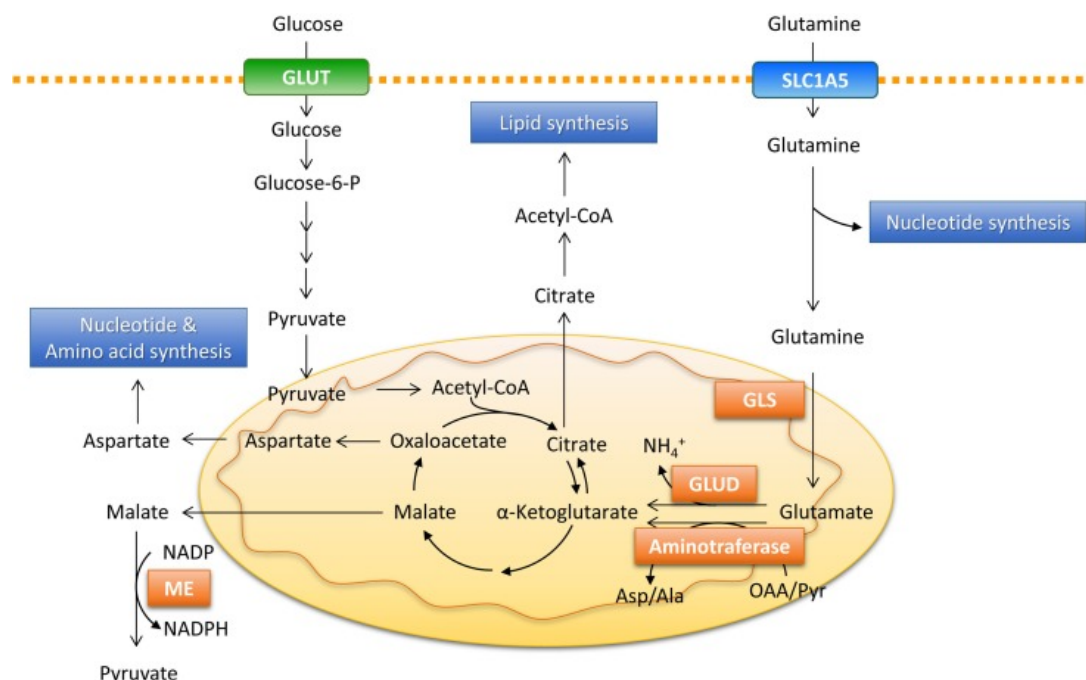


Figure 10. Major metabolic pathways involved with glutamine metabolism.

Glutamine uptake by mammalian cells occurs via a sodium-dependent neutral amino acid transporter (SLC1A5). After its uptake, glutamine can be directed for the synthesis of nucleotides, or be converted to glutamate by mitochondrial glutaminase (GLS). Aminotransferases catalyzes the redistribution of nitrogen between amino acids, and can convert glutamate to α -ketoglutarate, producing alanine and aspartate. Glutamate can also be converted in α -ketoglutarate by glutamate dehydrogenase (GLUD), and α -ketoglutarate enters the TCA cycle to generate energy for the cell. In the TCA cycle, α -ketoglutarate can be converted back into citrate, which can be exported from the mitochondria to the cytoplasm to be metabolized in acetyl-CoA and oxaloacetate, and acetyl-CoA is a key precursor for lipid synthesis. In the TCA cycle, α -ketoglutarate is converted to malate, which can exit the mitochondria and produce pyruvate and NADPH through the actions of the malic enzyme (ME). Malate in the TCA cycle is converted to oxaloacetate, which can be converted to aspartate for supporting nucleotide and amino acid synthesis.

Figure from [163]

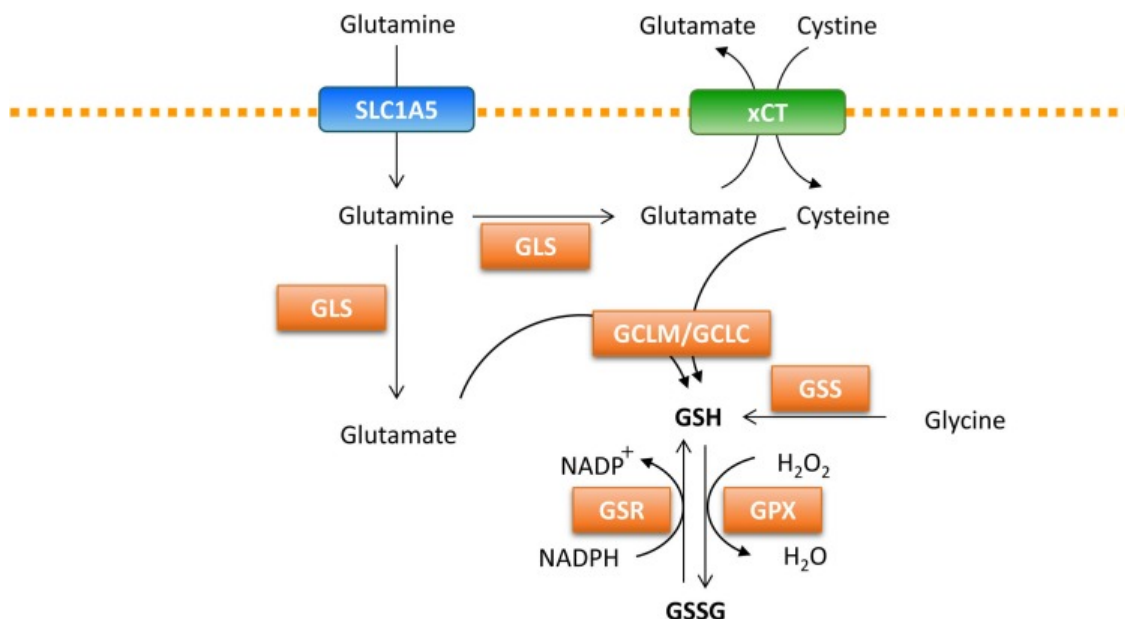


Figure 11. Glutamine metabolism and regulation of oxidative stress.

The conversion of glutamine to glutamate is catalyzed by glutaminase (GLS). Glutathione (GSH), a major antioxidant in mammalian cells, is a tripeptide composed of glutamate, glycine and cysteine. The first step in GSH synthesis is the ligation of glutamate and cysteine to form γ -glutamylcysteine, a process catalyzed by glutamate-cysteine ligase (GCL), which is a heterodimer composed of a modifier (GCLM) and a catalytic (GCLC) subunit. The second step in GSH synthesis is the coupling of γ -glutamylcysteine and glycine, which is mediated by glutathione synthetase (GSS). Glutathione peroxidase (GPX) converts hydrogen peroxide (H₂O₂) to water (H₂O), forming glutathione disulfide (GSSG), the oxidized form of GSH. GSSG can be converted back to GSH, through the conversion of NADPH to NADP⁺, in a reaction catalyzed by glutathione reductase (GSR). Figure from [163]

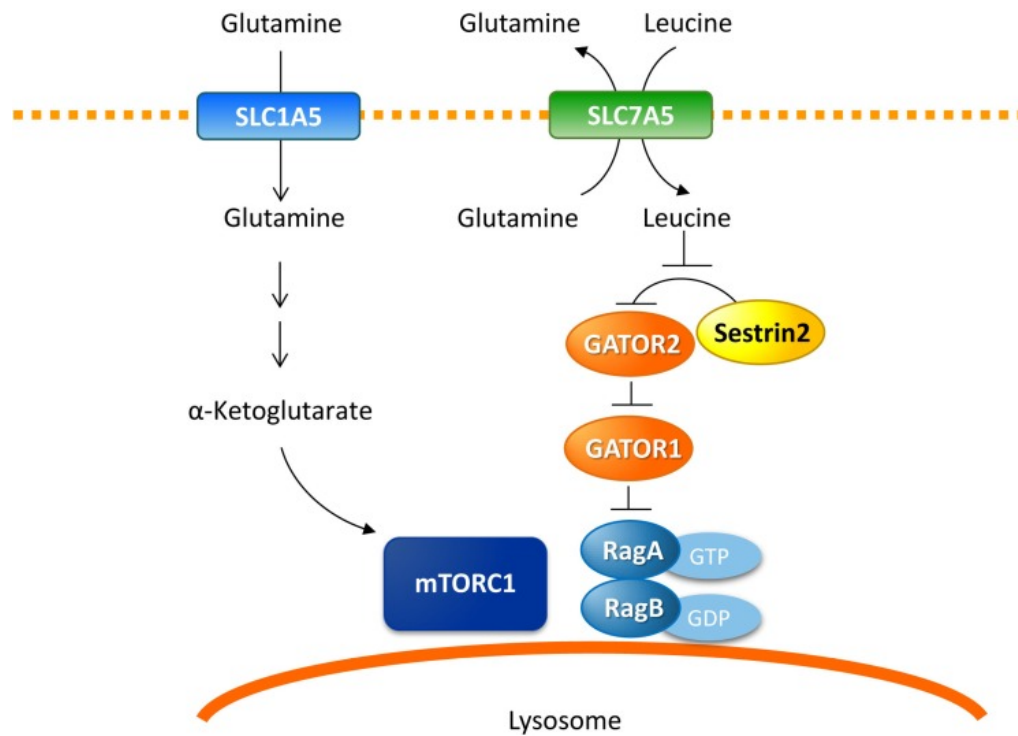


Figure 12. Activation of the mechanistic target of rapamycin complex 1 (mTORC1) by glutamine and leucine.

The active form of mTORC1 is localized in the surface of lysosomes, and α -ketoglutarate stimulates the recruitment of mTORC1 to the lysosome. Additionally, the amino acid transporter SLC7A5 mediates leucine efflux in exchange with glutamine, and leucine has been shown to disrupt the binding between Sestrin2 and GATOR2, an inhibitor of mTORC1, thus promoting mTORC1 activation. Figure from [163]

Lipids

Lipids in histotroph are hypothesized to play key roles in supporting embryonic development, because they serve as energy source and storage, provide substrates for membrane biogenesis, and act as signaling molecules [169].

The availability of lipids in uterine cells fluctuates throughout the estrus cycle in several species [170]. In sheep, lipid droplets in the surface of endometrial LE have been reported to be high during estrus, decreases during early diestrus (days 1-6), and as the corpus luteum develops and progesterone concentrations increases, increasing abundance of lipids are detected on the surface LE cells [171] (Figures 13 A). Similarly, concentrations of neutral lipids in the cytoplasm of endometrial epithelial cells increases substantially after day 8 of the estrus cycle in sheep (Figure 13 B). Thus, the available evidence suggests that onset of conceptus elongation (day 12) occurs when concentrations of lipids are increased in the endometrium epithelium and uterine lumen. Additionally, lipid droplets are reduced in the endometrium of pregnant than cyclic ewes between days 14 and 16 (Figure 13 B), suggesting that the elongating conceptus is utilizing the LE uterine lipids to support its growth [172, 173].

Lipids likely participate in the crosstalk between the conceptus and endometrium during early pregnancy, as a diverse class of lipids can serve as ligands of peroxisome proliferator-activated receptors (PPARs) [172, 174-178]. Both the bovine conceptus and endometrium express PPARs [94, 179]. 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 [175] (Figure 14). PPAR/RXR are involved in the transcriptional regulation of lipid uptake [180], fatty acid oxidation and metabolism [181], glucose homeostasis [182], and cell proliferation and

differentiation [183], which are particularly important biological processes during conceptus elongation. Of note, PPARG expression increases during conceptus elongation in cattle, and its expression is correlated with the expression several genes involved in lipid metabolism [94].

Fatty acids are common components of complex lipids, and serve as major energy source during embryonic development [184]. In eukaryotes, fatty acid catabolism (β -oxidation) occurs in the mitochondria, and generates acetyl-coA, NADH and FADH₂, which are used in the TCA cycle and electron transport chain (ETC) for the synthesis of ATP (Figure 15) [185]. Additionally, fatty acids are precursors of important lipid mediators with critical function during early pregnancy, such as classical eicosanoids (prostaglandins, thromboxane, and leukotrienes) and lysophospholipids [120, 186]. Fatty acid biosynthesis and catabolism in mammalian cells involves dynamic processes in which the number of carbons and double bonds change under the influence of elongation, desaturation, and β -oxidation reactions. Fatty acid elongases (ELOVL) regulate fatty acid length by catalyzing carbon chain extensions [187], and desaturases controls the degree of fatty acid unsaturation, as it catalyzes the insertion of double bonds into the fatty acid chain [188] (Figure 16).

The transport of lipids from the endometrium to the uterine lumen are thought to be mediated by distinct mechanisms involving carrier proteins, lipoproteins, and extracellular vesicles (EVs) [189] (Figure 17). Albumin is the main fatty acid binding protein in extracellular fluids [190], and nearly 100% of the fatty acid detected in plasma are bound to albumin [191]. Albumin is also the most abundant protein in the uterine luminal fluid of cows [81] and sheep [192], and therefore possibly a major carrier of fatty acids to the uterine lumen.

Available evidence suggests that lipids are also carried to the uterine lumen by lipoproteins (e.g. chylomicrons, very low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), high density lipoprotein (HDL)) [193]. Argraves and Morales [194] suggested that high-density lipoprotein (HDL) is synthesized by the endometrium GE in rats, as their uterine glands express apoproteins J and A1, which are important components for HDL synthesis. Additionally, Koch et al. [195] observed that apoprotein A1 was more abundant in uterine fluid of pregnant than open ewes on day 16 of the estrus cycle/pregnancy. Importantly, the elongating bovine conceptus expresses lipoprotein lipase (LPL) [179], and therefore is able to hydrolyze lipoproteins for fatty acids uptake [196].

The mechanism of fatty acid uptake involves membrane-bound carrier proteins, such as membrane-associated fatty acid binding protein (FABPa), and scavenger receptors (SRs, CD36), as well as cytoplasmic FABPs, which receives the fatty acid from the membrane bound transporters and facilitates their transfer between extra- and intracellular membranes [197]. Expression of FABP3 in endometrial epithelial cells has been reported to decrease from days 13 to 19 of the estrus cycle but not during pregnancy in heifers [198], suggesting that the rate of fatty acid transport remains high during the pre- and peri-implantation period of pregnancy.

Extracellular vesicles, which includes microvesicles and exosomes, are also thought to be an important lipid source during early pregnancy in ruminants [189]. EVs are lipid bilayer-enclosed nano-sized particles that are present in all body fluids, and can carry a large number of active molecules, such as lipids, proteins, and nucleic acids [199, 200]. The ovine conceptus and endometrium secrete EVs during early pregnancy, and the conceptus is able to incorporate uterine-derived EVs [201]. Thus, regardless of the cargo,

the lipid bilayer of uterine-derived EVs by itself constitute an important source of complex lipids for the elongating conceptus, as the cellular membrane of EVs are enriched in cholesterol, sphingolipids, glycerophospholipids and ceramides [202].

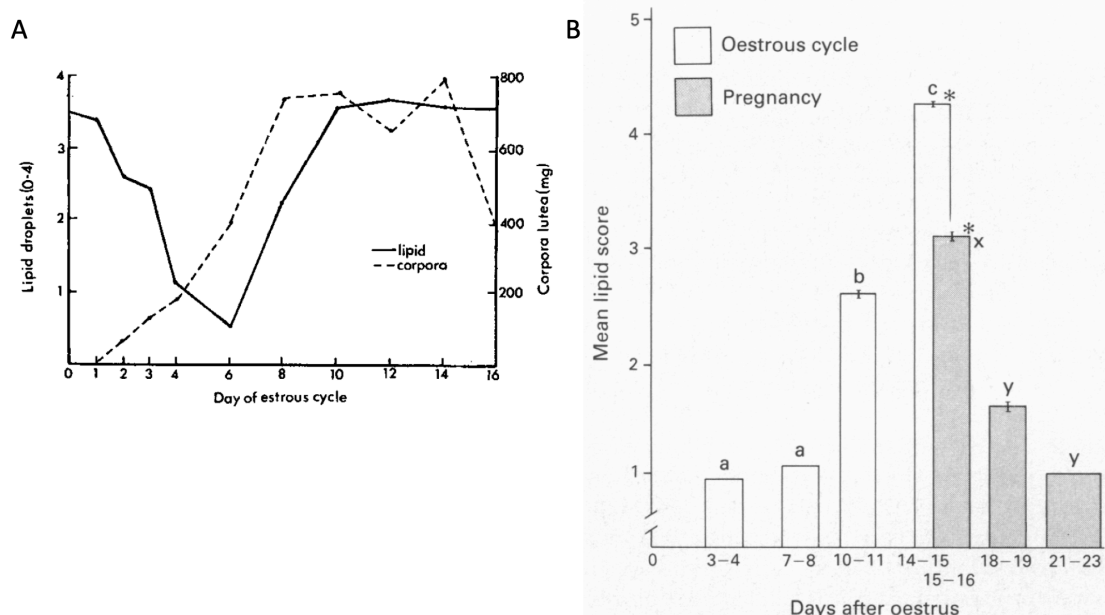


Figure 13. Lipid accumulation in the uterine lumen and within endometrial epithelial cells.

(A) Relationship between the abundance of lipid droplets (solid line) in the surface of uterine luminal epithelial (LE) cells and corpus luteum (CL) weight (dashed line) during the estrus cycle in sheep. The increase in CL volume is associated with increased concentrations of lipid droplets in the surface of uterine LE cells. Figure from [171]. (B) Mean lipid score in endometrial LE of cyclic and pregnant sheep. Concentrations of intra-epithelial lipids are reduced in pregnant than cyclic endometrium between days 14 and 16, and endometrial lipids decreases as pregnancy advances, suggesting that the conceptus is utilizing uterine lipids to support its growth. Figure from [173].

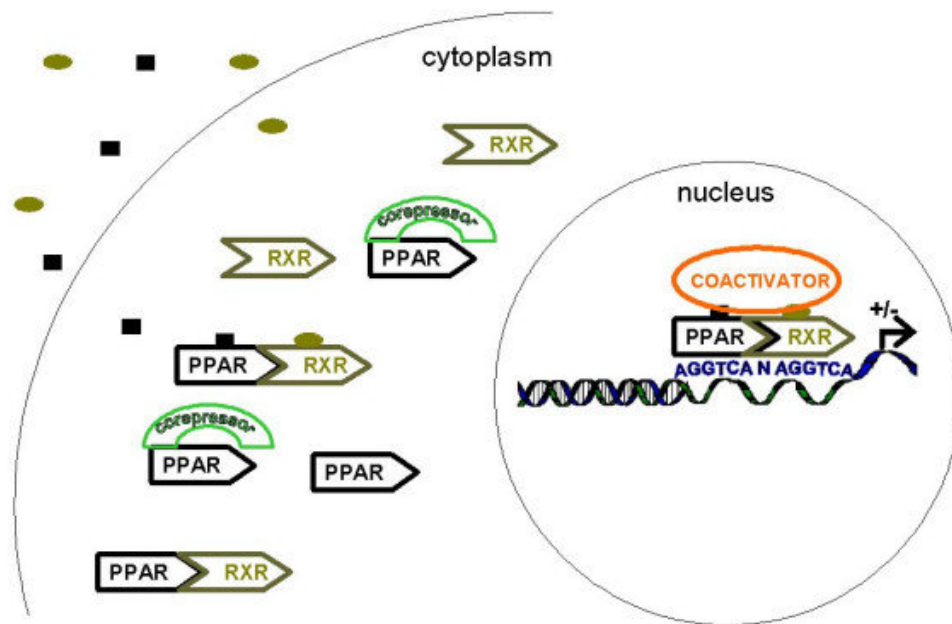


Figure 14. Mechanism of transcriptional activation of peroxisome proliferator-activated receptors (PPARs).

PPARs can heterodimerize with retinoid X receptor (RXR) independently of ligand binding. Upon ligand binding, PPARs undergo conformational change dissociating from corepressors and recruiting co-activators. A diverse class of lipids can serve as ligands of PPARs. PPAR signaling is mediated through binding of PPAR/RXR heterodimers to PPAR response elements (PPRE) present in the promoter region of PPAR responsive genes, activating transcription. Cellular processes regulated by PPAR signaling include lipid metabolism, glucose homeostasis, and cell proliferation and differentiation. Figure from [203].

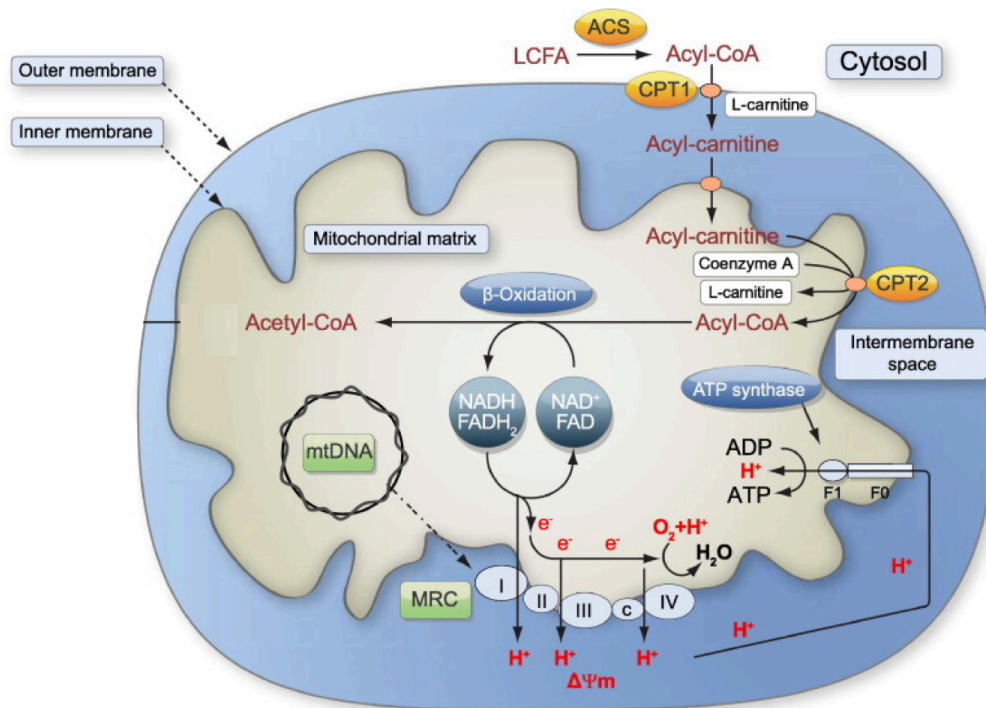


Figure 15. Mitochondrial fatty acid β-oxidation.

Long-chain fatty acids (LCFA; C11-C20) cannot diffuse through the mitochondrial membranes, thus a specific transport system is required for β-oxidation. First, LCFA are bound to coenzyme A by long-chain acyl-CoA synthetases (ACS) located in the outer mitochondrial membrane. Then, the enzyme carnitine palmitoyltransferase-1 (CPT 1) catalyzes the binding of long chain acyl-CoA with L-carnitine, forming acyl-carnitine, which can cross the mitochondrial inner membrane in a process mediated by carnitine-acylcarnitine translocase. Finally, carnitine palmitoyltransferase-2 (CPT 2), an enzyme located on the matrix side of the inner mitochondrial membrane, breaks down acyl-carnitine forming Acyl-CoA, coenzyme A and L-carnitine. Acyl-CoA is then oxidized into Acetyl-CoA, in a process that generates NADH and FADH₂, which are used in the electron transport chain (ETC) for the synthesis of ATP. Acetyl-CoA can also be directed to the TCA cycle for energy production. Figure adapted [185]

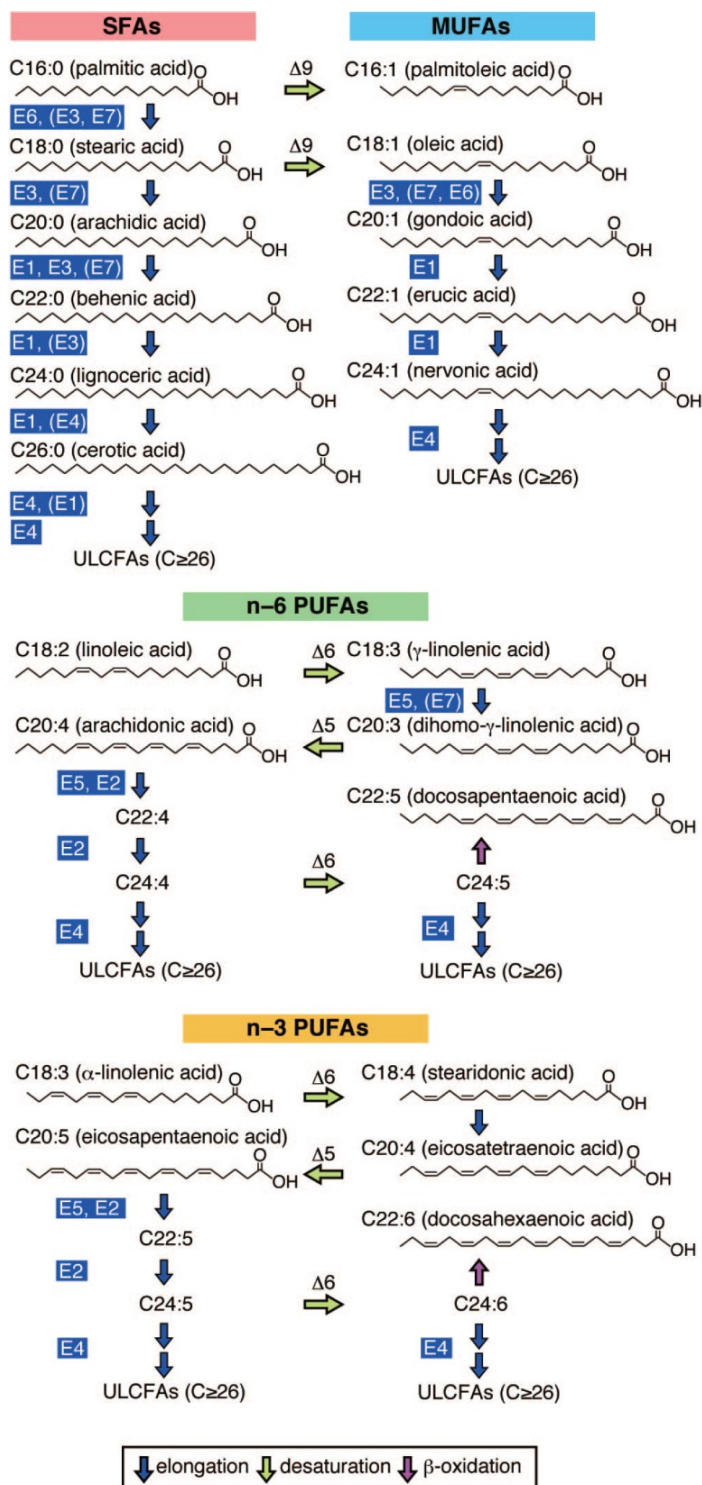


Figure 16. Fatty acid elongation and desaturation.

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 β -

oxidation reactions. ELOVL isozymes (E1-E7), are enzymes responsible for the elongation step, catalyzing carbon chain extension. Desaturases ($\Delta 5$, $\Delta 6$ and $\Delta 9$) are enzymes that catalyzes the removal of 2 hydrogen atoms from fatty acids, creating carbon/carbon double bonds. Fatty acids are shortened by two carbons each round of β -oxidation, as they are released in the form of acetyl CoA. FA: fatty acid; SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA. Figure from [188]

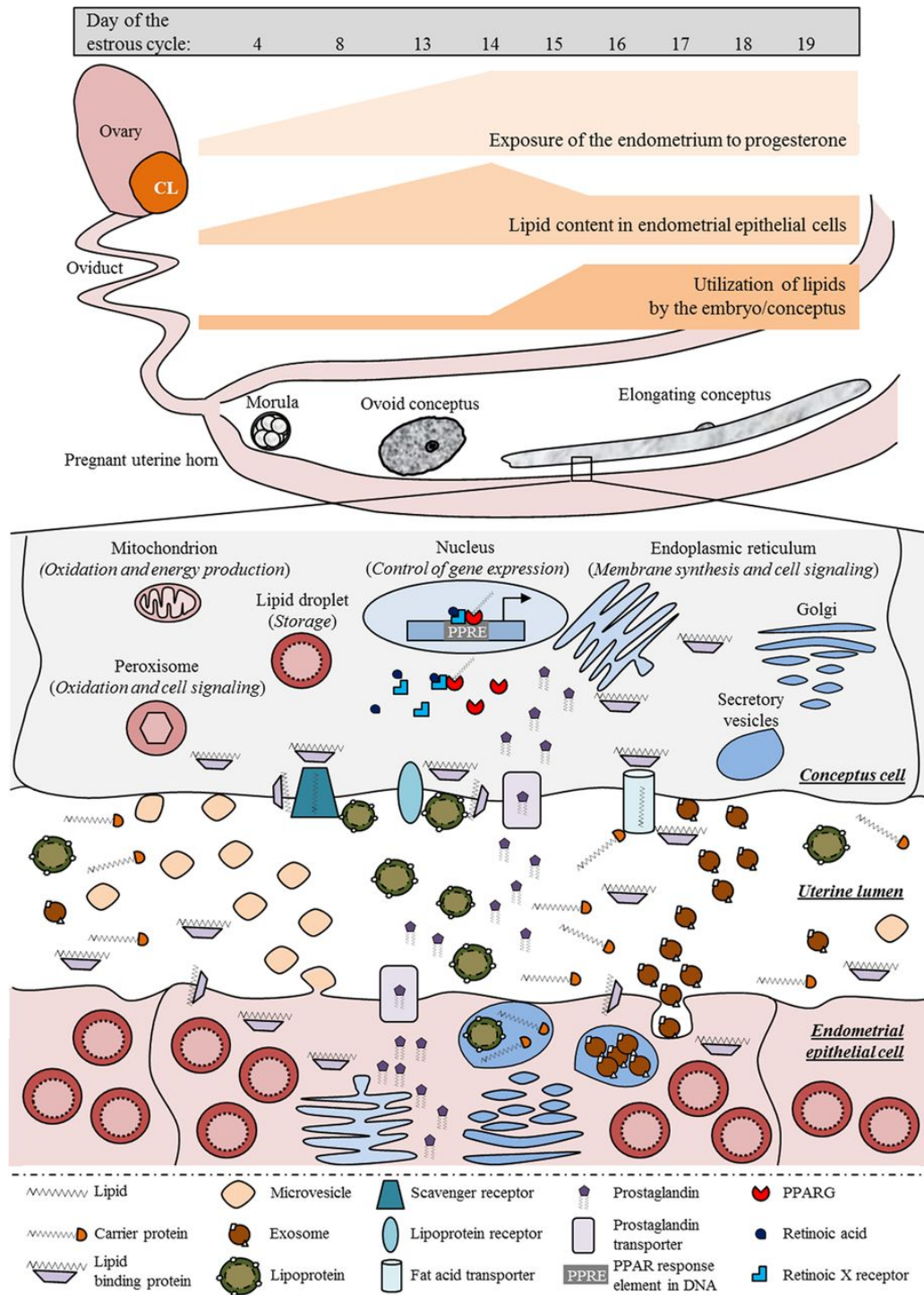


Figure 17. Proposed model for lipid metabolism during early pregnancy in cattle.

Ribeiro et al. [189]. Ovarian progesterone induces lipid accumulation in epithelial cells, which serve as lipid source to support conceptus development during early pregnancy. Endometrial lipids are transported to the uterine lumen by exosomes, microvesicles,

lipoproteins and carrier proteins. The conceptus utilizes endometrial lipids as energy source, for membrane biogenesis, and for cell signaling. Conceptus PPARG activation by uterine derived lipids is essential to regulate conceptus gene expression to support elongation.

Prostaglandins

Prostaglandins (PGs) are the most well-studied class of lipid mediators, and the ruminant uterus and conceptus secrete a variety of PGs [120, 204, 205]. The first step of PG synthesis (Figure 18) is the release of arachidonic acid from membrane phospholipids, a process mediated by phospholipase A2 (PLA2). Arachidonic acid can then be converted into PGG2 by prostaglandin synthases I (PTGS1/COX1) or II (PTGS2/COX2). PGG2 exits the active site of PTGS1/2 and undergoes a two-electron reduction, forming PGH2, which can be metabolized into the five primary prostanoids (PGD2, PGF2 α , PGI2, thromboxane A2 (TXA2), and PGE2) through the action of specific PG synthases. Prostaglandin signaling is mediated by membrane-bound G protein-coupled receptors as well as nuclear receptors [206-208]. Membrane bound receptors includes PGD2 receptor (DP, or PTGDR), PGF2 α receptor (FP or PTGFR), PGI2 receptor (IP or PTGIR), TXA2 receptor (TP or TBXA2R), and 4 subtypes of PGE2 receptors (EP1/PTGER1, EP2/PTGER2, EP3/PTGER3, and EP4/ PTGER4) [209-211]. Different types of PGs act through their specific receptor(s), and therefore regulates distinct biological processes [212, 213]. The nuclear receptors involved with PG signaling includes PPARD and PPARG, as PGI2 can signals through PPARD [214], and 15-deoxy- $\Delta^{12,14}$ -PGJ2, a metabolite of PGD2, can activate PPARG [215, 216].

Prostaglandin synthase 2 is the major PG synthase expressed by both, the conceptus and endometrium during early pregnancy in sheep. Charpigny et al. [205] demonstrated that PTGS2 was highly abundant in day 8 to 17 blastocysts/conceptuses, whereas PTGS1 was undetectable during this period. Loss of PGR in the endometrial LE/sGE around day 10 of the estrus cycle in sheep is associated with changes in endometrial gene expression required for successful implantation, and PTGS2 becomes upregulated in the endometrial

LE/sGE during this period. Interferon tau secreted by the elongating conceptus further stimulates endometrial expression of PTGS2 [120, 205]. Thus, concentrations of PGs in the uterine lumen rises during early pregnancy in ruminants, as the endometrium and conceptus PG synthesis is upregulated during this period [138, 204].

Prostaglandin signaling in the endometrium is essential to establish uterine receptivity (Figure 19). In fact, PGs and IFNT stimulates a number of progesterone stimulated genes in the endometrial LE/sGE during early pregnancy, including glucose (SLC2A1, SLC2A5, SLC5A11) and amino acid (SLC7A2) transporters, secreted proteins (CST3, CTSL1, GRP, IGFBP1, LGALS15), and intracellular enzymes (HSD11B1) [217].

Intrauterine infusion of meloxicam, a selective inhibitor PTGS2, completely inhibits conceptus elongation in sheep [218], and endometrial expression of HSD11B1 and IGFBP1 is particularly reduced by meloxicam treatment [218, 219]. The enzyme hydroxysteroid 11- β dehydrogenase 1 (HSD11B1), also known as cortisone reductase, catalyzes the conversion of inactive cortisone to active cortisol, which signals through the glucocorticoid receptor (GR) to stimulate a number of elongation and implantation-related genes [220, 221]. Cortisol also has immunosuppressive effects, which are thought to be important for maintaining the immunologic homeostasis required for the survival of the semiallogeneic conceptus/fetus during pregnancy [222]. IGF binding protein 1 (IGFBP1), another important gene upregulated by PGs in the endometrium, mediates IGF1 activity [223], and IGF1 is a major regulator of embryonic growth and development [224]. Interestingly, IGFBP1 also acts independently of IGF1 stimulating trophoblast migration and attachment in the human placenta [225]. Thus, the downstream effects of PGs in the endometrium are clearly important for successful attachment and implantation mammals.

Angiogenesis and vascular permeability are among the essential processes regulated by PGs during early pregnancy. For instance, PGF2 α has been reported to increase the expression of vascular endothelial growth factor A (VEGFA) in the endometrial LE [226], and VEGFA is one of the most important proangiogenic factors, inducing vascular permeability, angiogenesis, and endothelial cell growth [227]. Additionally, PGE2 signaling through EP4 can stimulate angiogenesis [228, 229], and PGI2 signaling can increase vascular permeability by inducing vascular smooth muscle relaxation [230].

Brooks et al [120] proposed a model for the regulation of early pregnancy events in sheep by PGs and IFNT (Figure 19). During conceptus elongation, conceptus-secreted PGs may act in an autocrine manner through nuclear receptors (PPAR α , PPAR γ) to increase the transcription of genes involved with cell proliferation and metabolism. Additionally, conceptus-secreted PGs may exit the TE through prostaglandin transporters (PGT and MRP4) to regulate endometrial expression of elongation and implantation related genes. Prostaglandins produced by endometrial epithelial cells may also act in an autocrine manner mediating the expression of genes to support conceptus elongation and implantation, as well as in a paracrine manner controlling vascularization and immunomodulation in the stroma and deep GE [120, 231] (Figure 19). Endometrial-derived PGs may also activate conceptus-expressed membrane bound and nuclear receptors to further support conceptus development.

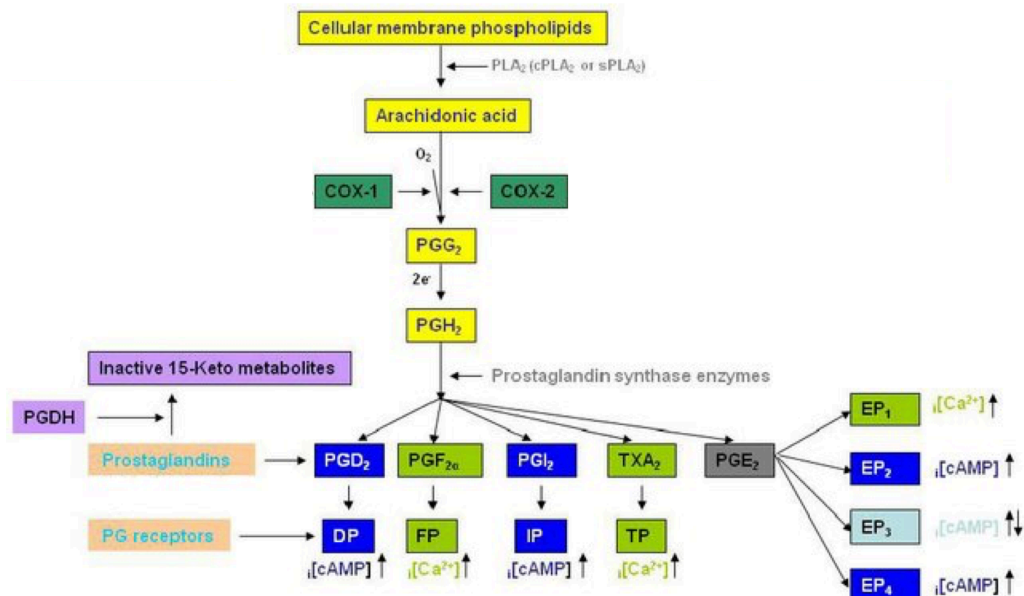


Figure 18. Prostaglandin synthesis from membrane phospholipids.

The first step of PG synthesis is the release of arachidonic acid from membrane phospholipids, a process mediated by phospholipase A2 (PLA2). Arachidonic acid can then sequentially be transformed into PGG2 by prostaglandin synthases I (PTGS1/COX1) and II (PTGS2/COX2). PGG2 then exits the active site of PTGS1/2 and undergoes a two-electron reduction, forming PGH2, which can be metabolized into the five primary prostanoids (PGD2, PGF2α, PGI2, thromboxane A2 (TXA2), and PGE2) through actions of specific PG synthases. The classical signaling of PGs is mediated by membrane-bound G protein-coupled receptors as well as nuclear receptors. Membrane bound receptors includes PGD2 receptor (DP, or PTGDR), PGF2α receptor (FP or PTGFR), PGI2 receptor (IP or PTGIR), TXA2 receptor (TP or TBXA2R), and 4 subtypes of PGE2 receptors (EP1/PTGER1, EP2/PTGER2, EP3/PTGER3, and EP4/PTGER4). The second messengers regulating PG signaling for each specific receptor are presented. The enzyme 15-hydroxyprostaglandin dehydrogenase (PGDH) catalyzes the first and rate-limiting step in the inactivation and degradation of PGs. Figure from [211]

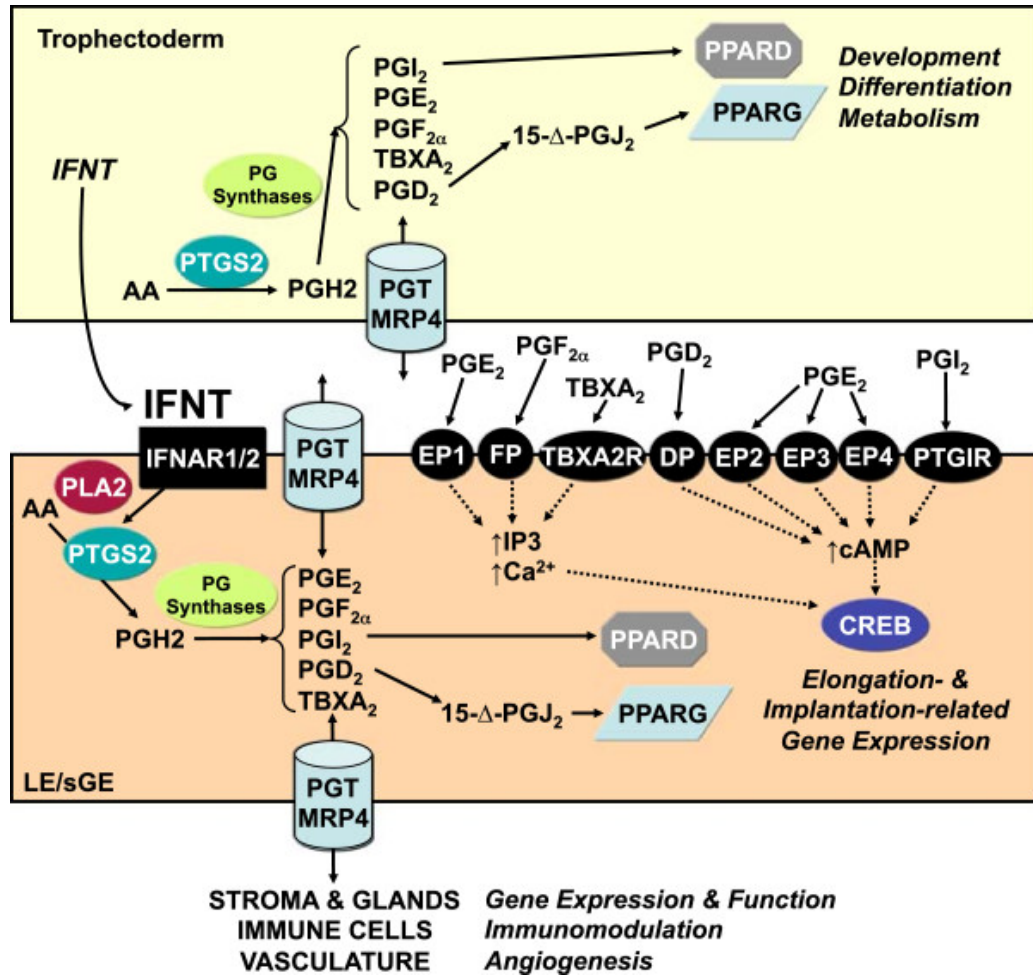


Figure 19. Proposed model for prostaglandin (PG) and interferon tau (IFNT) regulation of uterine function and conceptus elongation during early pregnancy in sheep.

Brooks et al [120]. Conceptus-secreted PGs may act in an autocrine manner through nuclear receptors (PPARD, PPARG) increasing the transcription of genes involved with cellular proliferation and metabolism. The expression of prostaglandin transporters (PGT and MRP4) by the conceptus facilitates the transport of conceptus-derived PGs into the uterine lumen. The endometrium LE/sGE express several classes of proteinoid receptors, and their signaling mediates the expression of elongation and implantation related genes. IFNT secreted by the elongating conceptus further increase the expression of prostaglandin

synthase 2 (PTGS2) in the endometrial LE/sGE, increasing the overall synthesis of prostanoids, and consequently their signaling. PGs also signals through nuclear receptors, as PGI₂ can activate PPAR delta (PPARD) [214], and 15-deoxy- $\Delta^{12,14}$ -PGJ₂, a metabolite of PGD₂, can activate PPAR gamma (PPARG). Additionally, prostanoids are transported from endometrial LE/sGE into the stroma and deep GE, where they regulate important biological processes during early pregnancy such as angiogenesis, immunity. Legend: ABCC4, ATP-binding cassette, sub-family C (CFTR/MRP), member 4; CREB, cAMP responsive element binding protein; IFNAR, interferon (alpha, beta and omega) receptor; DP, prostaglandin D receptor (PTGDR); EP, prostaglandin E receptor (PTGER); FP, prostaglandin F receptor (PTGFR); IP, prostaglandin I receptor (PTGIR); PLA₂, phospholipase A₂; PPARD, peroxisome proliferator-activated receptor delta; PPARG, peroxisome proliferator-activated receptor gamma; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); PG Synthases, prostaglandin synthases (AKR1C3, PTGDS, PTGES, PTGFS, PTGIS, TBXAS); ; SLCO2A1 (also known as PGT), solute carrier organic anion transporter family, member 2A1 (prostaglandin transporter); TBXA₂R, thromboxane A₂ receptor.

SECTION II:

Identification of Beef Heifers with Superior Uterine Capacity for Pregnancy

Thomas W. Geary, Gregory W. Burns, Joao G.N. Moraes, James I. Moss, Anna C. Denicol, Kyle B. Dobbs, M. Sofia Ortega, Peter J. Hansen, Michael E. Wehrman, Holly Neibergs, Eleanore O'Neil, Susanta Behura, and Thomas E. Spencer

This work has been published in:

Biology of Reproduction, Volume 95, Issue 2, 1 August 2016, 47, 1-12. doi:
10.1095/biolreprod.116.141390

ABSTRACT

Infertility and subfertility represent major problems in domestic animals and humans, and the majority of embryonic loss occurs during the first month of gestation that involves pregnancy recognition and conceptus implantation. The critical genes and physiological pathways in the endometrium that mediate pregnancy establishment and success are not well understood. In Study One, predominantly Angus heifers were classified based on fertility using serial embryo transfer (ET) to select animals with intrinsic differences in pregnancy loss. In each of the four rounds, a single in vitro-produced high-quality embryo was transferred into heifers on day 7 post-estrus and pregnancy was determined on days 28 and 42 by ultrasound and then terminated. Heifers were classified based on pregnancy success as high fertile (HF), subfertile (SF), or infertile (IF). In Study Two, fertility-classified heifers were resynchronized and bred with semen from a single high fertility bull. Blood samples were collected every other day from days 0 through 36 post-mating. Pregnancy rate was determined on day 28 by ultrasound, and was higher in HF (70.4%) than in heifers with low fertility (36.8%; SF and IF). Progesterone concentrations in serum during the first 20 days post-estrus were not different in non-pregnant heifers and also not different in pregnant heifers among fertility groups. In Study Three, a single in vivo-produced embryo was transferred into fertility-classified heifers on day 7 post-estrus. The uteri were flushed on day 14 to recover embryos, and endometrial biopsies were obtained from the ipsilateral uterine horn. Embryo recovery rate and conceptus length and area were not different among the heifer groups. RNA was sequenced from the day 14 endometrial biopsies of pregnant HF, SF and IF heifers (n=5 per group) and analyzed by edgeR robust analysis. There were 26 differentially expressed genes (DEG) in the HF compared to SF endometrium, 12 DEG for SF compared to IF endometrium, and 3 DEG between the HF

and IF endometrium. Many of the DEG encoded proteins involved in immune responses and are expressed in B cells. Results indicate that pre-implantation conceptus survival and growth to day 14 is not compromised in SF and IF heifers. Thus, the observed difference in capacity for pregnancy success in these fertility-classified heifers is manifest between days 14 and 28 when pregnancy recognition signaling and conceptus elongation and implantation must occur for the establishment of pregnancy.

INTRODUCTION

Infertility and subfertility are important and pervasive problems in agricultural animals and humans. In ruminants, embryo mortality is a major factor affecting fertility and thus production and economic efficiency[232-235]. There is a high degree of pregnancy loss in cattle between fertilization and term, ranging from 40% to 56%[235, 236]. The majority of embryonic loss (70-80%) occurs in the first 3 weeks of pregnancy in cattle[235-239]. Embryo mortality is greater in non-lactating cows than heifers[237], and early pregnancy loss is even greater in lactating dairy cattle and can approach 40% [232, 240, 241]. Infertility and subfertility also impact the embryo transfer (ET) industry[242]. Mean survival rate to calving following transfer of in vivo-derived embryos from superovulated donors ranges from 31% to 60%[243], whereas in vitro-produced embryo survival rate is lower and ranges from 30% to 40%[234, 243]. Failure of the embryo to survive and establish pregnancy is due to both embryonic and maternal factors[244]. Many of the pregnancy losses observed in natural or assisted pregnancies can be attributed to inadequate uterine receptivity, which can be defined as the physiological state of the uterus when conceptus growth and implantation for establishment of pregnancy is possible[245]. Understanding how embryo development is controlled is critical for determining ways to reduce the high rates of early embryonic mortality.

After fertilization (day 0), the zona pellucida-enclosed bovine embryo enters the uterus at the morula stage on days 4-5 of gestation and develops into a blastocyst. The spherical blastocyst hatches from the zona pellucida on days 7 to 10 and continues to grow, changing from spherical to ovoid in shape between days 12 and 14 during a transitory phase preceding elongation, after which it is termed a conceptus (embryo and associated extra-embryonic membranes)[246]. The conceptus grows from about 2 mm in length on day 13

of gestation to 6 mm on day 14, and reaches 60 mm by day 16 to 20 cm or more by day 19[247]. After days 16-17, the time of maternal recognition of pregnancy in cattle, the elongating conceptus begins the processes of implantation and placentation[248]. In both cattle and sheep, blastocyst growth into a conceptus and subsequent elongation has not been achieved in vitro and requires transfer into the uterus[249]. Progesterone action via the endometrium of the uterus is critical for conceptus growth and elongation in sheep and cattle[250-252]. Dynamic changes in endometrial gene expression occur between days 7 and 13 that are regulated by progesterone in nonpregnant and pregnant cattle and are associated with the onset of conceptus elongation[251-254]. A prevailing theory is that the gene expression changes modify the intrauterine milieu for support of the survival and growth of the blastocyst into an ovoid conceptus and then elongated filamentous conceptus. Uterine secretions in the lumen are not well defined in cattle, but are a complex mixture of proteins, amino acids, sugars, lipids and ions derived from genes expressed in the endometrium as well as selective transport of components (amino acids, glucose, albumin and other proteins) from maternal blood. Endometrial epithelial secretions are particularly important for conceptus survival and growth in ruminants, as uterine gland knockout (UGKO) ewes display recurrent early pregnancy loss due to defects in conceptus survival and elongation[255, 256]. Conceptus elongation is particularly critical for production of interferon tau (IFNT)[257, 258], the pregnancy recognition signal, that acts on the endometrium to sustain continued production of progesterone by the ovary and regulates genes implicated in implantation and placentation[245, 248, 259, 260]. Inadequate elongation of the conceptus results in low IFNT production, inability to maintain the corpus luteum, and early pregnancy loss[261]. Although much information is known about embryo development into a blastocyst from in vitro systems[262], post-hatching blastocyst

survival and growth as well as conceptus elongation remains under investigated. Available evidence supports an unequivocal role for progesterone-dependent endometrial secretions of the uterus as primary regulators of conceptus survival, growth and development throughout pregnancy[251, 263]. However, the essential endometrial genes and secretions that mediate survival and growth of the blastocyst and conceptus remain largely unknown in cattle[264, 265].

One of the major impediments to research on the physiology and genetics of early pregnancy success in cattle is the lack of animals with defined high and low rates of early pregnancy loss. McMillan and Donnison[266] summarized a unique approach for experimentally identifying high and low fertility in dairy heifers based on early pregnancy success. Contemporary yearling heifers (n=155) received two in vitro-produced (IVP) embryos on 6 separate occasions during a 26-month period. Sixty days after transfer, pregnancy and the number of fetuses were determined ultrasonically and then pregnancies were terminated with the process was repeated 6 times. That approach identified 25 heifers with high (76%) and low (11%) aggregate pregnancy rates. Of note, a failure in the mechanism involved in conceptus elongation and maternal recognition of pregnancy was suggested to be the cause of early pregnancy loss in the low fertility heifers[266, 267]. A similar approach was used here to fertility classify beef heifers based on natural variation in early pregnancy success. A series of studies were then conducted on fertility classified heifers to begin determining the physiological and genetic factors underpinning early pregnancy loss in beef cattle.

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, Washington State University, and the University of Missouri.

In Vitro Production of Embryos (Study 1)

Embryos were produced in vitro at the University of Florida to obtain blastocysts for transfer into recipients. Oocytes obtained from genetically-undefined ovaries (n=1699-2800 per replicate) were fertilized with frozen-thawed spermatozoa pooled from three bulls of various breeds. The total number of bulls used in the 8 replicates was 20 and represented Angus (n=7), Limousin (n=3), Brangus (n=3), Simmental (n=2), unknown (n=2), Hereford (n=2) and Polled Hereford (n=1) sires.

The procedures for in vitro production were as follows. Cumulus oocyte complexes (COCs) were obtained by cutting the surface of each ovary with a scalpel and vigorously rinsing the ovary through a bath of oocyte collection medium (tissue culture medium-199 with Hank's salts (Hyclone, Logan, UT, USA), 2% (v/v) of either bovine steer serum (Pel-Freez, Rogers, AR, USA) or adult bovine serum (Thermo Scientific HyClone, Logan, UT, USA), 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin and 1 mM glutamine). Groups of 10 COCs were matured in 50 µl droplets of oocyte maturation medium (tissue culture medium-199 with Earle's salts (Invitrogen, Carlsbad, CA, USA), 10% (v/v) bovine steer serum or adult bovine serum, 2 µg/ml estradiol 17-β, 20 µg/ml bovine follicle stimulating hormone (Bioniche Life Sciences, Belleville, Ontario, Canada), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate and 1 mM glutamine) covered

with mineral oil for 20 h at 38.5°C and in a humidified atmosphere of 5% (v/v) CO₂. Sperm were purified by centrifugation over a Percoll® (GE Healthcare, Pittsburgh, PA, USA) gradient (500 µl of a solution of 45% (v/v) Percoll (diluted 1:1 in HEPES-SOF) on top of 500 µl of a solution of 90% (v/v) Percoll [268]. Up to 250 matured oocytes were fertilized in a 35-mm dish with Percoll-purified sperm (1.0×10^6 /ml) for 8-10 h at 38.5°C in 1.7 ml of synthetic oviduct fluid-fertilization medium (SOF) [269]. Cumulus cells were denuded after fertilization by vortexing in 600 µl HEPES-TALP containing 10,000 U/ml hyaluronidase. Putative zygotes were then cultured in 50 µl microdrops of synthetic oviduct fluid-bovine embryo 2 (SOF-BE2) covered with mineral oil at 38.5°C in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂ with balance N₂. Cleavage rate was assessed at day 3 post-fertilization.

At day 4 or 5 post-fertilization, embryos were prepared for transport to Montana by placement of advanced embryos in groups of ~50 per tube in polypropylene-stoppered 10x63 mm polystyrene tubes containing 1 ml SOF-BE2 supplemented with 1 mM HEPES, pH 7.5 and 50 µM dithiothreitol. Tubes had been equilibrated overnight in a humidified incubator at 38.5°C, 5% O₂, 5% CO₂ and 90% N₂. After addition of embryos to the modified SOF-BE2, ~1.5 ml mineral oil was layered over the medium and tubes were placed in a Biotherm portable incubator (Cryologic, Blackburn, Victoria, Australia) at 38.5°C. Embryos were hand carried to Montana by airliner on day 5 or 6. Embryos were transferred to fresh 50 µl drops of SOF-BE2 and cultured at 38.5°C in a humidified atmosphere of 5% (v/v) O₂ and 5% (v/v) CO₂ with balance N₂ until day 7 (embryos shipped on day 4) or were allowed to develop in the shipping tubes until day 7 (embryos shipped on day 5).

Embryo Transfer (ET) and Pregnancy Determination by Ultrasonography (Study 1)

Crossbred beef heifers (n=275 Angus x Polled Hereford) were identified from a single population of cows at the USDA-ARS, Fort Keogh Livestock and Range Research Station and reared on a typical replacement heifer feedlot ration. Six heifers were culled at weaning based on extremely small body size. As illustrated in Figure 1, the remaining heifers were subjected to serial estrous synchronization and ET to classify heifers into fertility categories based on pregnancy outcome. Estrous cycles of heifers were synchronized beginning at approximately 14 months of age when heifers weighed 368 ± 2.8 kg and had a body condition score of 5.9 ± 0.1 (scale of 1 to 9: 1 = emaciated and 9 = obese) to prepare heifers for ET on day 7 after estrus (day 0). Estrus was synchronized using the PG-6d-CIDR protocol which includes prostaglandin F2 alpha (PGF) administration (Lutalyse, Zoetis Animal Health, Madison, NJ) on day -12, an injection of GnRH (100 µg, IM; Factrel, Zoetis Animal Health, Madison, NJ) concurrent with a intravaginal progesterone insert (CIDR) on day -9, CIDR removal and PGF on day -3, and estrus on day 0. Estrus detection patches (Estroject, Rockway, Inc., Spring Valley, WI) were affixed to the tail head of each heifer to aid in visual detection of estrus. Heifers were observed for signs of estrus 3 times a day beginning 24 h after CIDR removal. Heifers that did not exhibit standing estrus received GnRH (100 µg, IM; Factrel, Zoetis Animal Health, Madison, NJ) on day 0. During each ET round, heifers were randomly assigned to one of two groups on consecutive days to facilitate animal handling. All heifers received an in vitro produced embryo of high quality (blastocyst or expanded blastocyst) produced at the University of Florida on day 7 after estrus or induced ovulation by one of two experienced technicians. Ultrasonography was used to identify side and presence of the corpus luteum before ET by a single technician using an Aloka SSD 3500V and 7.5 MHz convex

transducer (Aloka, Wallingford, CT). The embryo was placed in the uterine horn ipsilateral to the ovary containing a corpus luteum using standard non-surgical techniques. Heifers without a corpus luteum on day 7 did not receive an embryo.

Heifers were diagnosed for pregnancy on days 28 and 44 using transrectal ultrasonography with an Aloka SSD 500V and 7.5 MHz (day 28) or 5.0 MHz (day 42) linear transducer. Viability was confirmed at each ultrasound by presence of a fetal heartbeat. At the second pregnancy diagnosis on day 44, heifers received a single injection of PGF and were allowed a minimum of 30 days to exhibit another estrous cycle before being synchronized for another round of ET. After three or four rounds of ET, Heifers were classified based on day 28 pregnancy success as high fertile (HF; 100%), subfertile (SF; 25-33%), or infertile (IF; 0%) based on results of 3 or 4 rounds of ET (Fig. 1).

Blood Collection and Progesterone Radioimmunoassay for the Initial Round of ET (Study 1)

Blood was collected from coccygeal vessels of heifers at the onset of synchronization (day -22 and -12) for the initial round of ET to assess serum concentration of progesterone as an indicator of pubertal status. Blood samples were allowed to clot for 1 h at room temperature, and then incubated at 4°C for approximately 24 h. Samples were centrifuged at 1,200 x g for 25 min at 4°C and serum was collected and stored at -20°C until radioimmunoassays were performed. Radioimmunoassay (RIA) was performed on serum samples to measure progesterone concentrations using the Coat a Count RIA kit (Siemens, Malvern, PA)[270]. Inter- and intra-assay CV for a sample with 1.5 ng/ml were 11.45% and 11.79%, respectively and assay sensitivity was 0.08 ng/ml.

Estrous Synchronization and Artificial Insemination (Study 2)

Estrous Synchronization and Pregnancy Determination. Estrous cycles of fertility-classified heifers were synchronized using the PG-6d-CIDR protocol described in Study 1. Artificial insemination was performed at 12 h and 24 h after onset of standing estrus with semen from a single bull of known high fertility. Pregnancy diagnosis was performed by transrectal ultrasonography (SonoSite EDGE equipped with a L52 10.0-5.0 MHz linear-array transducer; SonoSite Inc., Bothell, WA) on days 28 and 36 post-AI.

Blood Sampling and Serum Progesterone Determination. Blood samples for determination of circulating progesterone concentrations were obtained by coccygeal venipuncture using evacuated tubes without anticoagulant (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) at time of breeding (day 0) and every other day from day 0 through day 28. Blood samples from heifers determined to be pregnant at day 28 were also collected every other day from day 28 through day 36. Serum tubes were allowed to clot overnight at 4°C then centrifuged at 3,000 x g for 20 min at 4°C, and serum was collected and frozen at -20°C until analyzed. Serum concentrations of progesterone were determined in triplicate 100 µl aliquots of sample using manufacturer (MP Biomedical, Santa Ana, CA) reagents and recommendations for the liquid-liquid phase double antibody precipitation assay (MP Biomedicals cat# 07-170105), and validated as previously described[271]. Inter- and intra-assay coefficients of variation were <10%.

Embryo Transfer and Nonsurgical Uterine Flush on Day 14 (Study 3)

Synchronization and Embryo Transfer. Estrous cycles of donor cows (n=7) and fertility-classified heifers were tightly synchronized to optimize synchrony of embryos with uterine

environment of recipient heifers. All females received PGF on day -18 to pre-synchronize estrous cycles and increase ovulation response (follicular wave reset) to GnRH administration on day -15. On day -9, all females received an intravaginal progesterone insert (CIDR) to supplement blood progesterone to assist preparation of the uterus for pregnancy and an injection of 2.5 mg estradiol and 50 mg progesterone to regress all follicles larger than 0.5 mm and again reset follicular wave growth. Donor cows received twice daily injections of follicle stimulating hormone (Folltropin; Agtech, Inc., Manhattan, KS) in decreasing daily doses from day -4.5 to -1 to stimulate multiple follicular development. The CIDRs were removed and PGF administered on day -2.5 (recipients) and -1.5 (donors) to synchronize estrus. Donors were inseminated 12 h and 24 h after onset of estrus (day 0 and 0.5) with semen from the same sire. Recipient heifers that did not exhibit estrus by day 0 received GnRH and estrogen (1 mg estradiol benzoate). On day 7, each heifer received an in vivo-produced embryo placed in the uterine horn ipsilateral to the ovary containing a corpus luteum (as determined by ultrasound) using standard non-surgical techniques by a single technician. Ultrasonography was used to identify side and presence of the corpus luteum before ET. Three heifers did not receive embryos due to excess fluid in their uterus or lack of a CL on their ovaries. Transferred embryos were of excellent quality and either a blastocyst (n = 53) or morula (n = 8) produced through superovulation of 7 donor cows. All transfers were frozen-thawed direct transfer embryos blocked by donor and assigned to heifers equally across fertility classification group.

Nonsurgical Uterine Flush. The uterus of each heifer was flushed to recover embryos at 7 days post-transfer using non-surgical techniques. Briefly, a 2-way luer-lock catheter (cat. no. 19982/0104, Minitube of America, Verona, WI) was passed into the uterine horn

ipsilateral to the corpus luteum and 20 ml of flush medium (Vigro complete flush medium; Agtech, Inc., Manhattan, KS) was placed into the uterine horn. After approximately 30 sec of gentle massage, the initial flush medium was recovered in a syringe, transferred to a petri dish and searched for a conceptus. If a conceptus was not observed in the initial flush, additional medium (1 L) was subsequently used to thoroughly flush the uterine horn. The flushes were accumulated and filtered through a 75 μ m mesh nylon filter (VCI Filter, Agtech, Inc.) then rinsed with flush medium and searched again for an embryo.

Endometrial Biopsy. Following uterine flush, an endometrial biopsy was collected near the greater curvature of the uterine horn ipsilateral to the ovary containing the corpus luteum. Endometrial tissue was obtained using Jackson uterine biopsy forceps (GerMedUSA Inc., Garden City Park, NY). The forcep was sterilized prior to biopsy collection using a hot bead sterilizer (Fine Science Tools (USA), Inc., Foster City, CA). To collect the endometrial biopsy, the forcep was protected with a sanitary chemise (IMV Technologies, Maple Grove, MN), which was broken immediately before the biopsy tool was passed through the cervical os. The tip of the forceps was directed to the greater curvature of the ipsilateral uterine horn, and endometrial tissue (biopsy) was collected by closing the instrument jaw. The forcep was then removed, and the biopsy sections were immediately placed in 1.5 ml microcentrifuge tubes, frozen with liquid nitrogen, and stored at -80°C until RNA extraction.

Conceptus Morphology and Measurement. Embryos were imaged on a Zeiss Discovery V8 stereomicroscope with an AxioCam ICC 1 and AxioVision version 4.6 software.

Measurements of conceptus size (length and width) were collected using ImageJ (NIH, version 1.48).

RNA Sequencing

RNA Isolation. Total RNA was extracted from day 14 endometrial biopsies using the RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, frozen biopsy samples were disrupted and homogenized in RLT buffer with the use of a homogenizer (VDI 25, VWR International, Radnor, PA) and total RNA purified following the manufacturer's instructions. To eliminate DNA contamination, RNA was treated with DNase I during RNA purification using the RNase-Free DNase Set (Qiagen, Valencia, CA). RNA concentration was determined by quantitative high sensitivity RNA analysis on the Fragment Analyzer instrument (Catalog # DNF-472, Advanced Analytical Technologies, Inc., Ankeny, IA). RNA library preparation and sequencing was conducted by the University of Missouri DNA Core facility.

Illumina TruSeq RNA Library Preparation and Sequencing. High-throughput sequencing services were performed at the University of Missouri DNA Core Facility. Libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq mRNA stranded sample preparation kit. Briefly, the polyadenylated mRNA was purified from total RNA and fragmented. Double-stranded cDNA was generated from fragmented RNA, and the index containing adapters ligated. The final construct of each purified library was evaluated using the Fragment Analyzer instrument, quantified with the Qubit fluorimeter using the quant-iT HS dsDNA reagent kit (Invitrogen), and diluted

according to Illumina's standard sequencing protocol for sequencing on an Illumina HiSeq 2500 sequencer.

RNA-Seq Data Analysis

Raw sequence reads (fastq) were trimmed of adapter sequences using cutadapt [272] followed by a windowed adaptive trimming step for base quality of the reads. The quality trimming was performed using Sickle (<https://github.com/najoshi/sickle>) with default parameters for paired-end reads. The read data was deposited in the Gene Expression Omnibus (GSE81449). The trimmed paired-end reads were then mapped to the bovine reference genome UMD3.1.1 using SubRead aligner[273] followed by quantifying the number of reads mapped to exon sequences of annotated genes using FeatureCounts[274]. Genes significantly differentially expressed (FDR $P < 0.10$) between samples were determined by fitting the read counts to a generalized linear model implemented in edgeR-robust[275]. The fold-changes of differential expression were determined from $\log_2(\text{CPM})$ (counts per million reads), and the length-normalized FPKM (fragments per kilobase of exon per million reads) was determined from the sum of exon lengths of each gene. All statistical analyses and plotting were performed in R. The Pearson correlation coefficients were measured in pair-wise manner among samples to plot the correlation heat map of gene expressions. Conditional boxplots were generated using the Rlab package. The expression data of all genes across all the samples were used in exploratory maximum likelihood factor analysis by fitting the data to the factor analysis model using 'varimax' rotation option of 'factanal' in R. The plot was drawn using print method of R 'stats'. Violin plots were generated using ggplot2.

Statistical Analyses

Statistical analyses were conducted using SAS (SAS Institute Inc., Cary, NC). Statistical significance was defined as $P < 0.05$.

Study 2. Pregnancy success to AI at days 28 and 36 post-insemination was analyzed by logistic regression with Firth's bias correction using the LOGISTIC procedure. The proportions of heifers pregnant at days 28 and 36 were determined using FREQ procedure. Progesterone concentrations were analyzed by ANOVA for repeated measures using MIXED procedure. For the repeated measures, models included the effects of fertility classification, day of sample collection, the interaction between fertility classification and day of sample collection, and the random effect of heifer nested within fertility classification. For all analysis, orthogonal contrasts were used to compare groups with high (HF) versus low fertility (SF and IF) and to compare differences between the groups with low fertility (SF vs IF).

Study 3. Day 14 embryo recovery rates were analyzed by logistic regression with Firth's bias correction using the LOGISTIC procedure. The proportions of embryos recovered at day 14 flush were determined using FREQ procedure. Conceptus measurement data was subjected to least-squares analyses of variance (ANOVA) using the General Linear Models (GLM) procedure. Conceptus length, area and conception rate were tested for differences between fertility groups with embryo donor used as a covariate. Error terms used in the test of significance were identified according to the expectation of the mean squares for error. For all analysis, orthogonal contrasts were used to compare groups with high (HF) versus

low fertility (SF and IF) and to compare the differences between the groups with low fertility (SF vs IF).

RESULTS

Heifer Fertility Classification Using Embryo Transfer (Study 1)

A total of 275 heifers were identified for the study, and 6 were culled due to extremely small size at weaning. Of the 269 heifers, 4 heifers were removed due to anovulation, death, health, or inability to pass the cervix. At the onset of synchronization for the first round of ET, 10 heifers were not cycling based on serum progesterone concentrations less than 1.0 ng per ml in two samples collected 10 days apart. Estrous cycles were induced in 8 of 10 of these heifers with synchronization based on serum progesterone concentration on day 7 at ET. One of the anestrus heifers never began estrous cycles and was removed from the study; however, another anestrus heifer was pregnant each time she received an embryo and remained in the study.

In each round of ET, a single IVP embryo of high quality was transferred into heifers on day 7 post-estrus, and pregnancy determined on days 28 and 44 by ultrasound followed by pregnancy termination on day 44 (Fig. 1). Across the rounds of ET, 37 heifers did not have a CL at least once, and 5 heifers did not have a CL on two occasions. Pregnancy rates for each round of ET averaged 55% and 48% when diagnosed on day 28 and 44, respectively. Overall, serial ET was conducted in 260 heifers, and 4 rounds of ET data were generated on 228 heifers and 3 rounds of ET data on 32 heifers. As illustrated in Figure 1, this approach was used to classify heifers as high fertile (HF; 100%, n = 30), subfertile (SF; 25-33%, n = 53), or infertile (IF; 0%, n = 12) based on day 28 pregnancy success.

Pregnancy Rates after AI and Circulating Progesterone Concentrations (Study 2)

All HF and IF heifers and a subset of SF heifers were synchronized to estrus and bred 12 h and 24 h post-estrus with semen from a single bull of known high fertility. Pregnancy was determined on days 28 and 36 by ultrasound. Blood samples were collected every other day beginning on day 0 through 28 in all heifers and through day 36 post-mating in pregnant heifers. On day 28, pregnancy rates were 70.4% in HF ($n = 19/27$), 46.7% in SF ($n = 7/15$) and 0% in IF ($n = 0/4$) heifers. Orthogonal contrasts revealed that day 28 pregnancy rate was higher ($P = 0.04$) in HF than low fertile (SF and IF) heifers, but not different between SF and IF heifers ($P = 0.24$). On day 36, pregnancy rates were 63.0% in HF, 40.0% in SF and 0% in IF heifers. Day 36 pregnancy rates were not higher ($P = 0.06$) in HF than low fertile (SF and IF) heifers and not different ($P = 0.30$) between SF and IF heifers.

Progesterone concentrations during the first 20 days post-insemination were not different ($P = 0.93$) in all heifers regardless of fertility class (Fig. 2A). An effect of day ($P < 0.0001$) was detected, as progesterone concentrations increased after ovulation with formation of a corpus luteum. Further, there was an effect of day ($P < 0.0001$), but not fertility class ($P = 0.86$), on progesterone concentrations for HF and SF heifers diagnosed pregnant through day 36 (Fig. 2B). An interaction between fertility class and day of sample collection was observed ($P = 0.04$), which reflected the higher ($P < 0.01$) progesterone concentrations in SF than HF heifers on day 36.

Pre-implantation Embryo Development in Fertility-classified Heifers (Study 3)

Fertility-classified heifers were synchronized to estrus, and a single embryo of high quality produced in vivo was transferred into HF (n = 28), SF (n = 17), and IF (n = 11) heifers on day 7 post-estrus. Conceptuses were recovered on day 14 by non-surgical uterine flush, and endometrial biopsies collected from the ipsilateral uterine horn. If recovered, conceptus length and area were determined. Embryo recovery rate was 60.7% for HF, 52.9% for SF, and 45.5% for IF heifers. Embryo donor had no effect ($P = 0.94$) on embryo recovery rate. Orthogonal contrasts found that embryo recovery rate was not different ($P = 0.48$) in HF as compared to low fertility (SF and IF) heifers and not different ($P = 0.81$) in SF than IF heifers. Gross conceptus morphology was not different among fertility-classifications, and a spherical to ovoid conceptus was recovered with normal morphology and obvious embryonic disc from HF, SF and IF heifers (Fig. 3A). Conceptus length was not different ($P = 0.47$) in HF as compared to low fertility (SF and IF) heifers and not different ($P = 0.73$) in SF as compared to IF heifers (Fig. 3B). Similarly, conceptus area was not different ($P = 0.82$) between HF and low fertility (SF and IF) heifers and not different ($P = 0.73$) between SF and IF heifers (Fig. 3B).

RNA-Seq Analysis of Endometrial Biopsies (Study 3)

After non-surgical flush on day 14 (7 days post-ET), endometrial biopsies were obtained from the ipsilateral uterine horn. Histological analysis of selected endometrial biopsies determined that the biopsy method repeatedly obtained mostly intercaruncular endometrium with glands, stroma and luminal epithelium (Fig. 4A).

Transcriptional profiling of day 14 endometrial biopsies from pregnant heifers (n = 5 heifers per fertility class) was conducted using RNA sequencing. Sequencing of the libraries yielded more than 60 million quality reads for each sample. The reads used in data

analysis for each sample were of minimum length 30 bp with sliding window Phred scores of at least 30, and were mapped to the reference genome at 96-98% across samples with 70-80% of all the pair reads mapped concordantly. Between 10% to 18% of paired reads mapped discordantly, and they likely represent reads aligning to splice or fusion sites. Less than 1% of the read pairs mapped to the genome as singletons with unmapped mates; those reads may represent repeat sequences, sequences from foreign sources, such as microbial contamination, or regions of the genome that have not been well assembled, and is not unusual [276].

A ‘seed-and-vote’ mapping strategy was used to quantify read counts to individual genes annotated from the reference genome[273, 274]. On average, 63% of the mapped reads were associated with at least 5 reads to individual genes in each sample. About 14% of genes were not expressed in any samples based on absence of read counts. Such a result could be due to lack of coverage, but this is unlikely given the high coverage (>60 million) of reads per sample and consistency of zero-read mapping between samples. About 0.5% of mapped reads showed mapping of 1 read on average per gene per sample. Genes were then filtered that were associated with less than or equal to 1 read per million in each library prior to fitting the count data into the generalized linear model implemented in edgeR-robust [275]. Accordingly, 26 total DEG (≥ 2 fold change, FDR $P \leq 0.10$) were identified for the endometrium from HF as compared to SF heifers (Table 1). There were 12 DEGs in the endometrium from SF as compared to IF heifers (Table 2), and only 3 DEGs when HF were compared to IF heifers (Table 3).

The low number of DEGs detected by edgeR-robust analysis suggested low overall variation in gene expression across samples, which was confirmed by high pairwise correlation between samples (Fig. 4B) and lack of sample clustering as illustrated in the

multidimensional scaling (MDS) plot (Fig. 4C). Expression values in each group were then partitioned into non-overlapping bins and plotted as conditional box plots between groups (Supplemental Fig. 1A). These plots indicate reduced gene expression changes in the endometria of IF as compared to HF or SF heifers and was not observed in HF as compared to SF heifers. The violin plots presented in Supplemental Figure 1B present distribution of \log_2 fold changes of expression relative to \log_2 counts per million reads in pair-wise manner among samples. This analysis confirmed the reduced DEG in IF as compared to HF and SF heifers.

The DEGs identified by edgeR-robust analysis of endometrial biopsy RNA sequencing are presented in Figure 4 and summarized in Tables 1, 2 and 3 and Supplemental Tables 1, 2 and 3. Expression of 14 genes was higher and 12 genes lower in endometrial biopsies of HF as compared to SF heifers (Table 1 and Supplemental Table 1). Many of the 14 genes that were more abundant in the HF endometrium encoded proteins with antimicrobial activity (TAP, MUC1) or were immunoglobulins. In SF as compared to IF heifers, there were 12 genes with reduced expression in the endometrial biopsies (Table 2 and Supplemental Table 2). All 3 DEGs were lower in abundance in endometrial biopsies of HF as compared to IF heifers (Table 3 and Supplemental Table 3). Of note, 10 of the DEGs overlapped between the fertility-classified heifer endometrial biopsies.

DISCUSSION

The present study found that serial rounds of ET, each followed by pregnancy determination on days 28 and 42, is an effective strategy to identify beef heifers with high and low aggregate rates of early pregnancy loss. One might assert that by chance rates of heifers always or never being pregnant 12.5% of the time after 4 rounds of ET would be

correct if pregnancy rate were a random binomial event; however, pregnancy is not a random binomial event and rather a cumulative physiological process in which each successive day of gestation is dependent upon the additive successful events up to that time. Similarly, McMillan and Donnison[266] utilized serial ET to classify dairy heifers as high or low fertile. Failures in mechanisms involved in conceptus elongation and maternal recognition of pregnancy were hypothesized to be the major cause of pregnancy loss in the low-fertility dairy heifers[266, 267]. Indeed, the majority of pregnancy losses in beef and dairy heifers, non-lactating cows and lactating cows occur during the first three to four weeks of pregnancy[261, 277]. Studies in sheep, beef heifers and dairy cattle established that an early or delayed rise in circulating levels of progesterone after ovulation can advance or retard conceptus elongation[250, 251, 263, 278-280]. In the present Study 2, circulating levels of progesterone were not different in fertility classified heifers, regardless of pregnancy status. This result agrees with findings from the analysis of both dairy heifers and beef heifers that were fertility classified by serial ET or AI, respectively[266, 281]. Thus, differences in circulating levels of progesterone are not a factor in the fertility differences for the beef heifers in the present study. Although increasing concentrations of progesterone after ovulation clearly advances conceptus elongation in both beef and dairy cattle, supplementation with progesterone during early pregnancy has equivocal efficacy in increasing embryonic survival[282, 283]. In fact, strategies to improve postovulatory progesterone concentration such as treatment with GnRH or human chorionic gonadotropin following AI have resulted in inconsistent benefits in pregnancy outcomes in lactating dairy cows[284-286]. Further, progesterone administration is unlikely to rescue development of embryos with inherent genetic defects or during gestation in high-producing dairy cows[287-289].

In Study 3, fertility-classified heifers received a single high quality in vivo produced embryo on day 7 post-estrus and were non-surgically flushed to recover the embryo on day 14. Embryo recovery rate and embryo morphology development (length and area) was not different among HF, SF and IF heifers. The average embryo recovery rate on day 14 was 53% across HF, SF and IF heifers, which is not much lower than the 66% pregnancy rate found on day 16 in beef heifers bred by AI [7]. The lower pregnancy rate in Study 3 could be due to a failure of the non-surgical flush procedure to recover embryos from some heifers and also the use of in vivo produced embryo that was frozen and then thawed prior to transfer. Result of the present studies support the idea that pregnancy loss in the SF and/or IF heifers must occur between days 14 and 28, which encompasses the period of conceptus elongation, pregnancy recognition and implantation for establishment of pregnancy[248, 251, 263]. Given the lack of differences in conceptus survival and development, it is not surprising that substantial differences in gene expression for the endometrial biopsies from day 14 pregnant HF, SF and IF heifers were not observed. Indeed, the transcriptome difference was also minimal in other studies of fertility-classified heifers that evaluated endometria from beef heifers on day 13 post-estrus[281], endometrial cytobrush biopsies from beef heifers on day 14 post-estrus[290], or endometrial biopsies from lactating dairy cows on day 13 post-estrus[291]. The lack of conserved differences could be attributed to a myriad of factors including how fertility was classified, breed effects, and endometrial sampling. For instance, endometrium obtained at slaughter is generally a mixture of intercaruncular and caruncular endometrium, whereas the obtained biopsies in the present study are mostly intercaruncular endometrium. Of note, cytobrush sampling obtains mostly only LE from both caruncular and intercaruncular endometrium.

Although large numbers of DEG were not identified, RNA sequencing analysis did detect differences in endometrial gene expression among the fertility-classified heifers. The majority of genes that were more abundant in HF than SF, as well as IF than SF, endometrium encode factors involved in reproductive tract defense against pathogens. For instance, TAP was first identified in the bovine tracheal mucosa and found to be a member of a group of cysteine-rich, cationic, antimicrobial peptides found in animals, insects, and plants[292]. Purified TAP had antibacterial activity in vitro against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. In addition, TAP was active against *Candida albicans*, indicating a broad spectrum of activity. In the present study, MUC1 was also lower in SF heifers, and it forms part of the glycocalyx barrier that provides innate immune protection against bacterial infections[293]. Additionally, MUC1 regulates implantation in mice and perhaps other mammals. Walker and coworkers[294] found evidence that the embryo modulates the uterine immune system on day 17 and induces expression of molecules in the endometrium that function to suppress the immune response and/or promote tolerance to the embryo. During this period of immune suppression, the endometrium would be expected to be susceptible to infections; therefore, it actively expresses specific molecules for defense against foreign pathogens such as upregulation of genes of the innate immune response including antimicrobial response genes like TAP. It should be noted that none of the heifers used in the present study had evidence of metritis, and endometritis is not very prevalent in beef heifers.

Many of the down-regulated genes in SF as compared to HF endometrium encoded various immunoglobulins or a transcription factor (POU2AF1) that are expressed in B lymphocytes, which function in the humoral immunity component of the adaptive immune

system by secreting antibodies. Thus, B cells are likely higher in the endometrium of HF than SF heifers. Pregnancy changes the population of lymphocytes and macrophages in the bovine uterus[295-297]. The numbers of both cell types are significantly reduced between early and mid-pregnancy in cattle[298]. By mid-pregnancy, virtually no lymphocytes or macrophages are found in the caruncular endometrium although they are still present in the intercaruncular endometrium[299]. The lymphocyte population in the early pregnant cattle uterus is composed primarily of B cells, T cells and natural killer cells[300-302]. B lymphocytes are widely distributed throughout the endometrium, localizing in the stroma, myometrium, and the luminal and glandular epithelium. The B lymphocyte population in bovine endometrium was relatively large compared to the populations of $\gamma\delta$ T, CD4+, CD8+ and NK cells detected[300]. Although several genes were more abundant in the endometrium of SF than HF and SF than IF heifers, the function of most of those genes is unknown. Collectively, these results support the idea that the innate and adaptive immune system is different in the endometrium of SF as compared to that of HF and IF heifers, which could be involved in the observed fertility differences[297]. The lack of conserved differences in the endometrial transcriptome of the HF versus low fertility (SF and IF) heifers indicates that the biological mechanisms underlying subfertility and infertility are possibly different, and those differences may not manifest until after day 14 as the conceptus begins rapid growth for elongation. Future experiments will need to explore differences in the endometrial secretome, because histotroph has a major influence on growth and development of the conceptus[245, 251, 263].

The present study and others[266, 267, 281, 290, 291] support the hypothesis that natural variation in pregnancy rates can be utilized in cattle to identify animals with innate differences in uterine competence to support growth and development of the conceptus for

establishment of pregnancy. Although not addressed in the present study, it is also important to address the substantial loss of embryos that occurs prior to day 7, particularly in lactating dairy cows, due to problems with oocyte competence, sperm transport, fertilization and perhaps oviductal-uterine function[303, 304]. Studies of animals with natural variation in uterine competency for pregnancy could help define which genes and biological pathways in the endometrium are crucial to establish endometrial receptivity and support conceptus elongation in cattle. Further, the use of this animal model could discover genes and biomarkers that can be used to select animals for higher fertility and to diagnose subfertility and infertility.

ACKNOWLEDGMENTS

The authors appreciate the assistance of staff at the USDA-ARS Fort Keogh with animal handling and sample collection, William Rembert for collecting ovaries, the Chernin family and Central Packing (Center Hill, Florida) for donating ovarian tissue, and Scott Randel of Southeastern Semen (Wellborn Florida) for donating semen, and Zoetis for donation of synchronization products. They also appreciate the help of Drs. Mike Smith and Duane Keisler and the MU DNA Core facility of the University of Missouri-Columbia with completion of the studies.

FIGURES

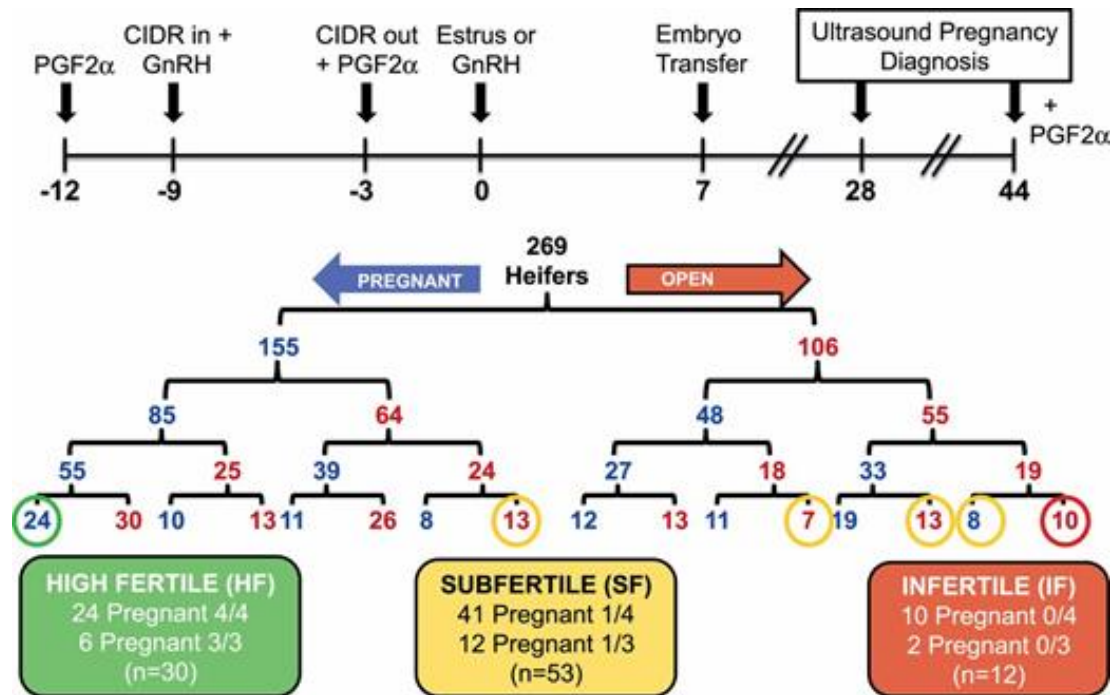


Figure 1. Experimental design and results for classification of fertility in beef heifers using serial embryo transfer (ET).

See text for detailed description of results. Each row of numbers will not add up to 269 because of occasional death loss or removal of heifers from the study for health reasons. Further, 37 heifers did not have a CL at least once, and 5 heifers did not have a CL on 2 occasions. Abbreviations: CIDR, controlled intravaginal drug releasing device; GnRH, gonadotropin releasing hormone; PGF2 α , prostaglandin F2 alpha.

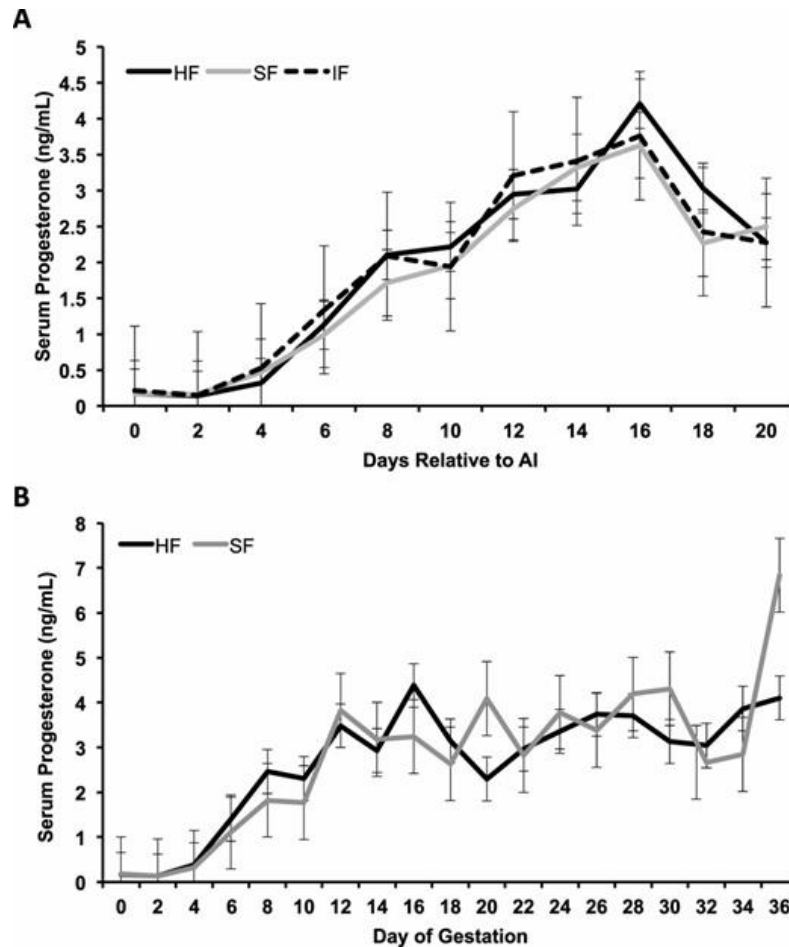


Figure 2. Circulating concentrations of progesterone in nonpregnant (A) and pregnant (B) fertility classified heifers.

Fertility classified heifers were synchronized to estrus and bred by artificial insemination (AI) at 12 h and 24 h post-estrus with semen from a high fertility bull. Pregnancy was determined on days 28 and 35 by ultrasound. An effect of day ($P < 0.0001$) was detected, as progesterone concentrations increased after ovulation. There was an effect of day ($P < 0.0001$), but not fertility class ($P = 0.86$), on progesterone concentrations for HF and SF heifers diagnosed pregnant through day 36. Progesterone concentrations were higher in SF than HF heifers on day 36 ($P = 0.04$, day x fertility class). Data is presented as least squares means (LSM) with standard error (SE).

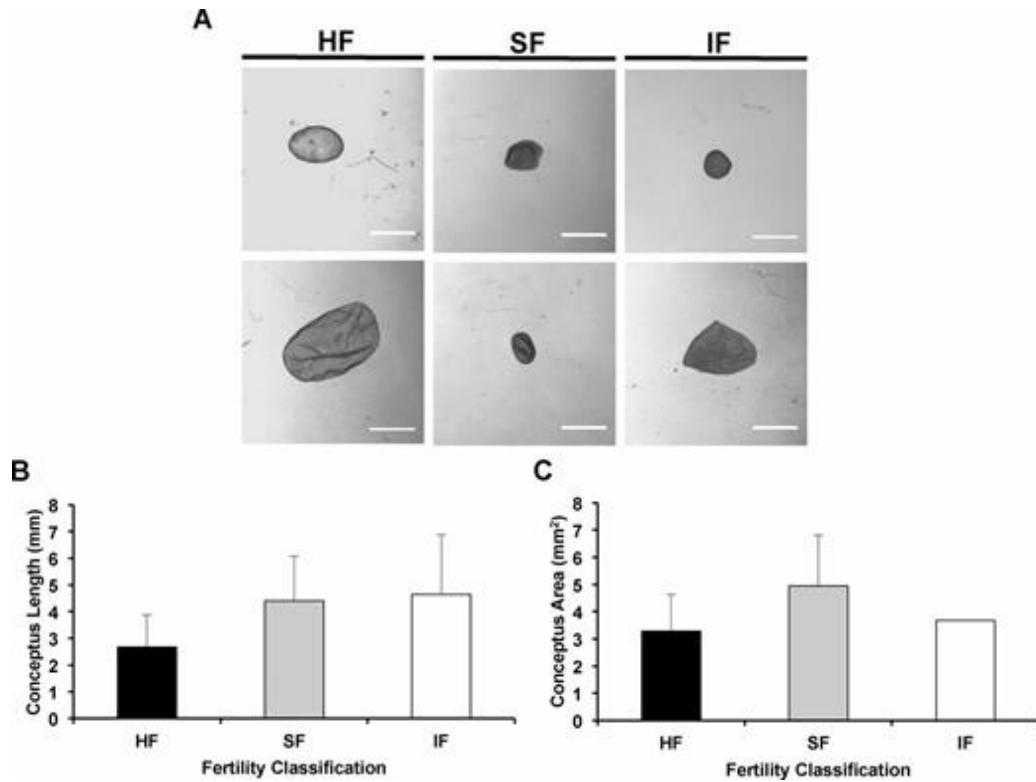


Figure 3. Day 14 conceptus morphology and measurements.

Fertility classified heifers were synchronized to estrus and received a high quality in vivo produced embryo on day 7 post-estrus. All heifers were nonsurgically flushed on day 14 (7 days post-ET) to recover the conceptus. If present, conceptus length and width were determined under a microscope. (A) Representative conceptus morphology recovered from high fertile (HF), subfertile (SF) and infertile (IF) heifers. Scale bar = 1 mm. (B and C) Conceptus length and area were not different ($P \geq 0.60$) among the fertility classified heifers. Data is presented as least squares means (LSM) with standard error (SE).

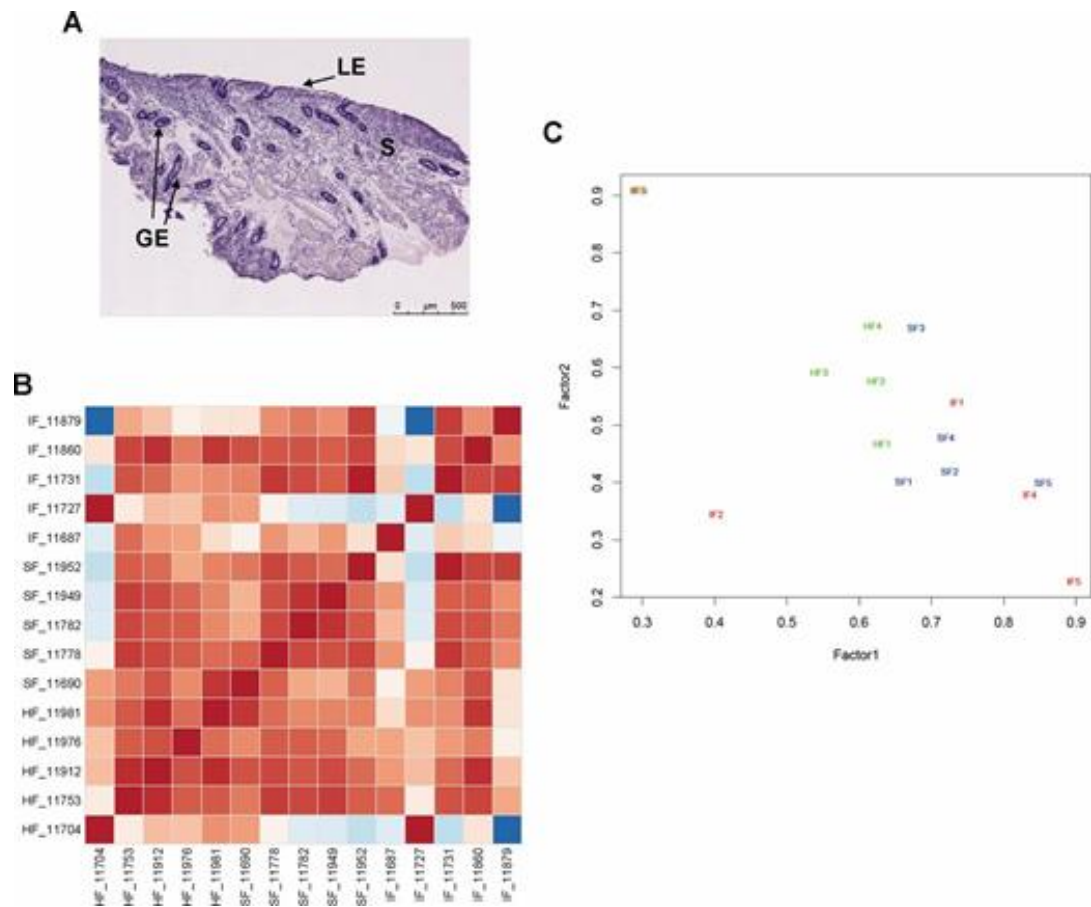


Figure 4. Endometrial biopsy histology and RNA sequencing analysis from day 14 pregnant heifers.

Fertility classified heifers were synchronized to estrus and received two high quality in vivo produced embryos on day 7 post-estrus. All heifers were nonsurgically flushed on day 14 (7 days post-ET) to recover the conceptus. If a conceptus was present in the uterine flush, an endometrial biopsy was obtained from the uterine horn ipsilateral to the corpus luteum. Total RNA was extracted from 5 biopsies of pregnant high fertile (HF), subfertile (SF) and infertile (IF) heifers and sequenced. Normalized and \log_2 transformed read count data were produced with edgeR-robust analysis. (A) Histological analysis of a representative endometrial biopsy. All biopsies were predominantly composed of intercaruncular endometrium. Sections were stained with hematoxylin and eosin. LE,

luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar = 500 μ m. **(B)** Pairwise correlation (Pearson) analysis of gene expression levels between endometrial biopsy samples. Each column represents one sample and shows the correlation to all samples (including itself) with red for lowest (0) distance and blue for the highest observed distance. **(C)** Multidimensional scaling (MDS) plot. A maximum likelihood factor plot of gene expression variation among the samples. The major factors (factor 1 and factor 2) that explain the expression changes among the samples are plotted in x- and y-axis respectively. The individual samples ($n = 15$) representing the three fertility groups (HF, SF and IF) are shown with different colors (green, blue and red, respectively).

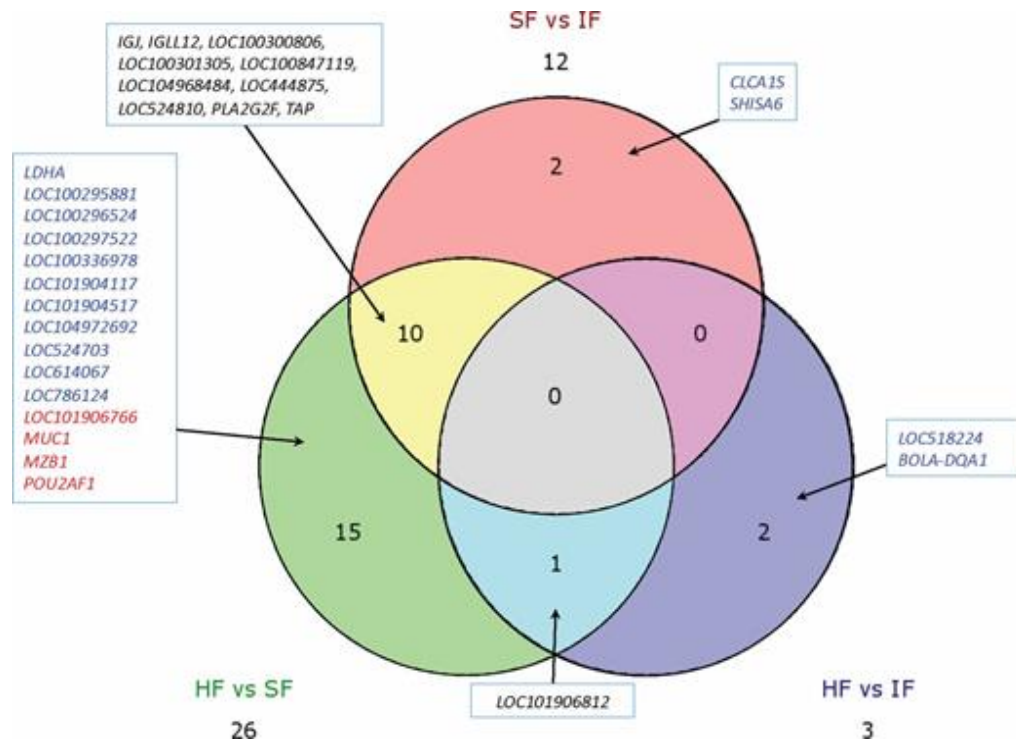


Figure 5. Venn diagram for differentially expressed genes in the endometrium of pregnant fertility-classified heifers.

Venn diagram showing the number of unique or common transcripts between the endometrium of fertility-classified heifers (HF, high fertile; SF, subfertile; IF, infertile). Increased (red) and decreased (blue) genes are presented (≥ 2 fold change and FDR $P \leq 0.05$).

TABLES

Table 1. Differentially expressed genes in endometrial biopsies from high fertile (HF) and subfertile (SF) heifers

Gene symbol	Gene name ^a	logFC ^b	FDR ^c P-value	Mean FPKM ^d	
				HF	SF
TAP	Tracheal antimicrobial peptide	7.25	0.01	7.36	0.00
LOC100847119	Ig lambda-7 chain C region-like	5.93	0.00	66.29	0.95
LOC524810	IgM	5.86	0.00	220.13	3.79
LOC100301305	Ig heavy chain V region MC101-like	4.49	0.00	10.84	0.41
LOC100300806	Ig heavy chain Mem5-like	4.08	0.00	53.94	3.18
IGLL1-2	Unknown	3.96	0.00	232.32	14.97
LOC104968484	Ig heavy chain Mem5-like	3.90	0.00	123.90	8.38
PLA2G2F	Phospholipase A2 group IIF	3.84	0.04	0.91	0.07
LOC444875	Ig heavy chain V region PJ14	3.66	0.00	66.72	5.31
LOC101906766	Ig lambda chain V-II region VIL-like	3.54	0.00	5.55	0.47
IGJ	Immunoglobulin joining chain	3.48	0.00	112.72	10.13
POU2AF1	POU class 2 associating factor 1	2.70	0.03	0.29	0.04
MZB1	Marginal zone B and B1 cell-specific protein	1.85	0.05	2.87	0.80
MUC1	Mucin 1, cell surface associated	1.00	0.05	55.64	27.30
LDHA	Lactate dehydrogenase A	-1.02	0.05	18.09	36.58
LOC101904117	Uncharacterized LOC101904117	-2.28	0.01	125.54	591.83
LOC614067	Ribosomal protein L21 pseudogene	-2.59	0.03	14.60	88.13
LOC104972692	Uncharacterized LOC104972692	-3.59	0.05	0.45	5.53
LOC524703	40S ribosomal protein S21 pseudogene	-3.66	0.04	0.52	6.62
LOC100336978	Multidrug resistance-associated protein 4	-3.76	0.03	0.46	5.55
LOC100297522	60S ribosomal protein L38	-3.77	0.02	2.72	37.20
LOC786124	Prostaglandin E synthase 3 pseudogene	-4.04	0.03	0.80	13.27
LOC101904517	Multidrug resistance-associated protein 4-like	-4.22	0.00	0.08	0.69
LOC100296524	60S ribosomal protein L39	-5.14	0.04	0.36	12.94
LOC101906812	Uncharacterized LOC101906812	-5.20	0.02	0.47	2.65
LOC100295881	Ribosomal protein S27-like pseudogene	-5.36	0.01	2.38	98.15

^a Ig, immunoglobulin.

^b Log2 fold change (logFC) in HF as compared to SF endometrial biopsy samples.

^c FDR, false discovery rate.

^d Data are presented as mean fragments per kilobase of exon per million (FPKM) mapped reads.

Table 2. Differentially expressed genes in endometrial biopsies from subfertile (SF) and infertile (IF) heifers

Gene symbol	Gene name ^a	logFC ^b	FDR ^c P-value	Mean FPKM ^d	
				SF	IF
IGJ	Immunoglobulin joining chain	-2.22	0.01	10.1	72.1
LOC104968484	Ig heavy chain Mem5-like	-2.58	0.00	8.4	57.0
IGLL1-2	Unknown	-2.61	0.00	15.0	106.1
LOC444875	Ig heavy chain V region PJ14	-2.68	0.00	5.3	34.5
LOC100300806	Ig heavy chain Mem5-like	-2.71	0.00	3.2	26.2
LOC100301305	Ig heavy chain V region MC101-like	-3.18	0.00	0.4	4.3
LOC100847119	Ig lambda-7 chain C region-like	-3.26	0.00	1.0	11.1
CLCA1	Chloride channel accessory 1	-3.59	0.00	0.1	1.5
LOC524810	IgM	-4.64	0.00	3.8	115.4
PLA2G2F	Phospholipase A2 group IIF	-4.85	0.00	0.1	1.5
SHISA6	Shisa family member 6	-5.15	0.02	0.0	0.2
TAP	Tracheal antimicrobial peptide	-8.15	0.00	0.0	7.9

^a Ig, immunoglobulin.

^b Log2 fold change (logFC) in SF as compared to IF endometrial biopsy samples.

^c FDR, false discovery rate.

^d Data are presented as mean fragments per kilobase of exon per million (FPKM) mapped reads.

Table 3. Differentially expressed genes in endometrial biopsies from high fertile (HF) and infertile (IF) heifers

Gene symbol	Gene name	LogFC ^a	FDR P-value	Mean FPKM ^b	
				HF	IF
LOC518224	Membrane cofactor protein	-4.01	0.05	0.2	0.9
BOLA-DQA1	Major histocompatibility complex, class II, DQ alpha, type 1	-4.67	0.02	0.6	19.0
LOC101906812	Uncharacterized LOC101906812	-5.15	0.02	0.0	2.4

^a Log2 fold change (logFC) in HF as compared to IF endometrial biopsy samples.

^b Data are presented as mean fragments per kilobase of exon per million (FPKM) mapped reads.

SECTION III:

Uterine Influences on Conceptus Development in Fertility-Classified Animals

Joao G.N. Moraes, Susanta K. Behura, Thomas W. Geary, Peter J. Hansen, Holly L. Neibergs, and Thomas E. Spencer

This work has been published in:

PNAS February 20, 2018 115 (8) E1749-E1758; doi: 10.1073/pnas.1721191115

ABSTRACT

A major unresolved issue is how the uterus influences infertility and subfertility in cattle. Serial embryo transfer was previously used to classify heifers as high fertile (HF), subfertile (SF), or infertile (IF). To assess pregnancy loss in those animals, two *in vivo* produced embryos were transferred into HF, SF and IF heifers on day 7, and pregnancy outcome assessed on day 17. Pregnancy rate was substantially higher in HF (71%) and SF (90%) than IF (20%) heifers. Elongating conceptuses were about two-fold longer in HF than SF heifers. Transcriptional profiling detected relatively few differences in endometrium of nonpregnant HF, SF and IF heifers. In contrast, there was a substantial difference in the transcriptome response of the endometrium to pregnancy between HF and SF heifers. Considerable deficiencies in pregnancy-dependent biological pathways associated with extracellular matrix structure and organization as well as cell adhesion were found in endometrium of SF animals. Distinct gene expression differences were also observed in conceptuses from HF and SF animals with many of the genes decreased in SF conceptuses known to be embryonic lethal in mice due to defects in embryo and/or placental development. Analyses of biological pathways, key players, and ligand-receptor interactions based on transcriptome data divulged substantial evidence for dysregulation of conceptus-endometrial interactions in SF animals. These results support the ideas that: the uterus impacts conceptus survival and programs conceptus development; and ripple effects of dysregulated conceptus-endometrial interactions elicit loss of the post-elongation conceptus in SF cattle during the implantation period of pregnancy.

INTRODUCTION

Infertility and subfertility are important and pervasive problems in agricultural animals and humans [305, 306]. In ruminants, embryo mortality is a major factor affecting fertility and thus production and economic efficiency [2, 15]. Pregnancy loss in cattle ranges from 30% to 56% with the majority of losses occurring in the first month of pregnancy [2, 36, 307]. Infertility and subfertility also impact the success of embryo transfer (embryo transfer) in cattle and humans [308, 309]. In cattle, mean survival rate to calving following transfer of in vivo-derived embryos from superovulated donors ranges from 31% to 60%, and in vitro-produced embryo survival rate is lower [36, 310]. Failure of the embryo to survive and establish pregnancy is due to paternal, maternal and embryonic factors [311-313]. Many of the pregnancy losses observed in natural or assisted pregnancies may be attributed to maternal factors such as an inability of the uterus to support conceptus growth and implantation [314, 315].

After fertilization (day 0), the zona pellucida-enclosed bovine embryo enters the uterus at the morula stage by day 5 and develops into a blastocyst. The spherical blastocyst hatches from the zona pellucida on days 7 to 10 and continues to grow, changing from spherical to ovoid in shape between days 12 and 14 after which it can be termed a conceptus (embryo and associated extra-embryonic membranes) [316]. The conceptus undergoes elongation involving exponential growth from about 2 mm in length on day 13, 60 mm by day 16, and 20 cm or more by day 19 [95]. After days 16-17, the time of maternal recognition of pregnancy (when the embryo signals to the mother to prevent regression of the corpus luteum), the conceptus begins the processes of implantation and placentation that involves apposition, attachment and adhesion of the trophectoderm to the endometrial luminal epithelium and onset of trophoblast giant binucleate cell differentiation [99].

Blastocyst growth into an elongated conceptus has not been achieved in vitro and requires transfer into the uterus [97], as the endometrium secretes or transports a myriad of factors critical for conceptus growth and elongation [110, 315]. Dynamic changes in the endometrial transcriptome occur between days 7 and 13 that are primarily regulated by progesterone in both nonpregnant and pregnant cattle [110, 253, 315, 317]. Those changes in the endometrium presumably establish a uterine environment conducive to blastocyst survival and conceptus growth into an elongated, filamentous type conceptus and subsequently implantation and placentation. Conceptus elongation is required for production of interferon tau (IFNT) [318], which is the pregnancy recognition signal that acts on the endometrium to sustain continued production of progesterone by the ovary and regulates genes implicated in conceptus growth, implantation and placentation [319, 320]. Inadequate elongation of the conceptus presumably results in lower production of IFNT, inability to maintain the corpus luteum, and thus pregnancy loss [320-322]. Although much information is known about embryo development into a blastocyst from in vitro systems [323], the essential endometrial genes and biological pathways important for conceptus survival, growth and implantation remain largely unknown [324, 325].

One of the major impediments to understanding maternal influences on pregnancy loss has been a lack of animals with defined high and low rates of early pregnancy loss. McMillan and Donnison [326] summarized a unique approach for experimentally identifying high and low fertility in dairy heifers based on serial transfer of in vitro-produced embryos. Their approach identified animals with high (76%) and low (11%) aggregate pregnancy rates, and a failure in the mechanism involved in conceptus elongation and thus maternal recognition of pregnancy was suggested to be the cause of early pregnancy loss in the low fertility-classified heifers [326, 327]. We recently used a similar

approach to identify beef heifers with intrinsic differences in pregnancy loss [328]. Serial transfer of a single in vitro-produced embryo was used to classify animals as high fertile (HF), subfertile (SF), or infertile (IF) based on day 28 pregnancy rate. In those heifers, no difference in pregnancy rates were observed on day 14 after transfer of a single in vivo-produced embryo on day 7 post-estrus [328]. Thus, the observed difference in uterine competence for pregnancy in fertility-classified heifers is hypothesized to manifest during maternal recognition of pregnancy and implantation. The present study tested this hypothesis and revealed that embryo survival to day 17 is compromised in IF heifers and that asynchronous conceptus-endometrial interactions in SF animals leads to post-elongation embryonic loss by day 28 during the implantation period of pregnancy.

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. In our recent work [328], 269 beef heifers were classified based on fertility using serial embryo transfer to select animals with intrinsic differences in pregnancy loss. In each of the four rounds, a single in vitro-produced high-quality embryo was transferred into heifers on day 7 post-estrus and pregnancy was determined on days 28 and 42 by ultrasound and then terminated. Heifers were classified based on pregnancy success as high fertile (HF=100%), subfertile (SF=25-33%), or infertile (IF=0%). In the present experiment, 41 heifers that have been previously [328] classified for fertility (HF, n = 21; SF, n = 14; IF, n = 6) were used.

Embryos. In vivo produced embryos were generated at the USDA-ARS, Fort Keogh Livestock and Range Research Station using 7 Angus donor cows using previously described methods [328] and a total amount of FSH equivalent to 320 mg NIH-FSH-P1 (Folltropin-V, Vetoquinol, Quebec, Canada). Donors cows were inseminated 12 h and 24 h after onset of estrus (day 0 and 0.5) with semen from a single high fertile Holstein sire, and were flushed on day 7 after breeding for embryo recovery. Recovered embryos were classified by stage of development and graded based on morphological appearance (Manual of the International Embryo Transfer Society, 4th edition). Two quality grade 1 or 2 embryos (a compact morula and a blastocyst) were loaded into a 0.25 cc polyvinyl straw containing ViGro Freeze Plus medium (Bioniche Animal Health, Belleville, Ontario) and frozen in a programmable embryo freezer using standard techniques for direct transfer [390].

Embryo transfer and Sample Collection. Estrous cycles of fertility-classified heifers were synchronized with an injection of PGF2 α followed in 3 days by an injection of GnRH concurrent with the insertion of a CIDR insert. Six days later, CIDR inserts were removed and an injection of PGF2 α was administered. Concomitant with CIDR removal, estrus detection patches (Estroject, Rockway, Inc., Spring Valley, WI) were applied on the tail head of each heifer to aid in visual detection of estrus. Heifers were observed for signs of estrus 3 times a day, beginning at 24 h after CIDR removal. On day 7 post-estrus, each heifer received two in vivo-produced embryos placed in the uterine horn ipsilateral to the ovary containing a corpus luteum using standard non-surgical techniques by a single technician. Ultrasonography was used to identify side and presence of the corpus luteum before embryo transfer. Only heifers that exhibited estrus signs after CIDR removal and

PGF2 α injection and that had a corpus luteum on day 7 post-estrus received embryos. For collection of nonpregnant animals, heifers were synchronized to estrus (day 0) but did not receive embryos on day 7.

The female reproductive tract was recovered on day 17 post-estrus, and their uteri were flushed with 20 mL of filtered sterile PBS (pH 7.2). If present, the state of conceptus development was assessed using a Nikon SMZ1000 stereomicroscope (Nikon Instruments Inc.) fitted with a Nikon DS-Fi1 digital camera. Conceptus size was determined using a ruler. The volume of the uterine flush was measured, and the flush clarified by centrifugation ($3,000 \times g$ at 4°C for 15 min). The supernatant was carefully removed with a pipette, mixed, aliquoted, and stored at -80°C until analyzed. The endometrium was physically dissected from the remainder uterine horn using curved scissors. Endometrial samples as well as conceptuses were frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction.

Circulating Progesterone Concentrations. For determination of circulating progesterone concentrations on the day of slaughter (day 17 post-estrus), blood samples were collected from the median coccygeal vein or artery into evacuated tubes containing K3 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Blood tubes were then centrifuged at $1,200 \times g$ for 20 min at 4°C and plasma collected and stored at -20°C until a radioimmunoassay was performed. Plasma concentrations of progesterone were determined in duplicate 100 μ L aliquots of sample using manufacturer (MP Biomedical, Santa Ana, CA) reagents and recommendations for the liquid-liquid phase double antibody

precipitation assay (MP Biomedicals cat# 07-170105), and validated as previously described (112). Intra-assay coefficient of variation was 2%.

Statistical analysis. Statistical analyses were conducted using SAS (SAS Institute Inc., Cary, NC). Statistical significance was defined as $P \leq 0.05$. Binomial data representing whether or not a conceptus was recovered on day 17 uterine flush (Yes = at least one conceptus was recovered; No = no conceptus was recovered) was analyzed by logistic regression with Firth's bias correction using the LOGISTIC procedure. The proportion of heifers that had at least one conceptus recovered on day 17 flush within each fertility classification was determined using FREQ procedure. The effect of fertility classification on the number of conceptuses recovered was analyzed by logistic regression using the GLIMMIX procedure with a multinomial distribution. Post-test comparisons were conducted using the contrast statement. The effect of fertility classification on conceptus length was estimated in a Poisson regression repeated measurements model using GENMOD procedure to account for the effect of more than one conceptus being recovered per heifer. The means of conceptus length were estimated using the MEANS procedure. Continuous variables were assessed for normality using the UNIVARIATE procedure. The effect of pregnancy status (pregnant vs nonpregnant) and fertility classification on plasma progesterone concentrations was determined by ANOVA using the GLM procedure. Post-test comparisons were conducted using the LSMEANS statement with the Fisher's protected LSD option. Only heifers which only one conceptus was recovered were used in the analysis to investigate the correlation of plasma progesterone concentration and conceptus size. Pearson's correlations were determined using the CORR procedure.

RNA Sequencing. Total RNA from day 17 endometrium samples of 25 heifers- HF nonpregnant (n= 5), HF pregnant (n=5), SF nonpregnant (n= 5), SF pregnant (n = 5, and IF nonpregnant (n = 5) was extracted using the Isol-RNA Lysis Reagent (5Prime, Hamburg, Germany). Briefly, frozen endometrium samples were disrupted and homogenized in Isol-RNA Lysis Reagent with the use of a homogenizer (VDI 25, VWR International, Radnor, PA) and total RNA purified following the manufacturer's instructions. Total RNA from 27 conceptuses (HF, n = 17; SF, n = 10) was extracted using the AllPrep DNA/RNA/Protein Mini kit (Qiagen, Valencia, CA).

To eliminate DNA contamination, total RNA from endometrium and conceptuses was treated with DNase I during RNA purification using the RNase-Free DNase Set (Qiagen, Valencia, CA). RNA concentration was determined by quantitative high sensitivity RNA analysis on the Fragment Analyzer instrument (Catalog # DNF-472, Advanced Analytical Technologies, Inc., Ankeny, IA). RNA library preparation and sequencing was conducted by the University of Missouri DNA Core facility. Libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq mRNA stranded sample preparation kit. Briefly, the polyadenylated mRNA was purified from total RNA and fragmented. Double-stranded cDNA was generated from fragmented RNA, and the index containing adapters ligated. The final construct of each purified library was evaluated using the Fragment Analyzer instrument, quantified with the Qubit fluorimeter using the quant-iT HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard sequencing protocol for sequencing on an Illumina NextSeq 500 sequencer. All the raw data and processed data from this study have been submitted to the Gene Expression Omnibus for public access (accession no. GSE107891).

Alignment of Sequences and Analyses of Differential Gene Expression. The raw sequences (fastq) were subjected to adapter removal and quality trimming using fqtrim (<https://ccb.jhu.edu/software/fqtrim/>). The quality reads were then mapped to the bovine reference genome UMD3.1 using Hisat2 mapper (<https://ccb.jhu.edu/software/hisat2/>) which is a fast and sensitive alignment program of next-generation sequencing data [391]. To produce a global gene annotation file (GTF) for comprehensive quantification of gene expressions across the samples, the sorted binary alignment maps of sequence reads from each sample were assembled to generate transcriptomes those were then merged, along with the gene annotations from reference genome assembly. The transcript assembly step was accomplished using the software ‘Stringtie’ (<https://ccb.jhu.edu/software/stringtie/>) which is considered as a highly efficient assembler of RNA-Seq mapping data [392]. The merged global transcriptome was then used in Stringtie to quantify transcript abundance of each sample. Differential expression analysis between sample groups were performed by robustly fitting the expression data to a weighted generalized linear model using edgeR robust [393].

GO, Pathway and Network Analysis. Additional analyses determined if DEGs were significantly enriched in specific pathways and expression networks. First, a model-based cluster analysis of differential gene expressions was performed using Bayesian Information Criterion to predict if DEGs are expressed in clusters and hence may establish expression networks. After predicting clusters, network analysis was performed based on mutual information of expression changes of genes using R package ‘minet’ [394]. Key genes within each network were predicted from centrality scores of DEGs within the networks using approaches of ‘key player analysis’, a method used in analyzing social networks

[394]. Functional prediction of genes corresponding to those expression modules were assessed by mapping the genes to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways and annotating gene ontology using DAVID (<https://david.ncifcrf.gov>). Significant impact of DEGs on signaling pathway were determined using a pathway perturbation algorithm called 'SPIA' [329]. This topology based pathway analysis was conducted in R using ToPAseq package [395]. Functional annotation analysis was also conducted for DEGs using ToppGene Suite for gene list enrichment analysis [396].

Ligand-Receptor Analysis. Determination of ligands and their receptors was conducted using curated ligand-receptor pairs in the FANTOM5 database for human protein-coding genes [333]. Tanglegram plots of ligand-receptor expression in endometrium and conceptus were generated by 'dendextend' [397] based on hierarchical clustering of ligand and corresponding receptor expression levels separately for endometrium and conceptus. The cluster branch and nodes were color coded and black lines were used to show the correspondence between the same pair of ligand and receptor in endometrium and conceptus. The hierarchical clustering performed based on distance measured by Ward's method from expression data.

RESULTS

Experiment Overview. This 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 (n=3-4 rounds) of a single in vitro-produced embryo (Grade 1) on day 7 followed by pregnancy determination on day 28 [328]. In the present study, those same 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 of the embryos were generated from superovulated donor cows using a single sire and were cryopreserved for direct thaw transfer. Each heifer received two grade 1 or 2 embryos from the same donor and developmental stages (a compact morula and a blastocyst). On day 17 (10 days post-embryo transfer), the entire female reproductive tract was obtained, and the uterine lumen flushed to recover the conceptus. If a conceptus was not observed in the uterine flush, the heifer was considered nonpregnant. As illustrated in Fig. 1A, the proportion of heifers from which one or two conceptuses were recovered was greater for HF ($P = 0.02$) and SF ($P = 0.03$) heifers compared to IF heifers, but was not different ($P = 0.91$) between HF and SF heifers. Accordingly, pregnancy rate was higher ($P < 0.05$) in HF (71%) and SF (90%) than IF (20%) heifers, but not different ($P > 0.05$) between HF and SF heifers (Fig. 1B). The morphology of the conceptuses recovered from HF and SF heifers was similar and ranged from tubular to elongated and filamentous (Fig. 1C). In contrast, a single small spherical hatched blastocyst (< 0.1 cm) was recovered from the uterus of only one IF heifer. Overall, the conceptus was longer ($P < 0.01$) 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 (Fig. 1D).

There were no differences in circulating progesterone concentrations on day 17 between pregnant and nonpregnant heifers ($P = 0.55$) or among pregnant HF, SF or IF heifers ($P = 0.23$) (SI Appendix, Fig. S1A & S1B). Similarly, there was no correlation of conceptus size and circulating progesterone concentrations ($R = -0.08$; $P = 0.80$) (SI Appendix, Fig. S1C).

Dysregulation of Endometrial Transcriptome in Response to Pregnancy.

Transcriptome analysis was performed using total RNA isolated from the day 17 endometria of nonpregnant and pregnant fertility-classified heifers. Sequencing of the 25 RNA-seq libraries ($n = 5$ per group) generated 27.2 to 42.8 million quality reads that mapped at a ~97% rate to the *Bos taurus* reference genome (assembly UMD3.1). EdgeR robust analysis was used to identify differentially expressed genes (DEGs) (FDR $P < 0.05$) in the endometria, and a gene was defined as expressed if it presented >1 fragments per kilobase of transcript per million mapped reads (FPKM). Similar to our previous study of endometrial biopsies from these fertility-classified animals on day 14 [328], there were relatively few genes that were different (FDR $P < 0.05$) in the endometrium of the nonpregnant HF, SF and IF animals (Fig. 2, Fig. 3A) (SI Appendix, Datasets S1, S2, S3). Functional analysis of those DEGs did not identify any significantly enriched gene ontology (GO) terms or biological pathways. Several of the DEGs in HF and SF as compared to IF endometrium encode proteins involved in immune responses or immunoglobulins (Fig. 3A). In SF compared to IF endometria, some of the 43 DEGs encode secreted proteins (GRP, IGFBP2, LYZ1, SERPINB7), intracellular enzymes (PLA2G2F), and transporters for water (AQP5) or amino acids (SLC2A3).

Comparison of the endometrial transcriptome of pregnant HF to pregnant SF animals detected 168 DEGs (Fig. 2) (SI Appendix, Dataset S4). Of note, none of the DEGs were classical ISGs (i.e. IFIT1, IFIT2, ISG15, MX2, RSAD2, OAS2) that are induced or considerably up-regulated by IFNT from the elongating conceptus [320] (Fig. 3B). The 168 DEGs were enriched for GO molecular function (extracellular matrix or ECM structural constituent), biological process (ECM organization, cell adhesion, collagen

catabolic process, locomotion, cellular response to endogenous stimulus), and cellular component (ECM, extracellular space, membrane region) terms (SI Appendix, Table S1). Pathway analysis found enrichment in ECM organization and collagen formation. Genes increased in pregnant HF as compared to SF endometrium included a water transporter (AQP8), lipid transporter (FABP3), and secreted protease (MEP1B). Genes decreased in HF as compared to SF endometrium include cell signaling (CAMK1G, IRS4), ECM constituents, and cell adhesion molecules (SI Appendix, Dataset S4).

Endometrial responses to pregnancy were assessed by comparing the transcriptome of nonpregnant to pregnant animals (Fig. 2, Fig. 3B) (SI Appendix, Datasets S5 and S6). This analysis found 3,422 DEGs (1,680 increased, 1,742 decreased) in HF heifers and 1,095 DEGs (833 increased, 262 decreased) in SF animals with 848 genes commonly responsive to pregnancy in HF and SF animals (Fig. 3B). Thus, ~20% of total expressed genes respond to pregnancy in the endometrium of HF animals, but only ~6% of genes in endometrium of SF animals. Of note, the number of up- and down-regulated genes specific to endometrium of HF animals was 6- and 20-fold greater, respectively, than in SF animals. The lower number of DEGs in SF animals was not due to higher levels of variation in the endometrial transcriptome, as gene expression was most variable in the endometrium of pregnant HF as compared to SF animals (SI Appendix, Fig. S2). An impact analysis of the DEGs was performed based on pathways topology [329]. This analysis found that specific signaling pathways were either negatively or positively impacted in response to pregnancy in HF and SF heifers (SI Appendix, Table S2). Of note, the ECM-receptor interaction pathway was negatively impacted by pregnancy only in HF animals. These findings

support the idea that the endometrial response to pregnancy, e.g. the conceptus, is considerably altered in SF animals.

Pathways Impacted by Pregnancy. Many of the 664 commonly responsive to pregnancy genes in HF and SF animals were classical type I ISGs stimulated by IFNT (MX2, ISG15, RSAD2, BST2, IFIT1, IFIT2, IDO1, ISG20, OAS2, IFIT3, etc.) or established progesterone- and conceptus-responsive genes (e.g. DKK1, FABP3, MEP1B, LGALS3BP) [108] (Fig. 3B) (SI Appendix, Datasets S5 and S6). Functional analyses of those 664 up-regulated genes found enrichment for many GO terms including molecular function (chemokine receptor binding, chemokine activity, peptidase activity), biological process (innate immune response, defense response, response to cytokine, response to type I IFN, response to virus), and cellular component (other organism, extracellular space, proteasome core complex) (SI Appendix, Table S3). As expected, pathways associated with the 664 commonly up-regulated genes included IFN signaling, IFNA/B signaling, cytokine signaling in the immune system, IFNG signaling, ISG15 antiviral mechanism, complement pathway, and toll-like receptor signaling (SI Appendix, Tables S1 and S3), as they are linked to IFNT actions within the endometrium. Analysis of the 184 commonly down-regulated genes found they were enriched for many GO terms including molecular function (ECM structural constituent, integrin binding, cell adhesion binding, glycosaminoglycan binding), biological process (ECM organization, collagen catabolic process, cell adhesion), and cellular component (proteinaceous ECM, ECM, collagen, basement membrane, extracellular space) (SI Appendix, Tables S1 and S4). Pathways associated with the 184 commonly down-regulated genes included ECM organization, collagen biosynthesis and modifying enzymes, collagen formation, ECM-receptor

interaction, ECM degradation, integrin signaling pathway and focal adhesion (SI Appendix, Tables S2 and S4).

The representation of enriched GO terms and pathways was substantially different between HF and SF heifers with respect to genes responsive to pregnancy (Fig. 3B) (SI Appendix, Datasets S5 and S6, Tables S1, S2, S5, S6 and S7). The 1,016 uniquely up-regulated genes in the endometrium of pregnant HF as compared to nonpregnant HF animals were enriched for GO terms including molecular function (ubiquitin-like protein ligase binding, enzyme binding, RNA binding), biological process (carboxylic acid metabolic process, cellular catabolic process, innate immune response), and cellular component (mitochondrion, proteasome core complex) (SI Appendix, Tables S1 and S5). The 1,558 uniquely down-regulated genes in the endometrium of HF pregnant as compared to nonpregnant HF animals were enriched for GO terms including molecular function (protein complex binding, ECM structural constituent, cell adhesion molecule binding), biological process (ECM organization, vasculature development, biological adhesion, cell adhesion), and cellular component (proteinaceous ECM, ECM, cell junction, cell-cell junction, focal adhesion) (SI Appendix, Tables S1 and S6). Pathways associated with the 1,558 down-regulated genes in pregnant HF endometrium included ECM, ECM organization, collagen formation, structural components of basement membranes, focal adhesion, ECM-receptor interaction, and integrin signaling pathway (SI Appendix, Tables S2 and S6).

As provided in SI Appendix, Tables S1 and S7, GO analysis of the 169 genes uniquely up-regulated in the endometrium of SF heifers in response to pregnancy revealed enrichment in GO terms including molecular functions (amide transmembrane transporter activity, peptidase regulator activity) and cellular component (cell surface, extracellular

space, plasma membrane). No biological pathways were enriched in those genes. The 78 genes uniquely down-regulated in the endometrium of SF heifers revealed no enrichment in GO terms or pathways. Collectively, these findings strongly support the idea that the endometrial response to pregnancy, e.g. the conceptus, is considerably altered in SF animals.

Alterations in the Transcriptome of HF and SF Conceptuses. Transcriptional profiling was conducted using individual conceptuses from HF (n=17) and SF heifers (n=10) (Fig. 2). RNA sequencing generated 36.5 to 65.5 million quality reads with a ~92% mapping rate to the *Bos taurus* reference genome. EdgeR robust analysis identified 1,287 DEGs (558 increased, 729 decreased) in HF as compared to SF conceptuses (SI Appendix, Fig. S4 and Dataset S7). In order to ensure that the observed differences were not related to size of the conceptuses [94, 330], conceptuses from only HF heifers were categorized as long (range = 9.8-32.2 cm; n = 10) or short (range = 1.2 - 6.9 cm, n = 7). Subsequent EdgeR robust analysis found only 18 DEG (7 increased, 11 decreased) between the long and short conceptuses from HF heifers (SI Appendix, Dataset S8). Thus, the differences in the transcriptome between HF and SF conceptuses is not due to their length [94, 330], but rather the fertility phenotype of the uterus.

Functional annotation of genes between HF and SF conceptuses identified many biological processes enriched in DEGs including growth and regulation of actin cytoskeleton in the DEGs increased in HF conceptuses (SI Appendix, Tables S8 and S9). GO analysis of the 558 genes increased in HF conceptuses found enrichment for molecular function (signal transducer activity, transcription factor activity, transcriptional activator activity) and biological process (embryo development, embryonic morphogenesis, animal

organ morphogenesis, epithelium development, receptor signaling, tissue morphogenesis, morphogenesis of an epithelium), but no cellular component terms (SI Appendix, Tables S8 and S9). The 558 genes increased in HF conceptuses were enriched for pathways including integrin-mediated cell adhesion, signaling by FGFR, signaling by NGF, signaling by FGFR, focal adhesion, FGF signaling, estrogen signaling, ErbB1 signaling, insulin signaling, and MAPK signaling. GO analysis of the 729 genes increased in SF conceptuses found enrichment for molecular function (molecular function regulator, receptor binding, sialyltransferase activity, heparin binding), biological process (organ development, cellular lipid metabolic process, cell adhesion, lipid metabolic process), and cellular component (extracellular space, ECM, apical part of cell, proteinaceous ECM) terms (SI Appendix, Tables S8 and S10). Pathways for the 729 up-regulated genes in SF conceptuses were enriched for ECM and ECM-associated proteins.

In order to illuminate possible mechanisms impacting loss of conceptuses in the SF animals, the Mouse Genomic Informatics Knockout database [331] was queried to determine if genes down-regulated in SF as compared to HF conceptuses are associated with embryonic lethality. As summarized in SI Appendix, Table S11, many of the genes decreased in SF conceptuses have knockout database annotation terms corresponding to “embryonic lethality during organogenesis, complete penetrance”, “embryonic lethality during organogenesis”, “abnormal vascular development”, “abnormal embryonic tissue morphology”, “abnormal prenatal growth/weight/body size“, “abnormal vitelline vasculature morphology”, and “lethality throughout fetal growth and development, complete penetrance”.

Network analyses of DEG in HF and SF Conceptuses. Mutual information based network analyses were conducted by implementing a model based cluster approach. Using Bayesian Inference Criterion as model selection [332], we identified 9 gene expression clusters in conceptus transcriptome and predicted gene networks of those clusters from DEGs discovered in conceptus of HF as compared to SF animals. Using network centrality method, we predicted top key players within each cluster that may play central role in gene expression networks in HF and SF conceptuses (SI Appendix, Table S12). This analysis revealed many key players including critical enzymes (FADS1, PTGS2), transcription factors (EFHD2, IRX4, ZBBTB7B) as well as several secreted proteins (TKDP1, TKDP4, SSLP1).

Conceptus-Endometrial Signaling. Conceptus-endometrial interactions during elongation primarily involve secreted factors from the trophoctoderm and endometrial epithelium [117, 315]. The GO extracellular space term (GO:0005615) was used to determine gene products secreted from the endometrium or conceptus into the uterine lumen based on DEGs in the endometrium (pregnant vs nonpregnant) and conceptus from HF and SF animals (SI Appendix, Table S13). This analysis revealed substantial differences in genes encoding secreted proteins. For instance, expression of 206 genes were increased and 296 were decreased in the endometrium by pregnancy in HF animals, but only 36 genes were increased and 5 decreased by pregnancy in SF animals. Further, 79 genes were increased in HF conceptuses and 173 were increased in SF conceptuses.

A network of ligand-receptor-mediated multicellular signaling from the FANTOM5 database of human cells [333] was used to determine ligands and receptors expressed by the endometrium and conceptus from HF and SF animals (SI Appendix,

Datasets S9 and S10) and is represented in Fig. 4. Tanglegram plots of ligand-receptor expression (SI Appendix, Fig. S3) found that SF heifers have relatively higher variation in branch lengths (0 through 300,000) of receptor-ligand clusters for conceptus than those of HF animals (0 through 250,000). Thus, ligand-receptor expression in SF conceptuses is more discordant in their correspondence with endometrium ligand-receptors compared to HF animals, which was measured from distance measure (Ward's method) of gene expression variation of receptors compared to ligands between endometrium and conceptus in SF and HF groups separately. As illustrated in Fig. 4 and SI Appendix, Fig. S3, some receptors are expressed with higher expression of ligands in both in conceptus and endometrium, but in the majority of cases there is a negative regulation of receptor-ligands both within and between endometrium and conceptus (SI Appendix, Table S14). However, there was a relative lack of receptor-ligand interactions between conceptus and endometrium in SF as compared to HF animals. Further, ligands and receptors were not as reciprocal in the SF as compared to HF animals and more ligands in the endometrium were uniquely increased or decreased by pregnancy in HF as compared to SF animals. The same result was found for receptors in the endometrium. Differential expression of ligands and receptors was also observed in the conceptus from HF and SF animals. The alterations in ligands and receptors in the endometrium and conceptus would impact paracrine and autocrine signaling for conceptus growth and endometrial receptivity.

Based on protein age estimates, protein ligands are generally evolutionary younger than the cognate receptors [333]. The age of proteins of *Bos taurus* were assessed from ProteinHistorian analysis [334] that predicts age from evolutionary conservation of proteins. Next, a contingency test of up-regulated versus down-regulated genes in day 17 HF and SF endometrium was conducted by differentiating them based on 'age' of the

encoded protein (SI Appendix, Fig. S5). This analysis revealed that the transcriptome response of HF and SF pregnant heifer endometrium is associated with protein age. In HF pregnant heifers, the endometrium transcriptome did not display age differences in encoded proteins, but age of proteins was different (Pearson Chi Square = 3.37, $P = 0.04$) in SF animals. The genes up-regulated in SF endometrium are biased ($P = 0.0064$) towards younger age. Collectively, these analyses of ligands and receptors further support the idea that the endometrium and conceptus is asynchronous in SF animals.

DISCUSSION

Our previous study of the fertility-classified heifers used here found no difference in pregnancy rate or conceptus development on day 14 (7 days post-embryo transfer) [328]. It has long been postulated that pregnancy loss in heifers and non-lactating cows was primarily due to defects in conceptus survival and elongation between conception or embryo transfer (day 7) and day 16 [36, 335]. In the present study, conceptus survival was substantially compromised in IF heifers on day 17 (10 days post-embryo transfer), but not in SF as compared to HF animals. Given that SF animals have substantially increased embryo mortality by day 28, these findings suggest that embryonic loss in heifers after embryo transfer occurs primarily between days 17 and 28 during the implantation period of pregnancy. True infertility occurs in only 5% or so of heifers, and SF is more pervasive and costly in beef and dairy animals [336-338]. Although pregnancy rates and embryo survival were not different on day 17 in SF and HF animals receiving two embryos on day 7, the conceptus was two-fold longer in HF than SF animals on day 17 in the present study. The variability in recovered conceptus size and morphology was striking (Fig. 1), but is consistent with many other studies of beef and dairy heifers and cows [36, 94, 95, 330, 339,

340]. In the present study, embryonic loss in the SF heifers had to occur between days 17 and 28, which encompasses the implantation period of pregnancy [341-343]. Recent data from dairy heifers and cows as well as somatic cell nuclear transfer pregnancies supports the hypothesis that embryonic loss is most prevalent during this period [2, 57, 344, 345]. However, little is known of the biological pathways and gene networks regulating implantation and placentation in cattle [342, 345, 346]. Progesterone action via endometrium is critical for conceptus development and elongation [315, 320] and increased post-ovulatory progesterone concentrations is associated with increased conceptus growth on days 13 and 14 [347, 348]. In agreement with our recent findings [328, 349], differences in levels of circulating progesterone concentrations were not associated with pregnancy outcomes. This reinforces our hypothesis that mechanisms other than circulating progesterone concentrations are associated with pregnancy loss in the fertility-classified SF and IF heifers.

A prevailing theory is that smaller conceptuses producing less IFNT are inferior in their ability to establish pregnancy as compared to longer conceptuses that produce more IFNT [350]. That theory has been difficult to test given the difficulties in retrieving elongating conceptuses and transferring them into a recipient uterus without damage [95]. Despite differences in the size of HF and SF conceptuses, the response of the endometrium to the conceptus was not different with respect to induction of classical ISGs by IFNT, such as ISG15, MX2, etc., which is not unexpected given that very little IFNT is needed to maximally stimulate expression of ISGs in cells and tissues [351]. In fact, the absolute amount of IFNT that must be produced by the conceptus to signal pregnancy recognition has never been established [108]. As the pregnancy recognition signal, IFNT acts in a paracrine manner on the endometrial luminal epithelium to regulate expression of the

oxytocin receptor (OXTR) gene, as OXTR are key for endometrial generation of luteolytic pulses of prostaglandin F2 alpha [108, 113]. In cattle, OXTR expression increases in the endometrial luminal epithelium after day 14 in nonpregnant but not pregnant cattle [352]. In the present study, endometrial OXTR mRNA levels were not different among nonpregnant HF, SF and IF heifers and, as expected, were lower in the endometrium of pregnant (mean 2.5 FPKM) as compared to nonpregnant (mean 19.5 FPKM) HF heifers. In SF heifers, OXTR mRNA levels were not different in the endometrium of pregnant (mean 3.8 FPKM) and nonpregnant (mean 2.1 FPKM) animals. Although OXTR expression was clearly different in SF heifers on day 17 post-estrus, no consistent difference in estrous cycle length was observed in our previous study of these SF and IF heifers [328].

Transcriptional profiling of the endometrium was conducted to begin understanding the biology of pregnancy loss in IF and SF animals. Minimal differences were observed in the nonpregnant endometrial transcriptome amongst IF, SF and HF heifers. Similarly, minimal gene expression differences were found in endometrial biopsies from these same fertility-classified heifers on day 14 (7 days post-embryo transfer) [328]. Few differences in the endometrium were also detected in endometria from fertility-classified heifers on day 13 post-estrus [349] or day 14 post-estrus [353] and also endometrial biopsies from lactating dairy cows on day 13 post-estrus [146]. The lack of conserved differences among these studies could be attributed to a myriad of factors including how fertility was classified, breed effects, and endometrial sampling technique. Nonetheless, RNA-seq analysis detect some genes increased in HF or SF as compared to IF endometrium that encode factors involved in reproductive tract defense against pathogens and immune system modulation (CTLA4, IFI47, IGJ, IGM, MUC13). In other

mammals, the embryo induces expression of molecules in the endometrium that function to suppress the immune response and/or promote tolerance to the embryo [345]; however, relatively little is known about the immunology of pregnancy in cattle [354]. In summary, analyses of the nonpregnant endometrium transcriptome did not provide significant insights into the biological pathways underlying embryo mortality in IF and SF animals. Similar to studies of humans with fertility problems [355], the gene expression signature in nonpregnant endometrium may not be useful to predict fertility phenotype and pregnancy outcome. Further studies on the genome [349] or uterine secretome of IF animals may provide insight into failure of conceptus survival and growth in those animals [356, 357].

Of the 44 genes decreased in pregnant SF as compared to HF heifers, several of them encode secreted factors (FABP3, MEP1B) and transporters (SLCO4C1, SLC7A1) implicated in conceptus implantation in ruminants. MEP1B is a zinc metalloendopeptidase that is expressed by and secreted from epithelial cells. In the bovine uterus, it is induced by progesterone in the endometrial glands and implicated in elongation of the conceptus [347]. FABP3 is involved in the uptake, intracellular metabolism and transport of long-chain fatty acids and up-regulated by progesterone in the endometrial luminal epithelium between days 13 and 16 in cattle [317]. Long chain fatty acids are important for cell growth and production of eicosanoids, which are important for conceptus elongation in ruminants [189, 358]. SLCO4C1 is an organic ion transporter that is involved in the membrane transport of eicosanoids. Analysis of the conceptus transcriptome data identified PTGS2 as a key player in the present study, and PTGS2 is critical for conceptus elongation and pregnancy establishment in sheep [359] and a predictor of embryo viability and pregnancy success in cattle [360]. SLC7A1 is an arginine transporter up-regulated in the luminal epithelium of

the ovine uterus during early pregnancy, and arginine stimulates trophoblast cell proliferation, migration and IFNT production in vitro [361]. Functional analysis of the 44 genes found enrichment for biological processes involved in the inflammatory response (MEP1B, S100A12, C2, IL17RC, CFI, IDO1, NUPR1, TFRC). Controlled inflammation is important for the establishment of pregnancy in other mammals, although the immunological underpinnings of pregnancy are not well understood in ruminants [354, 362].

Transcriptome analyses uncovered the remarkable finding that the endometrial response to the conceptus was extensively altered in SF animals with an approximate three-fold disparity in DEGs. Comparison of the nonpregnant and pregnant endometrium revealed 2,574 unique DEGs in HF animals and 247 unique DEGs in SF animals. Further, 848 DEGs commonly responded to pregnancy including classical ISGs up-regulated by IFNT (CLEC4F, MX2, ISG15, RSAD2, IFIT1, OAS2, etc.) as well as a number of known progesterone-induced and conceptus-stimulated genes (FABP3, MEP1B, LGALS3BP, SOCS3, S100A2). Thus, the endometrium of SF animals appears to respond appropriately to progesterone and IFNT, which are the major established regulators of endometrial gene expression and function to date in ruminants [108, 320]. Comparisons of the endometrium from pregnant HF and pregnant SF heifers as well as the differential response of the endometrium of HF and SF heifers to pregnancy revealed substantial alterations in genes regulating ECM structure and organization as well as cell adhesion, cell-matrix adhesion, and cell movement. Collectively, results support the idea that ECM remodeling is a normal response of the endometrium to the elongating and implanting conceptus, but this remodeling is substantially diminished in SF animals. Indeed, genes enriched in GO terms for cell adhesion, ECM, basement membrane and vasculature development were down-

regulated in pregnant as compared to cyclic cows [363, 364]. Differences in ECM remodeling were found in comparisons of the transcriptomes of chorionic, endometrial or placental tissues from natural as compared to embryo transfer or somatic cell nuclear transfer-derived pregnancies that are predominantly loss during implantation and placentation during the first few months [345]. Implantation and placentation involve remodeling of the endometrial and chorionic ECM [342, 365, 366]. Endometrial and fetal tissues undergo major tissue remodeling during this period to aid the proliferation, differentiation, and migration of binucleate cells, which are regulated by locally produced MMPs and tissue inhibitor of matrix metalloproteinase 1 [367-370]. The organization of the ECM is a complex process which involves interactions between the ECM components and extracellular proteoglycans that undoubtedly impact growth and development of the placentomes. The mechanisms of pregnancy loss in SF heifers appears to be associated with the process of ECM remodeling and impaired conceptus endometrium interactions. ECM and adhesion proteins increased in pregnant SF endometrium are normally decreased in HF endometrium by pregnancy. Trophoctoderm attachment and adhesion to the endometrial luminal epithelium is a fundamental event in implantation and pregnancy establishment in mammals [371] and thus likely disrupted in SF animals. Excessive ECM in SF animals could possibly inhibit the embryonic adhesion to endometrial tissue [372].

Many of the 729 genes down-regulated in SF conceptuses are enriched for GO biological processes that include embryo development, embryo morphogenesis, animal organ morphogenesis, epithelium development, and morphogenesis of an epithelium among others and associated with embryonic lethality or abnormal implantation in mice. Several genes down-regulated in the SF conceptus (GCM1, BMP2, FGFR2) are post-implantation embryonic lethal in null mice due to defects in placental development (SI

Appendix, Table S11). Of note, GCM1 is a critical transcription factor that regulates placental development and trophoblast differentiation in mice and humans [373]. Further, genes down-regulated in the SF conceptuses were enriched for GO molecular functions involving transcription factors activity and transcriptional activator activity; however, key transcription factors regulating conceptus elongation and placental development are not known in cattle [342]. A number of biological processes were also down-regulated in the SF conceptus included integrin-mediated cell adhesion, focal adhesion, FGF and FGF signaling, and MAPK signaling, which are all important pathways for conceptus growth and implantation [374]. Alterations in WNTs and DKK1, a WNT inhibitor, was observed in the SF conceptus, and canonical and noncanonical WNT signaling pathways are conserved regulators of conceptus-endometrial interactions in mammals and implicated in conceptus elongation and implantation in sheep and cattle [375, 376]. FGFR2 was decreased in the SF conceptus, and FGF2 from the endometrium activates FGFR2 in the elongating bovine conceptus and increases trophectoderm proliferation and IFNT production [135]. Factors involved in cell migration and elongation (GJA5, TSPAN1, GJB5, ITGB2, PECAM1) were also decreased in the SF conceptus. Thus, the developmental program of conceptus growth and differentiation is likely compromised in SF animals and leads to post-elongation embryonic mortality due to defects in extraembryonic tissues (placenta, allantois) and/or the embryo itself.

In addition, 558 genes up-regulated in SF conceptuses are enriched for GO molecular function (molecular function regulator, receptor binding, heparin binding) and cellular component (extracellular space, extracellular matrix). Altered secretion of proteins that are ligands for receptors expressed on the conceptus and/or endometrium could lead to defects in conceptus development and implantation [345]. This supposition is supported

by *in silico* determination of secreted factors and ligands and their differences in endometrium and conceptus of pregnant HF and SF animals. Indeed, the endometrium is an early biosensor and distinct endometrial responses are elicited by embryos produced by artificial means [345, 377, 378]. Results of the present study suggest that other factors besides IFNT are important mediators of conceptus-endometrial interactions during early pregnancy. Bioinformatics analysis identified several secreted proteins (TKDP1, TKDP4, SSLP1) as key players in conceptus gene expression networks. The trophoblast kunitz domain proteins (TKDP) are a multigene family that are predominantly expressed in the trophoblast cells of the bovine placenta during early pregnancy [379]. Other potential important signaling molecules from the conceptus include BMP2, BMP5, GDF6, and GDF11, which activate signaling pathways important for endometrial function and implantation in other mammals [380, 381].

Collectively, the transcriptional profiling results strongly support the idea that conceptus-endometrial interactions are dysregulated in SF animals and underlies the observed pregnancy loss by day 28. This hypothesis is strongly supported by studies in cattle, mice and humans finding that dysregulated interactions between the conceptus and uterus have adverse ripple effects resulting in pregnancy loss, miscarriage, or preeclampsia [382, 383]. Implantation requires carefully orchestrated interactions between the receptive endometrium and elongating conceptus, and studies of somatic cell nuclear transfer-derived pregnancies in cattle clearly support the idea that altered conceptus-endometrial interactions is a cause of post-elongation pregnancy loss [345, 378, 384]. Given that only minimal differences were observed on conceptus transcriptome when short and long HF conceptuses were compared, it is likely that the differences in the conceptus transcriptome of HF and SF conceptuses arise initially from influences of the endometrium.

Hypothesis for the Loss of Pregnancy in Infertile and Subfertile Animals. Pregnancy loss during the first trimester of gestation of pregnancies established by embryo transfer has been hypothesized to involve failures or delays in conceptus elongation and/or embryonic development resulting in loss of pregnancy by day 30 [2]. The present studies were conducted with beef heifers that were fertility-classified by embryo transfer and whose pregnancy rates were predicated on innate uterine competence to support pregnancy establishment. The defects in conceptus survival and elongation observed on day 17 in the IF heifers support the idea that an incompetent uterus is present in 5% or so of cattle, but they did not illuminate why the IF uterus fails to support pregnancy. Subfertility is a more prevalent issue in cattle, and studies here found that conceptus survival was not compromised in SF heifers, indicating that pregnancy loss occurs between days 17 and 28 in SF animals during the implantation period. Findings from the transcriptome analyses clearly support the idea that conceptus-endometrial interactions are dysregulated in the SF animals. Thus, our studies strongly support the adverse ripple effect hypothesis that aberrant communication between the endometrium and conceptus disrupts normal implantation and placentation processes leading to pregnancy loss and later pregnancy complications [382]. Based on the experimental approach of fertility classification here, we propose that the alterations in the SF conceptus transcriptome result from inappropriate responsiveness of the endometrium to the conceptus in SF animals. The alterations in endometrial and conceptus gene expression are likely caused, in part, by alterations in DNA methylation [385-389]. Subsequent pregnancy loss occurs due to the ripple effects of dysregulated conceptus-endometrial interactions due to insufficient: (a) conceptus attachment and adhesion to the endometrium; (b) placental development as a consequence

of inadequate endometrial remodeling or defects in allantois growth and development; and/or (c) embryogenesis. In summary, the studies here provide an important foundation to understand implantation and early placentation phase pregnancy loss and develop genetic and physiological approaches to improve the outcome of natural and assisted pregnancies.

ACKNOWLEDGMENTS

This work was supported by 1 R01 HD072898 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

FIGURES

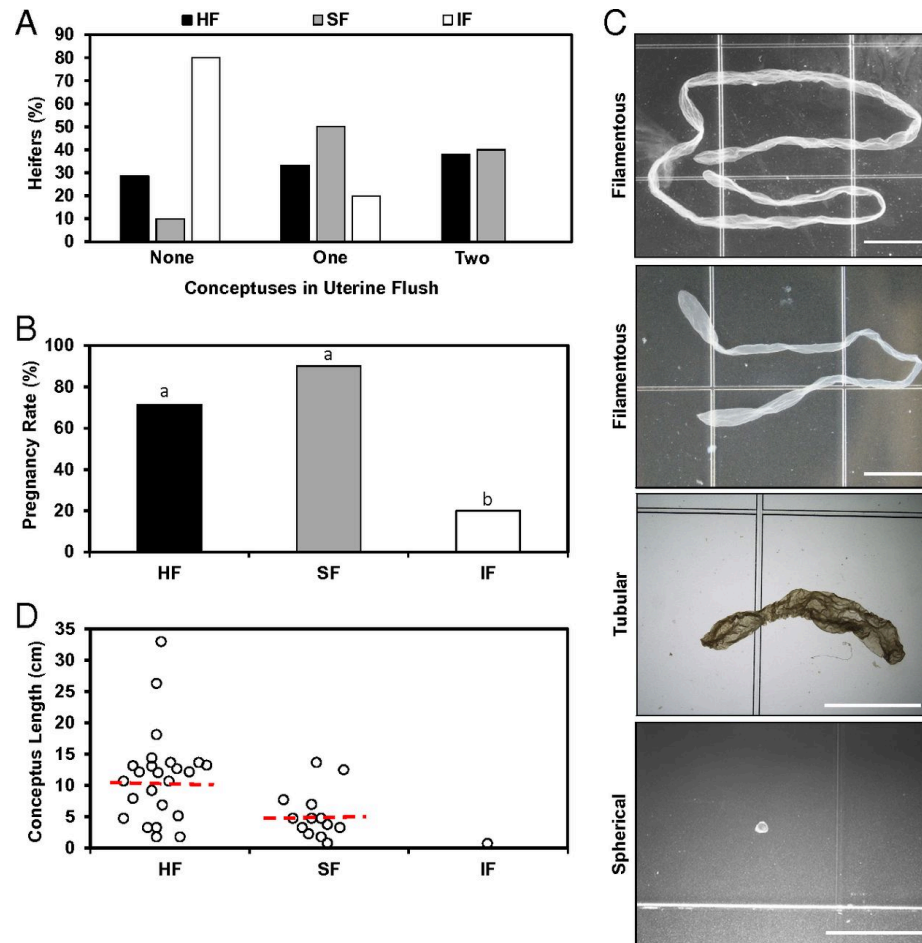


Figure 1. Day 17 pregnancy outcome in fertility-classified heifers.

High fertile (HF), subfertile (SF) and infertile (IF) heifers were synchronized and received two in vivo produced embryos on day 7 post-estrus. The reproductive tract was acquired on day 17 (10 days post-embryo transfer) and flushed to recover the conceptus(es). If present, conceptus morphology and length were determined using a stereomicroscope. A) Percentage of heifers with an identifiable conceptus in the uterine flush. B) Column graph showing pregnancy rate (y-axis) of the fertility classified heifers (x-axis). C) Representative conceptus morphology recovered from heifers. Note a single spherical embryo of less than 1.5 mm was recovered in one IF heifer. Bar = 1 cm. D) Conceptus

length was longer ($P < 0.05$) from HF as compared to SF heifers. Individual conceptus length is plotted with the mean length denoted by the dashed red line.

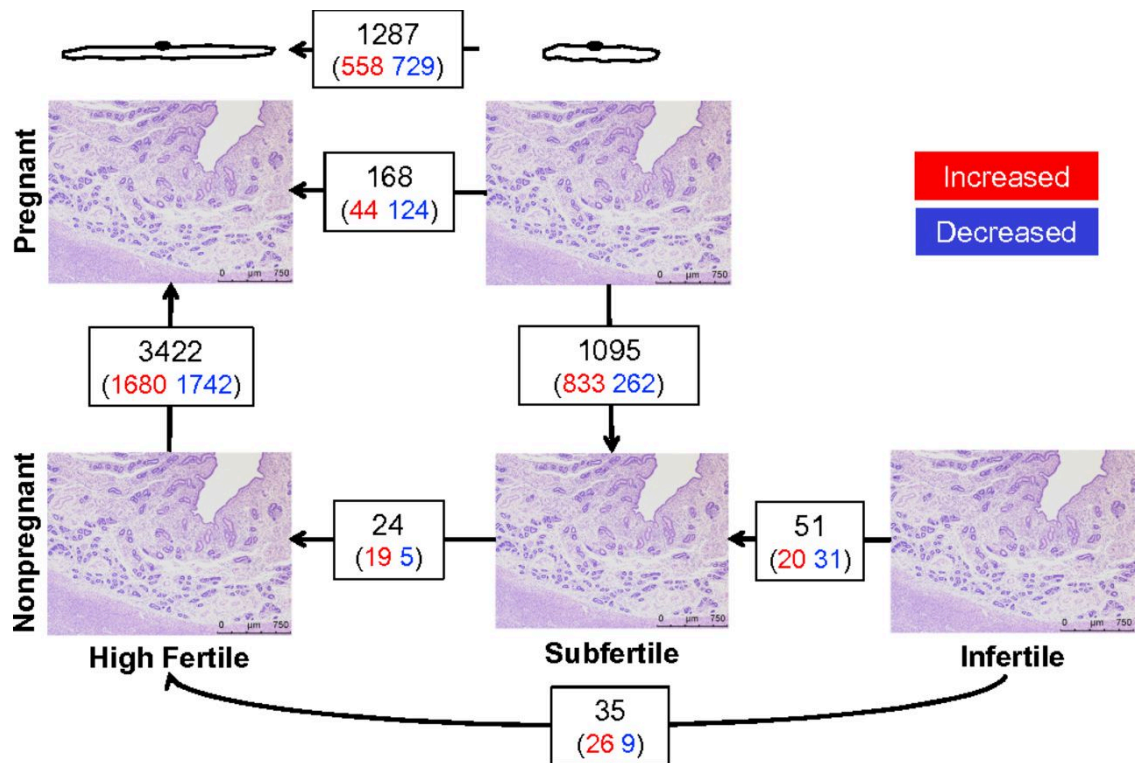
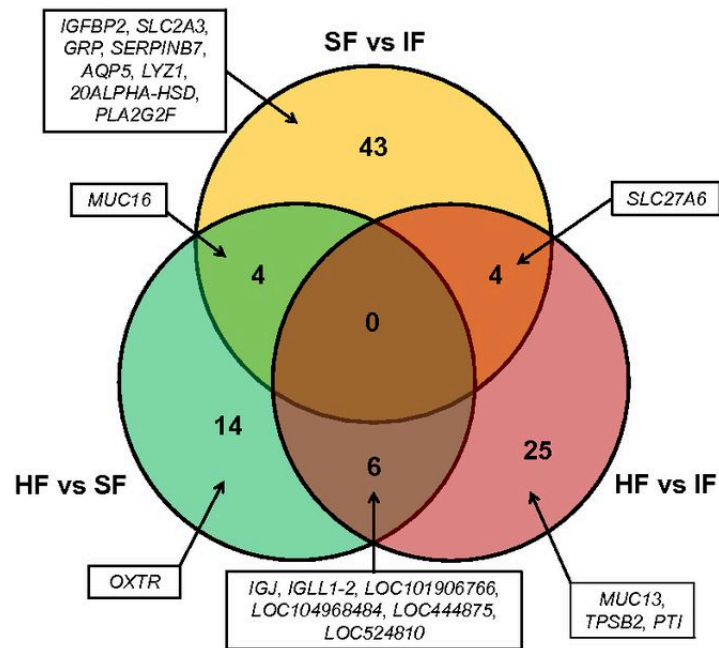


Figure 2. Summary of endometrium and conceptus transcriptome analyses.

Differentially expressed genes (FDR $P < 0.05$) were determined by EdgeR robust analysis, and the number of increased and decreased transcripts are indicated in red and blue, respectively, for each comparison.

A Nonpregnant Endometrium



B Pregnant vs Nonpregnant Endometrium

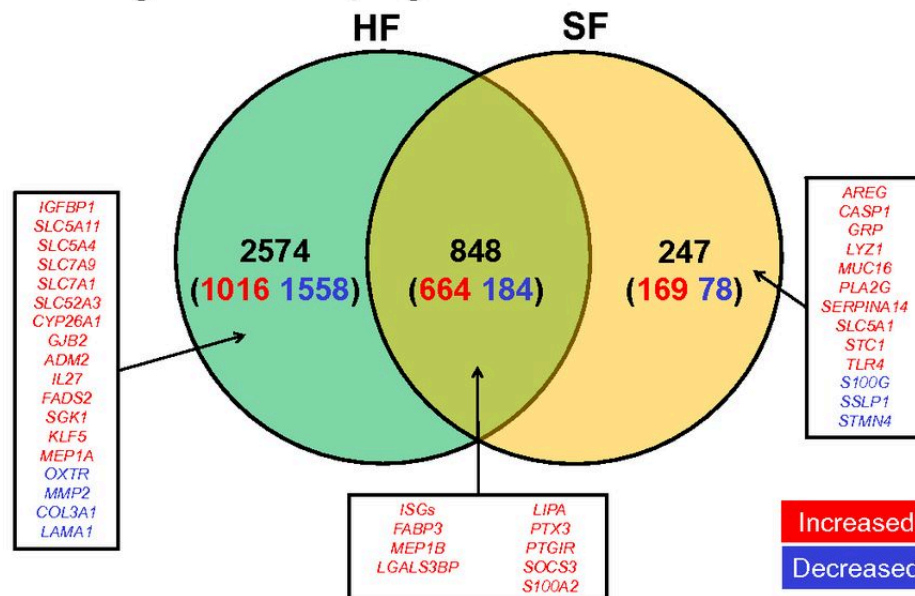


Figure 3. Transcriptome analysis of endometrium from nonpregnant and pregnant day 17 heifers.

Total RNA was extracted from five nonpregnant high fertile (HF), subfertile (SF), and infertile (IF) heifers and five pregnant HF and SF heifers. Normalized and log2 transformed

read count data and differentially expressed genes (FDR $P < 0.05$) were determined by edgeR-robust analysis. A) Venn diagram showing the number of unique or common transcripts between the endometrium of nonpregnant HF, SF and IF heifers. B) Venn diagram showing the number of unique or common pregnancy-regulated transcripts in the endometrium of HF and SF heifers. For this analysis, differentially expressed genes were determined in the endometrium of nonpregnant and pregnant HF or SF heifers and used to determine number of unique or common DEGs.

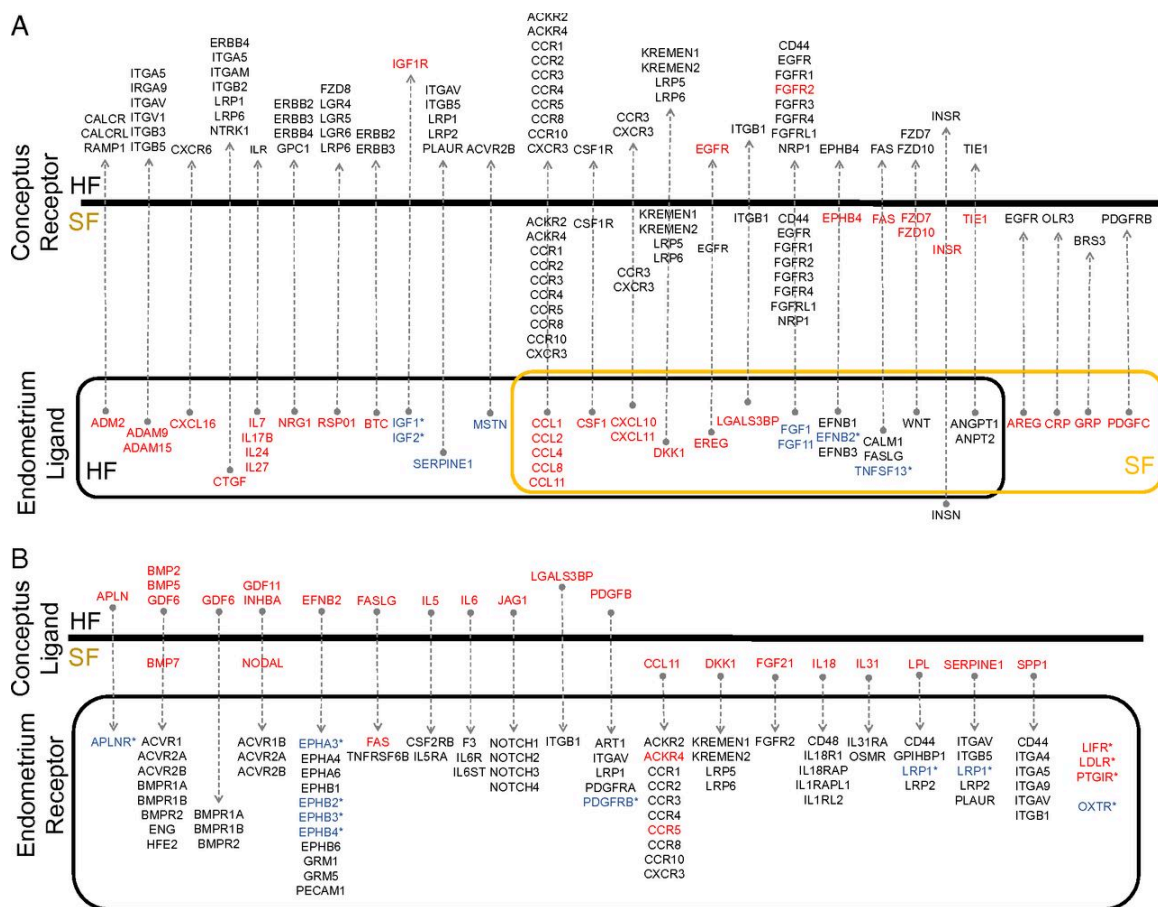


Figure 4. Receptor-ligand interactions between conceptus and endometrium at day 17 of pregnancy in high fertile (HF) and subfertile (SF) heifers.

(A) Diagram of select ligands from the endometrium and receptors in the conceptus. The endometrium ligand (on bottom) are shown as either commonly differentially expressed in both HF and SF endometrium (overlapping box) or exclusively to pregnant HF (black box) or SF (orange box) endometrium. (B) Diagram of select ligands from the conceptus and receptors in the endometrium. Genes shown in red have higher expression levels than those in black, and those in blue have lower expression than those in black.

SECTION IV:

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

Joao G.N. Moraes, Susanta K. Behura, Thomas W. Geary and Thomas E. Spencer

ABSTRACT

Preimplantation growth of the conceptus (embryo and associated extraembryonic membranes) is highly dependent on the substances present in the uterine lumen. Here, we investigated glucose, prostaglandins (PGs) and lipids in the uterine lumen of pregnant and nonpregnant fertility-classified heifers to explore the biological mechanisms regulating conceptus growth and survival during the time of maternal recognition of pregnancy (day 17). The transcriptional profile of endometrium and conceptuses were utilized to further explore the metabolism of glucose, PGE₂, PGF₂ α and PGI₂ during early pregnancy. Differences in the transcriptome were observed between conceptuses collected from heifers classified as high fertile (HF) or subfertile (SF) for the expression of genes encoding glucose transporters and among genes involved with glycolysis and gluconeogenesis. Uterine luminal concentrations of glucose were increased by pregnancy, but did not differ among fertility-classified heifers. PGE₂ and PGF₂ α were increased in the uterine lumen by pregnancy, and HF heifers tended to have higher uterine luminal concentrations of PGE₂, PGF₂ α and 6-keto-PG₁ α than SF heifers. Differences were observed in expression of genes regulating PG signaling, arachidonic acid metabolism, and PPAR signaling, among conceptuses and endometrium from fertility-classified heifers. Phosphatidylcholine was the main lipid class which increased in the uterine lumen of HF heifers by pregnancy. Expression of several genes involved with lipid metabolism differ between HF and SF conceptuses, and a number of fatty acids were differently abundant in the uterine lumen of pregnant HF and SF heifers. The observed differences in uterine luminal biology during early pregnancy are hypothesized to influence uterine receptivity and pregnancy establishment in cattle.

INTRODUCTION

The uterus clearly impacts conceptus (embryo/fetus and associated extraembryonic membranes) survival and programs its development, thus affecting pregnancy success [179, 398-401]. After hatching from the zona pellucida (days 9–10), the bovine blastocyst slowly grows into an ovoid or tubular form on days 12–14 and is then termed a conceptus [36]. The conceptus is only about 2 mm in length on day 13, about 6 mm by day 14, reaches about 60 mm (6 cm) by day 16, and 20 cm or more by day 19 [36, 95]. The preimplantation growth of the conceptus is highly dependent on the substances present in the uterine lumen. Uterine glands are present in the endometrium of all mammals [105], and their secretions constitute an important component of the histotroph, which is essential for preimplantation conceptus development in sheep [76, 77]. Histotroph in the uterine lumen of cattle has been characterized [81-85] and is a complex mixture of amino acids, glucose, lipids, proteins, vitamins, ions, cytokines, hormones, growth factors, among other substances. Little is known about how different levels of specific constituents in the uterine lumen regulates pregnancy success in cattle.

Glucose is an essential nutrient for preimplantation conceptus development [138, 139] and its concentration in the uterine lumen rises around time of conceptus elongation in sheep [139] and cattle [141]. Although the capacity of the uterus to generate free-glucose through gluconeogenesis is debatable [144-146], glucose transporters are thought to play an important role modulating intrauterine concentrations of glucose during pregnancy [138]. Mammalian cells express three families of glucose transporters, the facilitative glucose transporter family (solute carriers SLC2A/GLUT), the Na⁺/glucose cotransporter family (solute carriers SLC5A/SGLT) [136], and the recently discovered SWEET (SLC50) sugar transporter [149, 150], and the uterus expresses several members of these families

[151], including SLC2A1, SLC5A1 and SLC5A11 whose expression increase in pregnant compared to cyclic endometrium in sheep [138].

Prostaglandins (PG) are lipid hormones derived from arachidonic acid [402], and are essential for conceptus development. In sheep, intrauterine infusion of meloxicam, a selective inhibitor of prostaglandin-endoperoxide synthase 2 (PTGS2), inhibited conceptus elongation [218]. Concentrations of PGs in the uterine lumen increase during early pregnancy in ruminants [138, 204], because both the conceptus and endometrium produce prostaglandins, and endometrial production of PGs is further stimulated by interferon tau (IFNT) [120, 205], the signal for maternal recognition of pregnancy which is secreted predominantly by the trophoctoderm of the elongating bovine conceptus after day 15 [109]. The classical signaling of prostaglandins 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 [212, 213]. Nonetheless, selected PGs can also activate nuclear receptors [206-208]; PGI₂ can signals through the peroxisome proliferator-activated receptor (PPAR) delta (PPARD) [214], and 15-deoxy- $\Delta^{12,14}$ -PGJ₂, a metabolite of PGD₂, can activate PPAR gamma (PPARG) [215, 216]. 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 [175].

Lipids are another important component present in the uterine lumen. In addition to being a precursor of eicosanoids [403], they play many critical roles in the body, such as serving as energy source and storage, providing substrates for membrane biogenesis, and acting as signal molecules [169]. The lipid content of the uterus fluctuates throughout the estrus cycle in several species [170] and the accumulation of lipid droplets in the

endometrium luminal epithelium (LE) during the estrus cycle in sheep is attributed to progesterone from the ovarian corpus luteum (CL) [171, 173, 404], and this lipid accumulation has been hypothesized to serve as a reservoir to allow an increase in lipid availability in the uterine lumen during early pregnancy [172, 173]. Progesterone also increases the number of extracellular vesicles (EV) in the uterine lumen during the period of conceptus elongation in sheep [405], and the lipid component of EVs is hypothesized to be important to support the growth of trophoblast cells during conceptus elongation [201]. 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 [172, 174-177], which are expressed by both, the bovine conceptus and endometrium [94, 179]. Although fatty acids are essential biological components, uterine lumen fatty acid constitution 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 (n=3-4 rounds) of a single IVP embryo (Grade 1) on day 7 followed by pregnancy determination on day 28. Interestingly, conceptus development and survival by 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 [328]. Nonetheless, in a subsequent study, we demonstrated 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 [179]. Because differences in endometrial transcriptome can translate into differences in the uterine lumen constituents [192], the focus of the current study was to investigate concentrations of glucose, prostaglandins (PGE₂, PGF₂ α and 6-keto-PG₁ α metabolite of PGI₂ [406, 407]) and lipids in the uterine lumen from fertility-classified heifers, to gather information related to the mechanisms influencing conceptus development and survival in heifers with distinct uterine capacity to support pregnancy, and to detect differences in uterine lumen constituents that are induced by pregnancy.

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=22; 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), and the uterine lumen gently flushed with 20 ml of sterile and filtered 1X PBS. 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 pregnant (HF, n=16; SF, n=9; IF, n=1) and open (HF, n=6; SF, n=5; IF, n=5) ULF and blood plasma were measured using the MyQubit Amplex® Red Glucose Assay with Qubit® 3.0 Fluorometer, following the manufacture instructions. The Amplex® Red/UltraRed stop reagent was used to terminate the fluorescence signal-generating reaction in the enzymatic assay, and fluorescence was measured 15 minutes after the stop reagent was added.

Eicosanoids Analysis. The assay was conducted at the Eicosanoid Core Laboratory of Vanderbilt University on ULF samples collected from pregnant (HF, n=5; SF, n=5) and open (HF, n=5; SF, n=5; IF, n=5) heifers. To quantify PGs, a total of 100 uL of each sample was placed in a microcentrifuge tube containing 25% methanol in water (500 uL) and internal standard (d₄-PGE₂ and d₄-LTB₄, 1 ng each). The sample was vortexed and then extracted on an Oasis MAX µElution plate (Waters Corp., Milford, MA) as follows. Sample wells were first washed with methanol (200 uL) followed by 25% methanol in water (200 uL). The sample was then loaded into the well and washed with 600 uL 25% methanol. Eicosanoids were eluted from the plate with 30 uL 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., Milford, 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.

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 pregnant (HF, n=5; SF, n=5) and open (HF, n=5; SF, n=5; IF, n=5) heifers.

Unless otherwise stated, all reagents used were of LC-MS grade and obtained from Fisher Scientific (Fairlawn, NJ). A sample of 100 mg was weighed into a clean 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 0-0.5 min with a linear increase to 90%B from 0.5 to 7 min, a further increase to 95% B from 7-8 min and then held constant at 95% B from 8-21 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 100-700. 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, 100C probe temperature, 300C 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.

Untargeted Lipidomic Analysis. Global lipidomics was conducted on ULF from open (n=5) and pregnant (n=5) HF heifers. 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 μ m, 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 $\mu\text{L}/\text{min}$ with a column temperature of 50°C . 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 Analyses. Statistical analyses for measurements of glucose, prostaglandins, and fatty acids were conducted using SAS (SAS Institute Inc., Cary, NC). All continuous data was assessed for normality using the UNIVARIATE procedure of SAS. Glucose concentrations in ULF were not normally distributed and were log transformed. The effect of pregnancy status (pregnant vs non-pregnant), conceptus number (1 vs 2 conceptuses), and fertility classification on glucose, prostaglandins, and fatty acids concentrations were determined by 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, and the correlation between conceptus size (for heifers that had only one conceptus recovered) and glucose in the ULF were determined using the CORR procedure.

For the lipidomics data, statistical analysis was performed on MetaboAnalyst 4.0 [408-410]. 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 [411]. Fold change (FC) analysis was conducted to identify features that increased or decreased with pregnancy, and the standard fold change threshold

of 2 was used. A t-test was used to investigate if features were differently ($P < 0.05$) abundant between pregnant and open ULF.

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 [179]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>) was used for identifying genes associated with glucose, prostaglandins and lipid metabolism.

To explore the relationship of endometrium transcriptome and ULF composition, we first compared data from day 17 endometrium from pregnant (HF, $n=5$; SF, $n=5$) and open (HF, $n=5$; SF, $n=5$) heifers, to detect differences in endometrium gene expression associated with the metabolism of glucose, PGs and lipids that are normally induced by pregnancy. Then, we compared the endometrium transcriptome of only pregnant heifers (HF, $n=5$ versus SF, $n=5$), to explore differences in endometrium gene expression that could be associated with the detected differences in ULF composition, and possibly, related to the pregnancy loss occurring in SF heifers.

A similar approach was used to investigate the association 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 [29, 36, 94, 328, 330] and the conceptus transcriptome changes as it develops [94], 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. Then, 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.

RESULTS

Glucose Concentrations in ULF. Glucose concentrations in ULF were higher ($P < 0.01$) in pregnant compared to nonpregnant heifers on day 17 (Figure 1A). The number of conceptuses in the uterus of pregnant heifers did not influence ($P = 0.43$) glucose concentrations in the ULF (Figure 1B). Among pregnant heifers, there was no effect ($P = 0.19$) of fertility classification on ULF glucose concentrations (Figure 1C). Furthermore, ULF glucose concentrations did not differ ($P = 0.66$) among open fertility-classified heifers (Figure 1D).

Circulating concentrations of glucose in plasma were not different ($P = 0.35$) between pregnant and nonpregnant heifers (Figure 1E), between heifers with one versus two ($P = 0.90$) conceptuses present (Figure 1F), or among pregnant ($P = 0.82$) fertility-classified heifers (Figure 1G). Among open animals, SF heifers had higher ($P = 0.02$) plasma glucose concentrations than IF heifers (Figure 1H).

In pregnant heifers, glucose concentration in ULF was correlated (Figure 2A) with glucose concentrations in plasma ($r = 0.43$; $P = 0.03$), but there was no correlation ($r = 0.44$; $P = 0.13$) between conceptus size and ULF glucose concentrations (Figure 2B).

Transcriptome analysis of endometrium and conceptuses for glucose transport and metabolism genes. Expression of genes encoding glucose transporters (Dataset S1) that

increased in pregnant compared to open endometrium (SLC2A1, SLC5A1, SLC5A11, SLC35D2 and SLC5A4) were not different ($\text{FDR} \geq 0.68$) between pregnant HF and SF endometrium (Table 1). Interestingly, expression of other glucose transporters (SLC2A10, SLC2A3 and SLC37A4) decreased ($\text{FDR} < 0.05$) with pregnancy, but their expression was also not different ($\text{FDR} \geq 0.65$) in pregnant endometrium of HF and SF heifers (Table 1).

No differences were observed in 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 in the elongating conceptuses were SLC2A1, SLC2A5, SLC37A1, SLC2A8 and SLC2A13 (Dataset S2). Interestingly, expression of SLC2A2 (GLUT2) and SLC2A4 (GLUT4) was higher in SF than HF conceptuses (Dataset S3).

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 S4 and Table 2), and expression of one gene (FBP1, fructose-bisphosphatase 1) was increased in pregnant endometrium of HF compared to SF heifers (Dataset S5 and Table 2). None of the selected genes involved with glycolysis and gluconeogenesis were differently expressed in short vs long conceptuses (Dataset S6), but expression of HK1, FBP1 and GALM was increased in HF conceptuses, and ALDOB and ALDH3B1 was increased in SF conceptuses (Dataset S7 and Table 3).

Prostaglandins in the ULF. Pregnant heifers tended ($P = 0.07$) and had ($P < 0.01$) greater concentrations of PGE2 and PGF2 α in the ULF than nonpregnant heifers (Figures 3A and 3E, respectively). However, ULF concentrations of 6-keto-PG1 $\square\square$ was not affected ($P = 0.18$) by pregnancy status (Figure 3I), because 6-keto-PG1 \square concentrations did not differ

among pregnant and open SF heifers ($P = 0.61$; LSMEANS open = 29.7 ± 8.3 ng/mL, pregnant = 23.5 ± 8.3 ng/mL), even though 6-keto-PG1 α was increased by 5.4 fold in the ULF of pregnant than open HF heifers ($P < 0.01$; LSMEANS open = 8.5 ± 6.4 ng/mL, pregnant = 45.8 ± 6.4 ng/mL). Furthermore, among pregnant heifers, the number of conceptuses present in the uterus (1 vs 2) did not influence ULF concentrations of PGE2 ($P = 0.39$; Figure 3B), PGF2 α ($P = 0.14$; Figure 3F), and 6-keto-PG1 α ($P = 0.50$; Figure 3J). Similarly, ULF PG concentrations were not different ($P \geq 0.32$) among open fertility-classified heifers (Figures 3D, 3H and 3L). Interestingly among pregnant heifers, ULF concentrations of PGE2, PGF2 α , and 6-keto-PG1 α tended ($P \leq 0.10$) to be higher in HF compared to SF heifers (Figures 3C, 3G and 3K).

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. Endometrium expression of PTGR1, PTGR3, PTGFR, PTGIR and PPARG was very low (FPKM < 1). 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 also 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 upregulated 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 pregnant endometrium (CYP2J2, PLA1A, ALOX12, CBR3, PLA2G3, HPGDS, LOC506594 (prostaglandin F synthase 1-like), PTGES3, CBR1 and PLA2G2A-2) (Dataset S8). However, none of the genes associated with arachidonic acid metabolism were differently expressed in endometrium of pregnant HF and SF heifers (Dataset S9). In conceptuses, expression of only one gene (LOC506594; prostaglandin F synthase 1-like) was increased in short compared to long conceptuses (Dataset S10), and expression of one gene (PLA2G15) increased and three (PLA2G1B, GPX1 and CBR1) decreased in HF compared to SF conceptuses (Dataset S11).

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 S12). 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 S13).

In conceptuses, expression of one gene (FADS2) in the PPAR signaling pathway increased in long compared to short conceptuses (Dataset S14), and expression of one gene increased (PDPK1) and 6 decreased (FABP3, APOA5, SORBS1, ACADL, LPL and ACADM) in HF compared to SF conceptuses (Dataset S15).

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 4A. 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 4A), accounting for about 90% of the total fatty acid content detected in the ULF (Figures 4B and 4C). 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% octacosaeptaenoic (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 4B and 4C). The remaining 28 fatty acids together accounted for only around 1.1% of the total fatty acid detected in ULF (Dataset S16). A heatmap for fatty acid concentration in the ULF across samples is presented in Figure 5, and 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 comparisons of only pregnant HF and SF heifers, concentrations of 8 fatty acids increased and one decreased in the ULF of HF compared to SF heifers (Table 5). Among 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 S17). 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 S18).

In conceptuses, expression of six genes associated with lipid metabolism differ between short and long conceptuses (Dataset S19). 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 differ between HF and SF conceptuses (Dataset S20). 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 (Dataset S21) and 2,020 features detected in the negative mode (Dataset S22). Only features identified based on fragmentation spectra (ddMS2) was 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 5 of the 9 lipids which increased by pregnancy in the FC analysis were significantly different in the t-test (Table 6). The differently abundant lipids included phosphatidylcholines, triacylglycerides and acylcarnitines.

DISCUSSION

The present study was conducted to investigate uterine luminal concentrations of glucose, prostaglandins and lipids in fertility-classified heifers to gain insights in the mechanisms regulating uterine receptivity. Conceptus elongation is characterized by rapid proliferation of trophectoderm cells, therefore sufficient energy and anabolic precursors are required to support cellular replication and specialization [153]. 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 [412, 413]. The blastocyst preferably uses glucose as energy source compared to embryos in earlier stages of development, and rather than oxidize the glucose consumed, aerobic glycolysis seems to be preferred pathway, which is a metabolic adaptation of rapidly proliferative cells known as Warburg effect [140, 154, 157]. This same pattern of glucose metabolism was observed during conceptus elongation in sheep [414]. Thus, glucose availability in the uterine lumen and the expression of glucose transporters by the elongating conceptus are essential for pregnancy success in ruminants.

Gluconeogenesis appears not to occur in the uterus of mice and humans, because all the necessary enzymes required for the synthesis of glucose from non-carbohydrate carbon substrates are not expressed in the uterus. The gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK, also known as PCK), which converts oxaloacetate into phosphoenolpyruvate and carbon dioxide, was not detected in mouse uterus [144], 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 [145]. In the present study, the expression of rate-limiting gluconeogenic enzymes (G6PC, PCK2, FBP1) increased in pregnant compared to open endometrium. Similarly, mRNA expression of those same 3 enzymes were found to increase in the intercaruncular endometrium from day 28 to 42 of gestation in cattle [146], 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. Nonetheless, the uptake of glucose into the uterine lumen through glucose transporters expressed by the endometrium appears to be the main pathway regulating uterine luminal concentrations of glucose in ruminants [138]. 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 [138, 139]. These glucose transporters are non-classical IFNT-stimulated genes [108] and were also found to be increased by pregnancy in the endometrium in the present study, thus likely contributed to the higher uterine luminal concentrations of glucose observed in the ULF of pregnant compared to open heifers. The moderate positive correlation observed between plasma and uterine luminal concentrations of glucose perhaps demonstrates the active transport of glucose from plasma into the uterine lumen through the up-regulated glucose transporters. Nevertheless, no differences in uterine luminal concentrations of glucose were 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. 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. Although no differences

were observed between short and long conceptuses for genes encoding glucose transporters, expression of SLC2A2 (GLUT2) and SLC2A4 (GLUT4) were higher in SF than HF conceptuses, which could indicate a dysregulated energy metabolism in SF conceptuses, as 5 genes involved with glycolysis and gluconeogenesis (HK1, FBP1, GALM, ALDOB, ALDH3B1) were differently expressed between HF and SF conceptuses.

Prostaglandins are major regulators of female reproductive function [415, 416] and modulate conceptus and endometrial gene expression during early pregnancy in ruminants [120]. In fact, PTGS2 was a key player regulating overall gene expression of day 17 bovine conceptuses [179]. In the present study, uterine luminal concentrations of PGE₂, PGF₂ α , and 6-keto-PG₁ α tended to be higher in the ULF of pregnant HF than SF heifers. Although no differences in endometrium and conceptus mRNA expression of selected prostaglandin 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 [109].

Prostaglandin E₂, PGF₂ α , and PGI₂ signals through their designated receptors, termed EP, FP, and IP, respectively. There are 4 subtype of PGE₂ receptors (EP1, EP2, EP3, and EP4) but only one PGF₂ α receptor (FP, also known as PTGFR) and one PGI₂ receptor (IP, also known as PTGIR) [209, 210]. Among the EP receptors, EP2 and EP4 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 signals through cAMP [417], 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 [231]. The luteotropic effects of PGE2 are well described [418], and it has been recently reported that intrauterine infusions of low doses of PGE1 blocked the luteolytic effects induced by intrauterine infusions of PGF2 α in cattle [419]. Because PGE1 and PGE2 acts through the same EP receptors [420], it is possible that the reduced uterine luminal concentration of PGE2 in pregnant SF heifers resulted in diminished luteotropic effects on the corpus luteum (CL), which might have contributed to the higher pregnancy loss in SF heifers.

In the present experiment, the expression of PGF synthase (PTGFS) by day 17 conceptuses was low, but the expression of the PGF2 α receptor (PTGFR) was the highest among the PG receptors investigated in the conceptuses, suggesting that paracrine actions of endometrial PGF2 α might be of particular importance regulating bovine conceptus elongation, and interestingly, HF conceptuses expressed higher PTGFR than SF conceptuses. Although the effect of PGF2 α on embryonic and trophoctoderm cell growth have not been investigated, PGF2 α has been shown to induce endometrial cell proliferation, angiogenesis, tissue remodeling, and to regulate the expression of genes involved with conceptus endometrium interactions [226, 421] which are important biological events occurring during conceptus elongation. Furthermore, higher expression of a PG transporter (SLCO2A1) was observed in HF than SF conceptuses, which could be a result of higher PGs available in the ULF of pregnant HF heifers, and may indicate higher PG signaling in HF conceptuses. The solute carrier organic anion transporter family member 2A1 (SLCO2A; also known as PGT) [422] is able to transport PGE1, PGE2, PGD2, PGF2 α and, to a lesser extent TXB2 [423]. 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 important role particularly 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) [424, 425]. The expression of another transmembrane PG transporter, the ATP-binding cassette, subfamily C member 4 (ABCC4; also known as MRP4) [426], which is expressed by the pig endometrium and conceptus [427] was not detected on day 17 endometrium or conceptus in the present study.

High uterine luminal concentrations of 6-keto-PG1 α was detected in open or pregnant heifers, and PGI₂ synthase was highly expressed by the endometrium in pregnant and open heifers and by conceptuses. 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 [428], the downstream effects of PGI₂ signaling in the endometrium and conceptus is poorly understood. PGI₂ is a strong vasodilator and a potent platelet inhibitor [429, 430]. Additionally, PGI₂ is essential for implantation and decidualization in mice [214], which is an event mediated particularly through the nuclear receptor PPARG. PGI₂ signaling through PPARG in sheep conceptuses does not seem to be important, as knock down of PPARG in trophectoderm cells using morpholino antisense oligonucleotides (MAO) did not disrupt conceptus elongation [431]. In contrast, the knock down of PPARG using MAO resulted in severely growth-retarded conceptuses [431] 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 [94]. Of note, PPARG which regulates FA catabolism in several tissues [432], was the highest PPAR expressed by day 17

endometrium, and perhaps 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 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 [433]. FABP3 modulates cell growth and proliferation [434] and is upregulated by pregnancy in the endometrium LE on days 15 and 18 of pregnancy in cattle [198].

Regarding the DEG involved with arachidonic acid metabolism, expression of glutathione peroxidase 1 (GPX1), a major antioxidant enzyme [435], was increased in SF than HF conceptuses, and may 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 [436]. PLA2G15 is localized in lysosomes and hydrolyses preferentially zwitterionic phospholipids (phosphatidylcholine - PC and phosphatidylethanolamine - PE) [436, 437], and PLA2G1B is described as a pancreatic lipase [436] 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 [438, 439]. This difference 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.

Surprisingly, day 17 ULF fatty acid content of pregnant and open heifers did not differ. Palmitic acid (16:0) and stearic acid (18:0) are the two most abundant saturated fatty acids in mammalian cells [188, 404], and together, accounted for around 90% of the total fatty acid detected in the ULF. Two unusual ultra long-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. Ultra long-chain polyunsaturated fatty acids (PUFAs) have also been found in the other mammalian tissues, such as brain, retina, skin, and testis [188, 440], and in the testis, 28:5 and 30:5 are essential for sperm maturation and male fertility [441].

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, although the differently abundant fatty acids had overall low abundance 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 [184, 442]. 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 β -oxidation reactions [188]. Fatty acid extension depend on elongases (ELOVL), which are enzymes that catalyzes carbon chain extension [443]. In the present study, ELOVL1 and ELOVL6 were upregulated in the endometrium by pregnancy. In addition, ELOVL5 was upregulated in HF conceptuses, and ELOVL7 was upregulated in SF conceptuses. Fatty acid desaturase 1 and 2 (FADS1 and FADS2) are essential enzymes for PUFA biosynthesis [444, 445], and expression of FADS1 was increased in HF and long conceptuses and decreased in SF and short conceptuses. Furthermore, FADS2 expression was increased in

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 biosynthesis of glycerolipids (GPAM) [446], phosphatidylcholines (CHKA) [447], ceramides (CERS1, ASAH1) [448, 449], steroid hormones (CYP11A1) [450], and with cholesterol esterification (ACAT1) [451]. The observed differences in lipid metabolism among HF and SF conceptuses likely contributed to the observed differences in fatty acid content in ULF among pregnant fertility-classified heifers.

PCs 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 [201, 405], as PC are a major component of the cell membrane in eukaryotes [452]. Although, PC have been reported as the most abundant phospholipid in sheep endometrium on days 3, 12 and 15 of the estrus cycle and pregnancy [453], and the main lipid component of uterine epithelial cells during the peri-implantation period in mice [454], pregnancy likely inhibits endometrium derived extracellular vesicles through interferon stimulated gene 15 (ISG15) signaling [455], thus conceptus derived EVs are likely responsible for the increased abundance of PC observed in pregnant ULF.

In summary, the current study describes the concentrations of glucose, PGs and lipids in the uterine lumen of fertility-classified heifers during the time of maternal recognition of pregnancy. 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, which consequently affects the uterine luminal contents and imparts conceptus survival and elongation.

ACKNOWLEDGEMENTS

The authors greatly appreciate the help of Dr. William R. Lamberson with statistical analysis, Kenneth Ladyman for his help caring for the animals, and Rick Disselhorst for coordinating the animal slaughter. This work was supported by NIH Grant 1 R01 HD072898 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

FIGURES

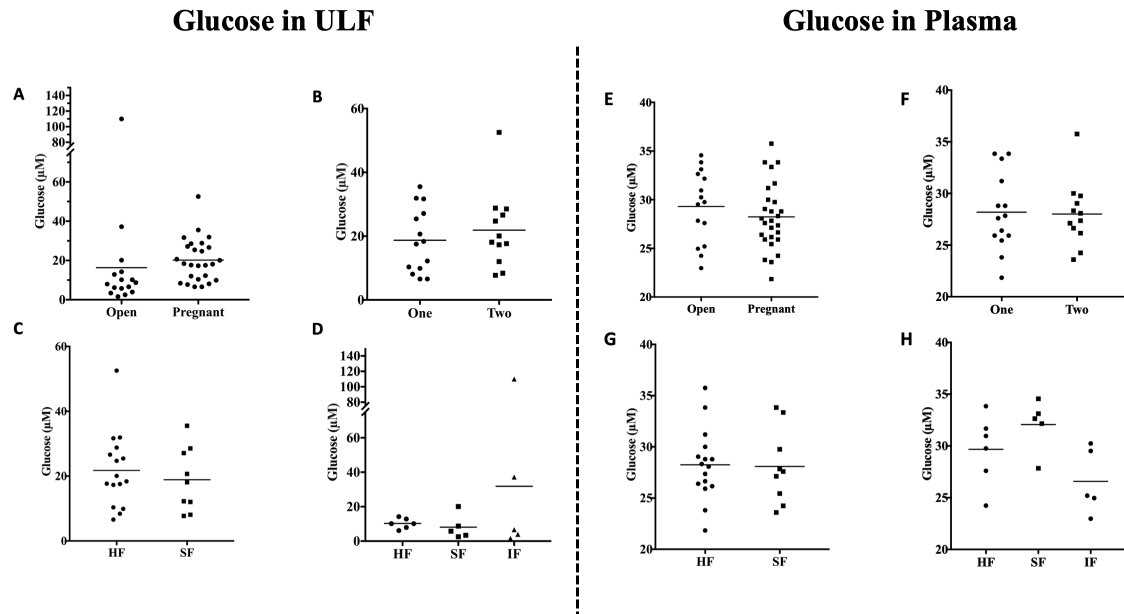


Figure 1. Glucose concentrations in the ULF and plasma of fertility classified heifers.

Glucose in the uterine luminal fluid (ULF) and plasma according to pregnancy status (**1A** and **1E**), among pregnant heifers with one or two conceptuses present in the uterine lumen (**1B** and **1F**), among pregnant fertility-classified heifers (**1C** and **1G**), and among open fertility-classified heifers (**1D** and **1H**). Statistical significance was only observed in two comparisons: (**1A**) Concentrations of glucose increased ($P < 0.01$) in pregnant than open ULF. (**1H**) Plasma glucose concentrations were higher ($P = 0.02$) in SF than IF heifers.

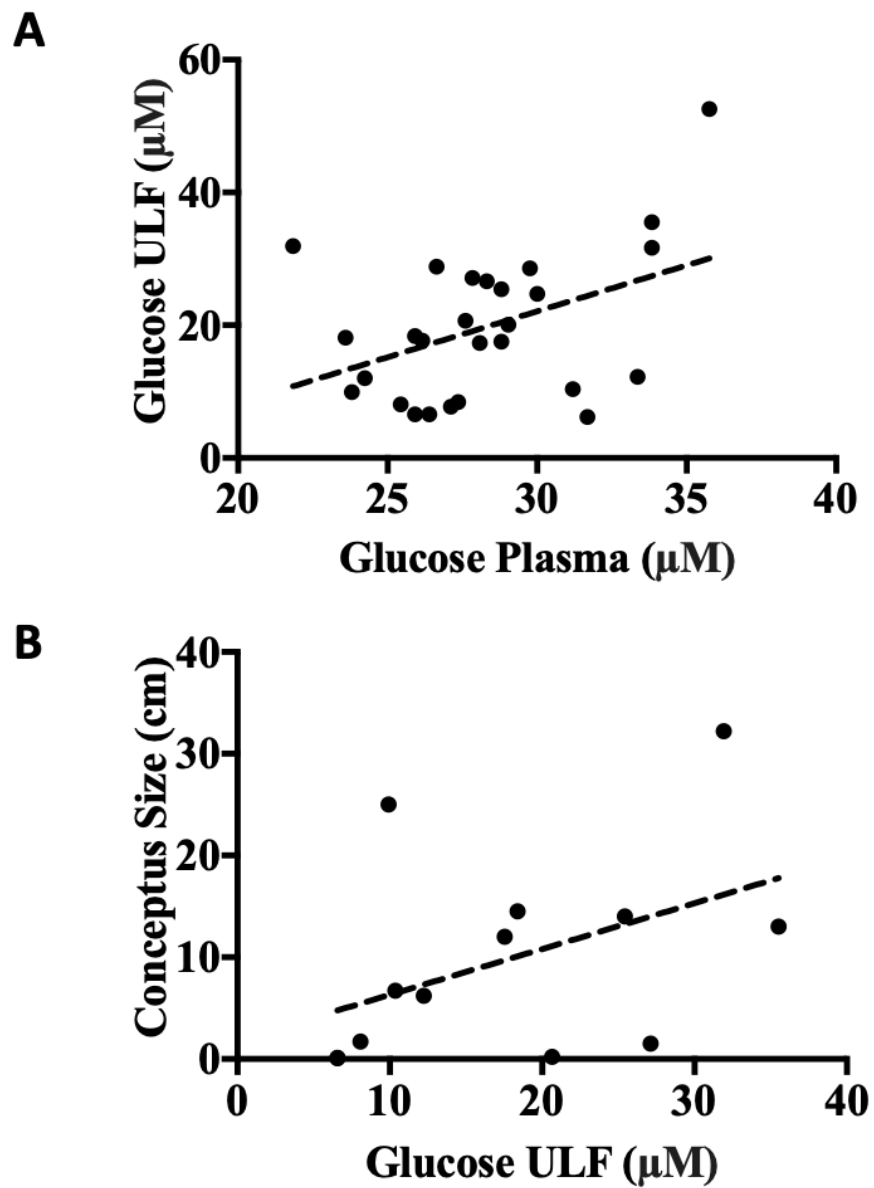
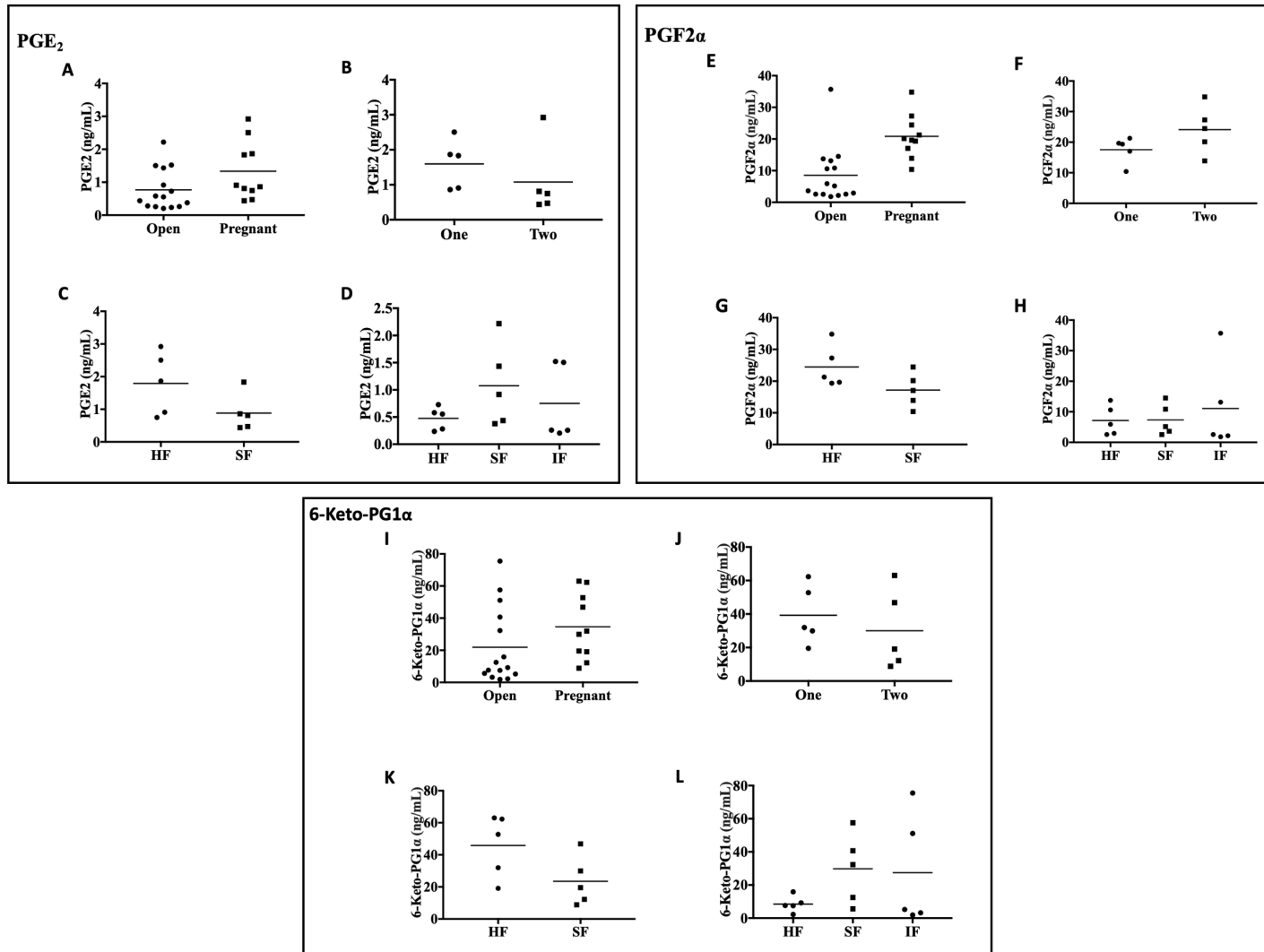


Figure 2. Correlations between ULF glucose, plasma glucose and conceptus size.

Pearson's correlation between glucose concentrations in plasma and in ULF ($r = 0.43$; $P = 0.03$) (A), and correlation between conceptus size and ULF glucose ($r = 0.44$; $P = 0.13$) (B).

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3 **Figure 3. Concentrations of prostaglandins in the ULF of fertility-classified heifers.**

4 ULF concentrations of PGE₂, PGF₂ α and 6-keto-PGF₁ α according to pregnancy status (**3A, 3E, and 3I**), among pregnant
5 heifers with one or two conceptuses present in the uterine lumen (**3B, 3F, and 3I**), among pregnant fertility-classified heifers
6 (**3C, 3G, and 3K**), and among open fertility-classified heifers (**3D, 3H, and 3L**).

7

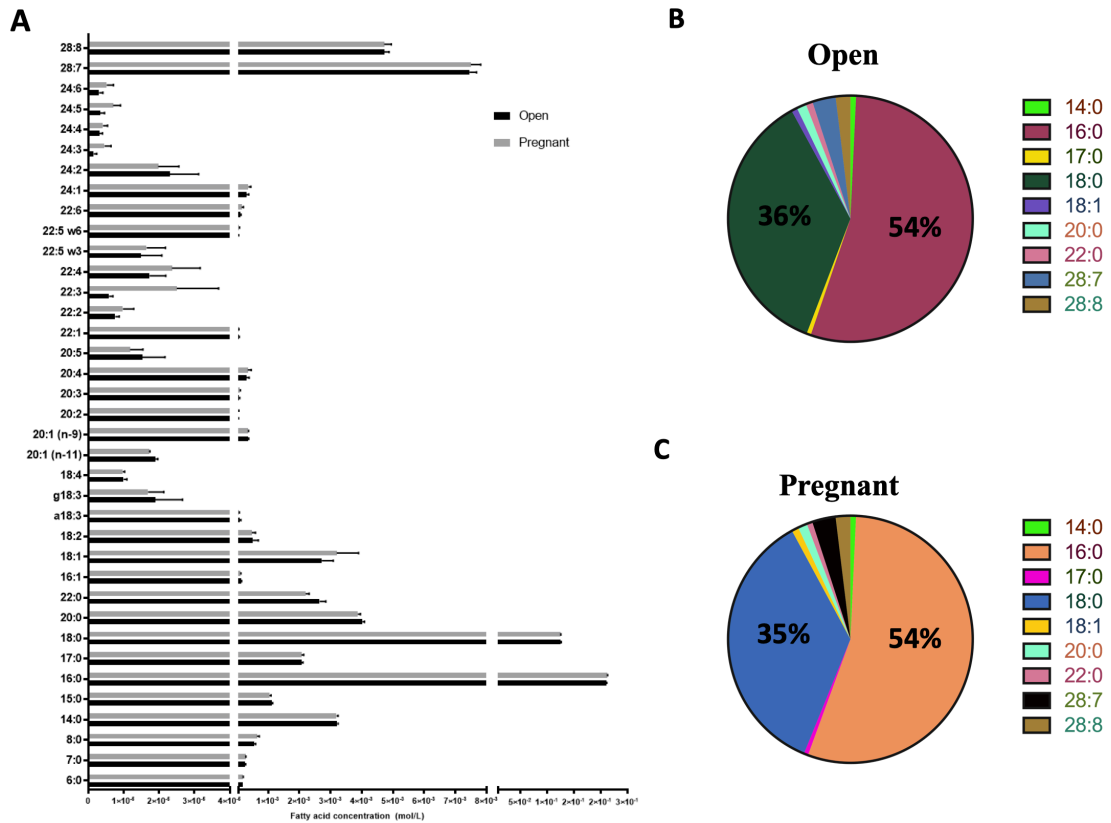
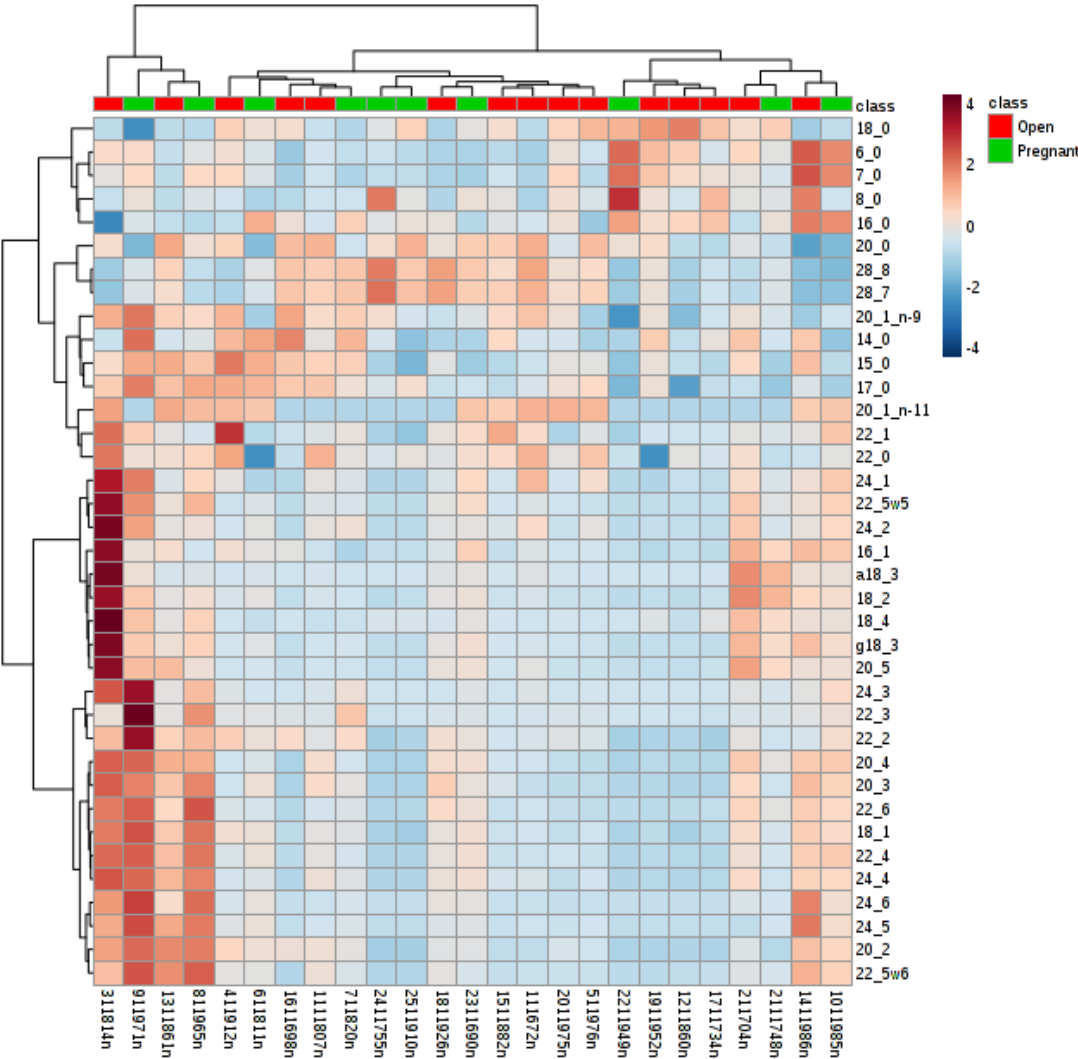


Figure 4. Fatty acids in ULF.

Concentrations of fatty acids identified in the ULF of the fertility-classified heifers (A), and the 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.

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17 **Figure 5. Heatmap for fatty acid concentration in the ULF across samples.**

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TABLES

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

	Gene name	Gene description	Pregnant vs Open (HF and SF)			Only Pregnant HF vs SF		
			FPKM ¹ OPEN	FPKM ¹ Pregnant	FDR	FPKM ¹ HF(P)	FPKM ¹ SF (P)	FDR
Increased in Pregnant	SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	68 ± 11	153 ± 19	0.00	174 ± 35	132 ± 13	0.68
	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
	SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	8 ± 0.5	5 ± 1	0.00	5 ± 1	6 ± 1	0.65
Endometrium	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

¹Data 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 of fertility-classified heifers for genes involved with glycolysis and gluconeogenesis using KEGG database

Comparisons	Gene name	Gene description	FPKM ¹	FPKM ¹	FDR
Pregnant vs Open			Open	Preg	
Increased in Open	LDHB	Lactate dehydrogenase B	125 ± 5	101 ± 2	0.04
	LDHA	Lactate dehydrogenase A	21 ± 2	13 ± 1	0.00
	PFKM	Phosphofructokinase, muscle	8 ± 0.4	5 ± 0.3	0.00
Increased in Pregnant	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
	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	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

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

Table 3. Expression of genes involved with glycolysis and gluconeogenesis in conceptuses from fertility-classified heifers using the KEGG database

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

¹Data 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		
		FPKM ¹ HF (P)	FPKM ¹ SF (P)	FDR	FPKM ¹ HF	FPKM ¹ SF	FDR
PG synthase							
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

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

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	22:0, 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, 24:2, 18:1, 22:5w6, 22:4, 22:2, 20:3, 20:2 8:0	Higher in HF than SF Higher in SF than HF
Only Open HF vs SF vs IF	22:1 22:0, 24:1	Higher in HF than SF Higher in HF than SF and IF

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

ID	Preg/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

SECTION V:

Analysis of the Uterine Lumen from Fertility-Classified Heifers: II Interferon-tau, Proteomics and Metabolomics

Joao G.N. Moraes, Susanta K. Behura, Jeanette V. Bishop, Thomas R. Hansen, Thomas

W. Geary, Thomas E. Spencer

ABSTRACT

The current experiment investigated pregnancy induced changes in uterine luminal fluid (ULF) of previously fertility-classified heifers with focus on interferon tau (IFNT), proteins and metabolites. IFNT concentrations in ULF were higher in pregnant heifers classified as high fertile (HF) than in subfertile (SF) animals. Although there were no differences in IFNT mRNA levels between HF and SF conceptuses, the increased IFNT abundance in ULF from HF heifers were presumably due to conceptus size. Nevertheless, no differences in endometrium 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, and not a major cause of reduced uterine receptivity in SF animals. As expected, pregnancy induced significant changes in the proteomics, and several proteins found to be increased by pregnancy, were also increased in the ULF of pregnant HF than SF heifers, including proteins involved in energy metabolism (ACAA2, ENO1, TKT, PGD), oxidative stress (GSS), amino acid metabolism (PSPH, GOT1, LAP3, WARS, AHCY) and cell proliferation and differentiation (RAC1, HSPB1, CDC42). In the metabolomics, pregnancy induced 271 differential metabolites in the ULF of HF heifers, but no differences were identified in ULF of SF heifers that were pregnant or open. The majority of the metabolites that increased in the ULF of pregnant HF than SF heifers were associated with energy and amino acid metabolism. Hence, the observed differences in ULF proteome and metabolome are hypothesized to influence uterine receptivity and consequently conceptus development and survival.

INTRODUCTION

During mammalian embryonic development, the oocyte is fertilized in the ampulla of the oviduct forming a zygote [88], which undergo several mitotic divisions to form a morula [89] that enters the uterus between days 4 to 6 post-mating in cattle [90]. The morula then develops into a blastocyst that contains an inner cell mass (ICM) and a blastocoele or central cavity surrounded by a monolayer of trophectoderm (TE) cells [91]. Upon blastocyst formation, two lineages of cells differentiate within the ICM: the epiblast (EPI) and the differentiated primitive endoderm (PrE) [456]. The cells from the ICM are pluripotent and able to form all three embryonic germ layers: the endoderm, mesoderm and ectoderm [92]. The transcription factor POU5F1 (also known as Oct-4) is a key regulator of pluripotency in mice embryos [457], and in ruminants, it has been reported to suppress the transcription of interferon tau (IFNT) by mononuclear TE cells during the blastocyst stage [458].

IFNT is the pregnancy recognition signal in ruminants, and it prevents luteolysis in cattle by suppressing expression of oxytocin receptor (OXTR) in the uterine luminal (LE) and glandular epithelia (GE), which abrogates the uterine luteolytic mechanism [108, 112, 113]. IFNT is a type 1 interferon (IFN) produced exclusively by mononuclear TE cells of the elongating conceptus in ruminants, and in cattle it is secreted predominantly between days 15-24 [109-111]. The transcriptional regulation of IFNT expression has been recently reviewed [122] and transcription factors such as POU5F1 [123] and T-box protein eomesodermin (EOMES) [124] have been reported to suppress IFNT expression, while others such as ETS2 [125-127], distal-less 3 (DLX3) [128], CDX2 [124, 129] and GATA2/3 [130, 131] have been implicated with increased IFNT transcription. Additionally, a co-activator, the cAMP-response element-binding protein-binding protein

(CREBBP), is expressed by the elongating ovine conceptus and can stimulate IFNT transcription [132]. Uterine derived factors also regulate IFNT transcription by the conceptus. For instance, colony stimulated factor 1 (CSF1), interleukin 3 (IL3), and fibroblast growth factor-2 (FGF2) are expressed by the endometrium LE and GE in sheep, and have been shown to induce conceptus expression of IFNT in vitro [133-135].

The denominated classical signaling of interferons (IFNs) is through the JAK/STAT (janus kinase/signal transduce and activator of transcription) pathway, but IFNs also activates non-classical signaling cascades, such as PI3K and p38 MAP kinase pathways [121]. The bovine endometrium and conceptus express the type I interferon receptor (IFNAR) [114, 115], which is a heterodimeric receptor composed of IFNAR1 and IFNAR2 subunits [116]. Thus, the elongating conceptus secretes IFNT that signals through IFNAR in the endometrium and stimulates the expression of classical and non-classical interferon-stimulated genes (ISGs), that along with progesterone induced changes in endometrium, modulate uterine gene expression which is essential for establishing uterine receptivity in ruminants [98, 117, 119, 120]. Although there are no detectable differences in uterine transcriptome of pregnant and cyclic heifers prior to day 14, by day 16, the endometrium transcriptome is greatly affected by the presence of a conceptus [253]. Pregnancy-induced changes in the endometrium transcriptome are hypothesized to influence uterine secretome composition, affecting conceptus development and pregnancy success [94, 192, 459]. 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 [85, 139, 460]. Additionally, pregnancy modulates the availability of proteins in the uterine lumen during

the preimplantation period in cattle [81, 459]. Alterations in the profile of uterine luminal components during early pregnancy are a result of complex conceptus-endometrium interactions, which regulates conceptus and endometrium gene expression, ensures corpus luteum (CL) maintenance, provides substrates for cell proliferation and differentiation while preventing cell damage through oxidative stress, and induces uterine receptivity, to support conceptus growth and pregnancy establishment. Thus, it is possible that heifers with reduced fertility have altered uterine environment which is less adequate to support conceptus growth and survival. Hence, in order to identify changes in the uterine luminal constituents which 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 (n=3-4 rounds) of a single IVP embryo (Grade 1) on day 7 followed by pregnancy determination on day 28. Interestingly, conceptus development and survival by 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 [328]. Nonetheless, in a subsequent study, we demonstrated 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 (Figure S1A and S1B, representative figure from [179]). Because differences in endometrial transcriptome can translate into differences in the components available in the uterine lumen [192], in the present study, we assay the uterine luminal fluid (ULF) for IFNT, and conducted global proteomics and untargeted metabolomics in the ULF, to gather information related to the

mechanisms influencing conceptus development and survival in heifers with distinct uterine capacity to support pregnancy, and to further explore the differences in uterine luminal components that are induced by pregnancy.

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.

Collection of Uterine Luminal Fluid. Fertility-classified heifers (HF, n=22; 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), and the uterine lumen gently flushed with 20 ml of sterile and filtered 1X PBS. The conceptuses were removed, if present, the ULF clarified by centrifugation (3000 x g at 4°C for 15 min), and the supernatant carefully removed with a pipette, mixed, divided into aliquots, frozen in liquid nitrogen, and stored at -80°C until analyzed.

IFNT Analysis. Bovine IFNT was generated as a glycosylated recombinant protein (rbIFNT) using bovine trophoblast protein 1 cDNA (bTP509) as template [461] and human HEK cells at 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 monoclonal antibody in mice (9.1.1; 16.2 µg/ml) and 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 validated to be specific for IFNT, and does not cross-react with IFN ω , IFN α/β or IFN γ . Uterine flush samples from open (HF, n=6; SF, n=1; IF, n=4) and pregnant (HF, n=15; SF, n=9; IF, n=1) heifers were analyzed in the same assay. Uterine flushing was 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.

ULF Proteomics. ULF proteomics was conducted by the Proteomics Center of the University of Missouri, Columbia, MO. Two procedures were used to investigate the proteomic profile of ULF from pregnant (n=5 for per group: HF and SF) and open (n=5 for per group: HF, SF and IF) fertility classified heifers. In the first procedure, ULF proteins were precipitated, and in-solution digestion performed. For a possibly increase in the number of proteins identified, a second procedure was conducted in which precipitated proteins were first loaded in a 12% acrylamide SDS-PAGE gel for size separation, and protein digestion was performed in slices of SDS-PAGE gels.

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 hours for protein precipitation. Samples were then centrifuged at 16,000 g for 10 minutes, and the supernatant discarded. Protein pellets were resuspended in a solution of urea/ammonium bicarbonate (6M urea, 100mM ammonium bicarbonate), digested with trypsin (www.osa.sunysb.edu/Proteomics/ProteinDigestPrep), and peptides purified by large-

format, 100uL, C18 tips (according to the manufacturer's instructions, Pierce/Thermo Scientific).

Description of the SDS-PAGE method. ULF protein concentrations were first determined using the Qubit® Protein Assay Kit with a Qubit® 3.0 Fluorometer, and variables volumes of ULF containing 200µg of protein were aliquoted. ULF proteins were precipitated as described in the in-solution method, and the protein pellet was resuspended in 20 uL of 1X laemmli buffer (60mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 100mM DTT), and loaded on one of three 12% acrylamide SDS-PAGE gels (Figure S2). After staining with colloidal coomassie blue, the gels were destained in water and each lane excised into 8 slices. Slices were arrayed in deep-well microtiter plates and 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 40µL solution of 5% acetonitrile and 1% formic acid. Half of the sample (20µL) was transferred to autosampler vials, and liquid chromatography tandem mass spectrometry (LC-MS/MS) performed on a LTQ Orbitrap XL™ mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA).

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 25cm x 150µm 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 2min ramp to 10%B. Gradient of 10-20%B over 35min, gradient of 20%B to 30%B over 40min, gradient of 30%B to 90%B over 5 min, hold at 90%B for 22 min, ramp back to (1min) and hold at (5min) initial conditions.

Fourier Transform Mass Spectrometer (FTMS) data were collected (30,000 resolution, 1 microscan, 300-1800m/z, profile, AGC 5e5) and then each cycle (approximately 3 seconds) the 9-most-abundant peptides (ignore +1 ions, ignore trypsin autolysis ions, pick peptides with > 1,000 counts) were selected for MSMS (2m/z mass window, 35% normalized collision energy, centroid). Dynamic exclusion was applied with following parameters: repeat count 1, repeat duration 30sec, exclusion list max 500, exclusion duration 180sec. 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 concatenated into a single FASTA file (253,732 total sequences). Sequest searches were performed with trypsin as enzyme, 2 missed cleavages allowed, carbamidomethyl cysteine as a fixed modification, oxidized methionine as variable mod, 25ppm 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 parts per million. 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 [462]. Further analysis was conducted on peptides identified as exclusive and unique to each protein following the removal of protein clusters. Total spectrum counts for proteins were used for comparisons and statistical analysis.

Untargeted Metabolomics. Global metabolomics analysis of ULF from day 17 pregnant (HF, n = 5; SF, n = 5) and open (HF, n = 5; SF, n = 5; IF, n = 5) heifers was conducted by the Southeast Center for Integrated Metabolomics (SECIM) at the University of Florida, Gainesville, FL. 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 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 ACE 18-pfp 100 x 2.1 mm, 2 μ m 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 have some limitations for the coverage of very polar species. The flow rate was 350 μ L/min with a column temperature of 25°C. A total of 4 μ L of each sample was

injected for negative ion mode analysis and 2 μ L was injected for positive ions mode analysis.

Statistical Analyses

Statistical analyses for measurements of IFNT in ULF were conducted using SAS (SAS Institute Inc., Cary, NC). IFNT concentrations were assessed for normality using the UNIVARIATE procedure, and because it was determined not normally distributed, it was 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 LSD option. Pearson's correlation for conceptus size and ULF IFNT concentrations were determined using the CORR procedure. Of note, the correlation analysis included only heifers which had only one conceptus present in the uterus, to remove the possible confounding effect of conceptus number on ULF IFNT concentrations.

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 when the analyses contained 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 C 4.0 (<http://www.metaboanalyst.ca>) [408-410, 463, 464]. For this analysis, a table matrix

of 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 standard fold change threshold of 2 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 patterns 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 [179] 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 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 [179].

To explore the relationship of endometrium transcriptome and ULF composition, we first compared data from day 17 endometrium from pregnant (HF, n=5; SF, n=5) and open (HF, n=5; SF, n=5) heifers, to detect differences in endometrium gene expression which are induced by pregnancy. Then, we compared the endometrium transcriptome of only pregnant heifers (HF, n=5 versus SF, n=5), to explore differences in endometrium gene expression that could be associated with the detected differences in ULF composition, and related to the pregnancy loss occurring in SF heifers.

A similar approach was used to investigate the association 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 [29, 36, 94, 328, 330] and the conceptus transcriptome changes as it develops [94], 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. Then, transcriptome of HF (n = 17) and SF (n = 10) conceptuses were compared for the same set of genes of interest, to investigate the relationship 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.

RESULTS

IFNT in the ULF. 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

concentrations (Figure 1B). Consistent with differences in conceptus length (Figure S1B), IFNT concentrations in ULF was higher in pregnant HF than SF ($P = 0.045$) and IF ($P = 0.02$) heifer(s) (Figure 1C). In addition, the amount of IFNT in the ULF was correlated ($r = 0.78$, $P < 0.01$) 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, or between HF and SF conceptuses (**Table 1**). In addition, endometrium expression of IFNT receptors (IFNAR1 and IFNAR2) was not affected by pregnancy and not different among pregnant HF and SF heifers. Expression of oxytocin receptor (OXTR) which was decreased in the endometrium by pregnancy, did not differ among pregnant HF and SF heifers. Furthermore, the expression of selected classical and nonclassical ISGs in the endometrium which increased by pregnancy were not different comparing only pregnant HF vs SF endometrium (**Table 1**).

The expression of selected genes involved with the transcriptional control of IFNT by the conceptus and endometrium [122] are presented in **Table S1**. Although the expression of POU5F1 by conceptuses was not significantly different between short and long ($FDR = 0.20$) or between HF and SF conceptuses ($FDR = 0.11$), a trend for higher expression of POU5F1 in less developed conceptuses was observed, as POU5F1 expression was increased by 2.1 folds in short than long conceptuses, and by 2.8 folds in SF than HF conceptuses. Additionally, EOMES expression by day 17 conceptuses was very low ($FPKM < 1$). Interestingly, conceptus from HF heifers had higher expression of IFNT transactivators DLX3 and GATA3 compared to SF conceptuses, and GATA3 and CREBBP also tended ($FDR < 0.1$) to be increased in HF than SF conceptuses.

Furthermore, among uterine secreted factors that stimulates IFNT transcription, colony stimulating factor 1 (CSF1) was increased in pregnant compared to open endometrium, but no differences were observed in the endometrium of pregnant HF and SF heifers (Table S1). Additionally, endometrium expression of FGF2 on day 17 was not affected by pregnancy, or between pregnant HF and SF heifers, and endometrium expression of CSF2 and IL3 was very low (FPKM<1).

ULF Proteomics. There was a total of 699 proteins identified using the in-solution method (Dataset S1) and 899 proteins identified using the SDS-PAGE method (Dataset S2). As expected, the SDS-PAGE method had higher sensitivity, and therefore a greater number of proteins were identified by this procedure.

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 differently abundant in both procedures (Table 2; Dataset S5).

In regard to the 103 different proteins in 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 3 cytoskeletal proteins (TUBB2B, TUBA1A, TUBB5), and regulation of insulin secretion, due to the increased abundance of 3 G-protein subunits (GNAI2, GNB2, GNAQ) in open ULF (Table S3).

Among all comparisons performed on the proteomics data (Table 2), 51% of the significant proteins identified using the in-solution method were also significant in the SDS-PAGE method. Thus, because the SDS-PAGE method had higher sensitivity, 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 pregnant than open ULF (Table 3) included embryonic secreted factors (TKDP1, PAG11), nuclear-envelop 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) and other membrane-bound proteins (PAS-6/7, FAM234A, RARRES1, GPC1), and a protein of the coagulation system (Factor V).

Furthermore, there were 221 proteins differently present in the ULF of pregnant HF and SF heifers, in which the abundance of 142 proteins increased and 79 decreased in pregnant HF than SF ULF (Dataset S6, Figure 2). Panther pathways associated with the 142 proteins that increased in pregnant HF than SF ULF 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 pregnant SF ULF compared to HF 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 endometrium and conceptuses. Among the 62 proteins commonly increased by pregnancy in both methods (**Dataset S5**), 56 were expressed by the endometrium based on RNA-seq data [179]. 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) which 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. Additionally, there were 11 proteins (ACO2, GDA, HSPA9, PREP, EEF2, HSD17B10, MTAP, PGM2, PSAT1 and TPI1) which increased in the ULF by pregnancy that were determined to be encoded only by genes expressed by the endometrium, not by conceptuses.

Additionally, 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 and GSS), but none of them were increased in pregnant SF than HF ULF (Dataset S6). Conversely, the expression of only 13% (5/39; 2 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. There was a total of 1,378 features detected (122 identified and 1,256 unknown) in the positive ion mode (Dataset S10) and 551 features 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. A summary of the differential metabolites (FDR < 0.05) detected in the ULF according to fertility classification and pregnancy status is presented on Table 4.

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

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 1 unidentified metabolite (m/z 109, retention time: 8.5 min) which was increased in the ULF of SF than HF and IF heifers (Dataset S18).

Results of FC analysis ($FC > 2$) comparing pregnant and open ULF are presented on Dataset S19 and S20, and Figures S3A and S3B. The top 20 metabolites with highest differences FC 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 luminal 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), and the top 5 pathways were urea cycle, glycine and serine metabolism, glutamate metabolism, and arginine and proline metabolism. Furthermore, the top 5 most significant pathways associated the differential metabolites between pregnant or open HF heifers included urea cycle, ammonia recycling, 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 ($\text{FDR} < 0.05$) and metabolites ($\text{FDR} < 0.05$) 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 Figure 6A). Interestingly, pathways associated with differentially expressed genes ($\text{FDR} < 0.05$) and metabolites ($\text{FC} > 2$) 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.

Endometrium expression of 5 genes encoding amino acid transporters was increased (SLC15A3, SLC7A1, SLC15A1, SLC7A9, and SLC3A2) and of 3 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) [465], was upregulated in HF than SF 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 5 most

expressed amino 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 were assessed by comparing the endometrium transcriptome of nonpregnant with pregnant animals. This analysis found 3,422 differently expressed genes (DEGs) in the endometrium of HF heifers, but pregnancy resulted in only 1,095 DEGs in the endometrium of SF heifers (Figure S1A). This diminished endometrial response to pregnancy was hypothesized as the main cause of reduced uterine receptivity in SF heifers [179]. The overall differences observed in 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 was observed among SF heifers that were pregnant or not. Several metabolites associated with glutamine metabolism were increased in pregnant HF than SF ULF, such as 3-hydroxy-3-methylglutarate by 6.6 folds, N-methyl-L-glutamate by 4.6 folds, and glutarate by 3.5 folds. 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 [159, 160]. Glutamine is 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) [466].

Activation of mTORC1 promotes cell growth and proliferation, and mediate the expression of several genes that regulates cell metabolism [467], playing important role during conceptus elongation [166].

Cancer cells and 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 [140, 154, 157]. 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 [156, 157]. This is a result of pyruvate being diverged from the TCA cycle and metabolized to form lactic acid [140], and glutamine metabolism provides a carbon source for the synthesis of lactic acid [158]. 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 diverts glucose to create macromolecular precursors for synthesis of fatty acids, amino acids, and carbohydrates [140]. In fact, the pentose phosphate pathway (PPP) plays a key role during in this process, producing ribose sugars for nucleotide synthesis, and generating sufficient levels of nicotinamide adenine dinucleotide phosphate (NADPH) for the biosynthesis of macromolecules [468]. Interestingly, in the present study, the abundance of two key enzymes which participates in the PPP were upregulated in the ULF of pregnant HF than SF heifers; transketolase (TKT) was increased by 47 folds, and 6-phosphogluconate dehydrogenase (PGD) was increased by 2 folds in the ULF of pregnant HF than SF heifers. Additionally, 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 uterine lumen, 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 [469]. Histidine availability in the uterine lumen during the preimplantation period has been observed to increase in cattle [85, 460] and sheep [139]. In the present experiment, histidine increased by 3.5 folds in pregnant than open ULF, and the expression of the histidine transporter (SLC15A3) increased by 4.6 folds in the endometrium by pregnancy. The SLC15A3 transporter is an ISG [460], and 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.

Glutamate and glutamine are precursors of arginine [470]. Arginine is a precursor of nitric oxide (NO) and polyamines (putrescine, spermidine, spermine and agmatine) which regulates key events during early pregnancy, such as angiogenesis, placentation and embryonic development [166, 471, 472]. The secreted phosphoprotein 1 (SPP1; also known as osteopontin), an extracellular matrix protein which is secreted by the uterine LE [77, 473], interacts with arginine and plays important role on conceptus elongation and attachment through the activation of mTOR complexes 1 and 2 (MTORC1 and MTORC2) [166, 474]. Interestingly, in the present study, the expression of a cationic amino acid (arginine, lysine and ornithine) transporter SLC7A1 [465] was 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 folds in the ULF of pregnant HF than

SF heifers. Lysine is another essential amino acid, and have been observed to induce cell proliferation in vitro and in vivo [475]. Lysine can be converted into carnitine [476], and two amino acids derivative of carnitine were upregulated in pregnant HF than SF ULF; the abundance of isovalerylcarnitine increased by 7.3 folds, and acyl-carnitine (5-OH) by 2.5 folds. The primary role of carnitine is associated with the transport of long-chain fatty acids from the cytosol into the mitochondria for β -oxidation, as acyl CoA (long chain fatty acid bound to coenzyme A) is not permeable to the inner mitochondrial membrane [477, 478]. 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. Additionally, L-carnitine also possess antioxidant [479] and anti-inflammatory [480] properties, which are important biological processes during early pregnancy [481, 482].

The importance of arginine and glutamine in porcine embryo development have been demonstrated [483-485]. Additionally, the knockdown of SLC7A1 in the ovine trophectoderm using morpholino antisense oligonucleotide (MAO) resulted in retarder conceptus development [470], indicating the importance of cationic amino acids during conceptus growth in ruminants. In the present experiment, the expression of SLC7A6, which mediates mediates arginine efflux in exchange with glutamine [486, 487] was increased in HF than SF conceptuses, and perhaps suggests abnormal arginine and in glutamine metabolism in SF conceptuses.

L-Methionine was increased by 13 fold in the ULF of pregnant than open heifers, and by 18 folds in the ULF of pregnant HF than SF heifers. Methionine is the initiating amino acid for the synthesis of proteins in eukaryotes [488, 489]. Preimplantation bovine embryos are able uptake methionine from its environment [490], and the absence of methionine in the culture media reduced blastocyst rate in bovine embryos produced in

vitro [491]. 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) [492, 493], a major antioxidant in mammalian cells [161]. Furthermore, GSH is also a key regulator of cell proliferation [494], and therefore might play important role during the exponential growth of the ruminant conceptus during elongation. Moreover, concentrations of GSH in the ULF have been observed to increase during early pregnancy in ewes [139]. In the present study, glutathione synthetase (GSS) was increased by 19 folds in the ULF of pregnant than open heifers, and by 3 folds in pregnant HF than SF ULF. Additionally, glutathione disulfide (GSSG), the oxidized form of glutathione (GSH), was increased by 5 folds in the ULF of open than pregnant heifers. The increase in GSSG in open ULF may be because nonpregnant heifers lacks conceptus derived glutathione reductase (GSR) and disulfide isomerases (PDI), which can converted GSSG back into GSH [161]. 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 [161].

The overall findings from the proteomics 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 suggests that HF heifer have improved uterine environment to support 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 regulated by conceptus signaling. Interestingly, 56% of these proteins were also increased in pregnant HF than SF ULF, and are involved in crucial biological events during the preimplantation period, such as energy metabolism (ACAA2, ENO1, MDH2, LOC615277, QPRT) [204, 495-498], glutathione synthesis (GSS) [499], attachment between trophoctoderm and uterine LE (HSPA9) [500], de novo purine biosynthesis (ATIC) [501], amino acid metabolism (PSPH, GOT1, LAP3, WARS, AHCY) [502-506], and cytoskeletal organization and cell signaling (ACTN4, CAP1) [507, 508].

Regarding the IFNT results, HF heifers had higher IFNT in the ULF than SF heifers, even though no differences in IFNT gene expression were observed between HF and SF conceptuses, or between short versus long conceptuses. Longer conceptuses have greater number of trophoctoderm cells, and therefore are able to secrete greater amounts of IFNT. As expected, IFNT in the uterine lumen was highly correlated with conceptus size ($r = 0.78$). Likewise, an earlier study reported no differences in IFNT mRNA level 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 [109].

We further explored conceptus expression of genes that regulate 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/co-activators also mediate important biological processes during preimplantation development. For instance, DLX3 and GATA2/3 play an important role mediating gene expression in the placenta, regulating trophoblast

differentiation, angiogenesis, and coordinating embryonic-extraembryonic signaling cross-talk [509-512], and CREBBP signaling controls vital cellular processes such as cell proliferation, differentiation and apoptosis [513-515].

Among uterine secreted factors that stimulates IFNT transcription, expression of CSF1 was increased in the endometrium of pregnant than open heifers, but no differences were observed between pregnant HF and SF heifers. Additionally, IFNT signaling in the endometrium appears to be similar between pregnant HF and SF heifers, as no differences in the endometrium transcriptome were observed for the expression of genes encoding the oxytocin receptor (OXTR) or selected classical and non-classical ISGs. This result was not surprising as very low concentrations of IFNT have been shown to induce ISGs expression in vitro [516, 517], and a lot more is secreted during conceptus elongation [518]. Thus, it is reasonable to speculate that IFNT actions in the endometrium to establish uterine receptivity [108, 117] were not substantially different between pregnant HF and SF heifers. Nonetheless, the adequate conceptus size or concentrations of IFNT that are necessary to successfully induce the state of uterine receptivity in cattle are still unknown, and may vary among individual animals.

Studies in sheep and cattle supports the idea that IFNT exits the uterus and induce the expression of ISGs in maternal tissues, such as on white blood cells (WBC), CL and liver [29, 519, 520]. Interestingly, IFNT infusions into the uterine or jugular vein in sheep during days 10-13 of the estrus cycle have been shown to protect the CL against luteolysis induced on day 11 by exogenous administration of PGF2 α , which demonstrates the luteotropic effects of IFNT [517]. Prostaglandin E2 is also luteotropic [418, 419], and PGE2 was similarly increased in the ULF of pregnant HF than SF heifers. Thus, although the CL was not investigated in the current study, it is reasonable to speculate that because

SF heifers had shorter conceptuses [179], the concentrations of IFNT and PGE2 in the ULF were insufficient to promote adequate luteotropic effects on the CL, which consequently, contributed to the observed reduced reproductive efficiency in SF heifers.

In summary, the current study found substantial differences in uterine luminal components of fertility-classified heifers. These differences are hypothesized to occur as a result of dysregulated conceptus-endometrium interactions in SF heifers, affecting conceptus growth and signaling, and consequently, leading to lower pregnancy rates in SF animals.

ACKNOWLEDGEMENTS

Authors would like to thank Dr. Brian Mooney and the Charles W Gehrke Proteomics Center of the University of Missouri for the support with the proteomics, Dr. William R. Lamberson for his help with the statistical analysis, Kenneth Ladyman for his help caring for the animals, and Rick Disselhorst for coordinating the animal slaughter. This work was supported by NIH Grant 1 R01 HD072898 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

FIGURES

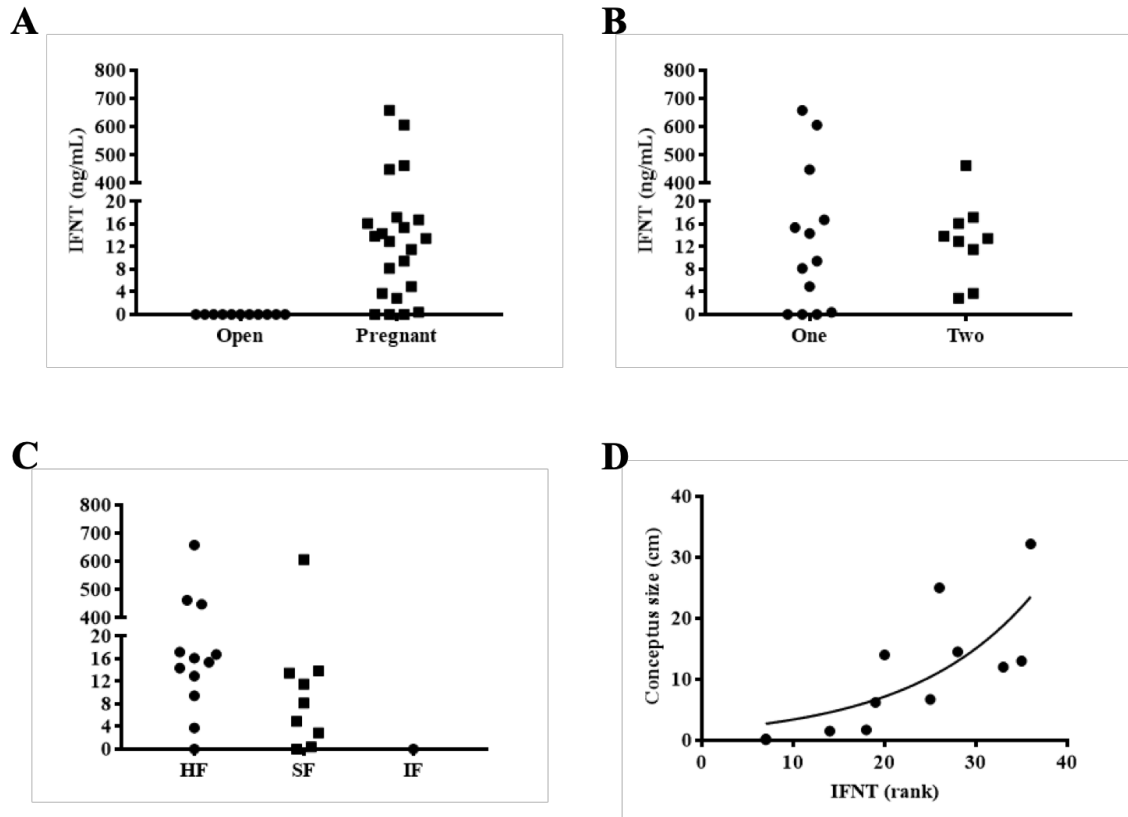


Figure 1. Concentrations of interferon tau in the ULF of fertility-classified heifers.

Interferon tau (IFNT) concentrations in the uterine luminal fluid (ULF) according to pregnancy status (**A**), among pregnant heifers with one or two conceptuses present in the uterine lumen (**B**), among pregnant fertility-classified heifers (**C**), and Pearson's correlation between IFNT in the ULF and conceptus size ($r = 0.78$, $P < 0.01$) (**D**).

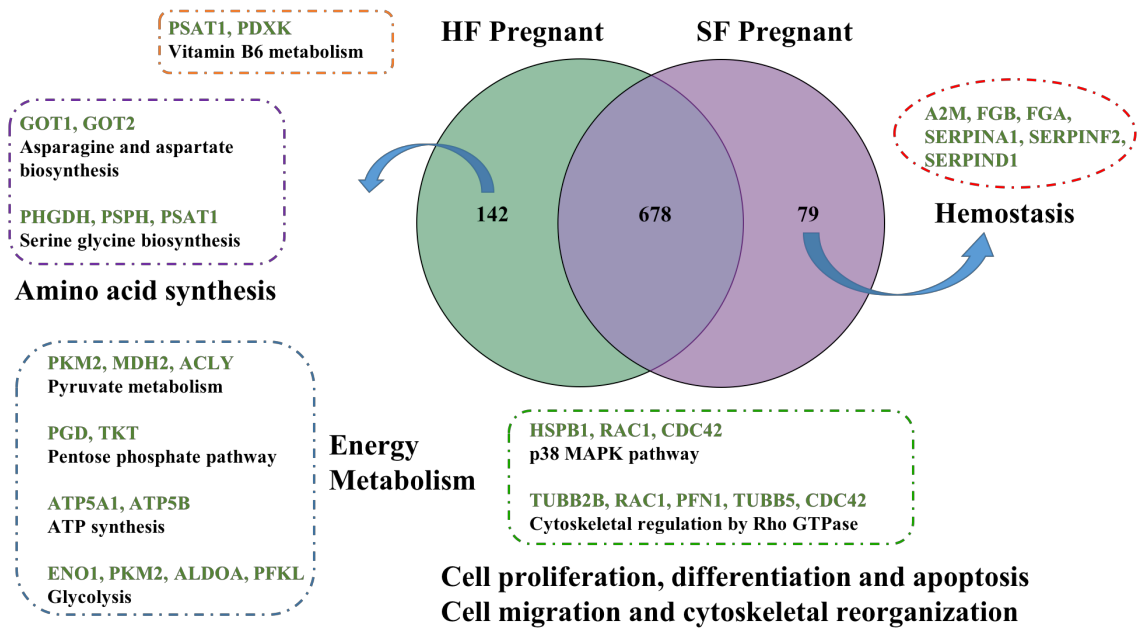


Figure 2. Venn diagram for proteins differently abundant in the ULF of pregnant HF and SF heifers according to the SDS-PAGE procedure.

Panther pathways associated with the differently abundant proteins are highlighted.

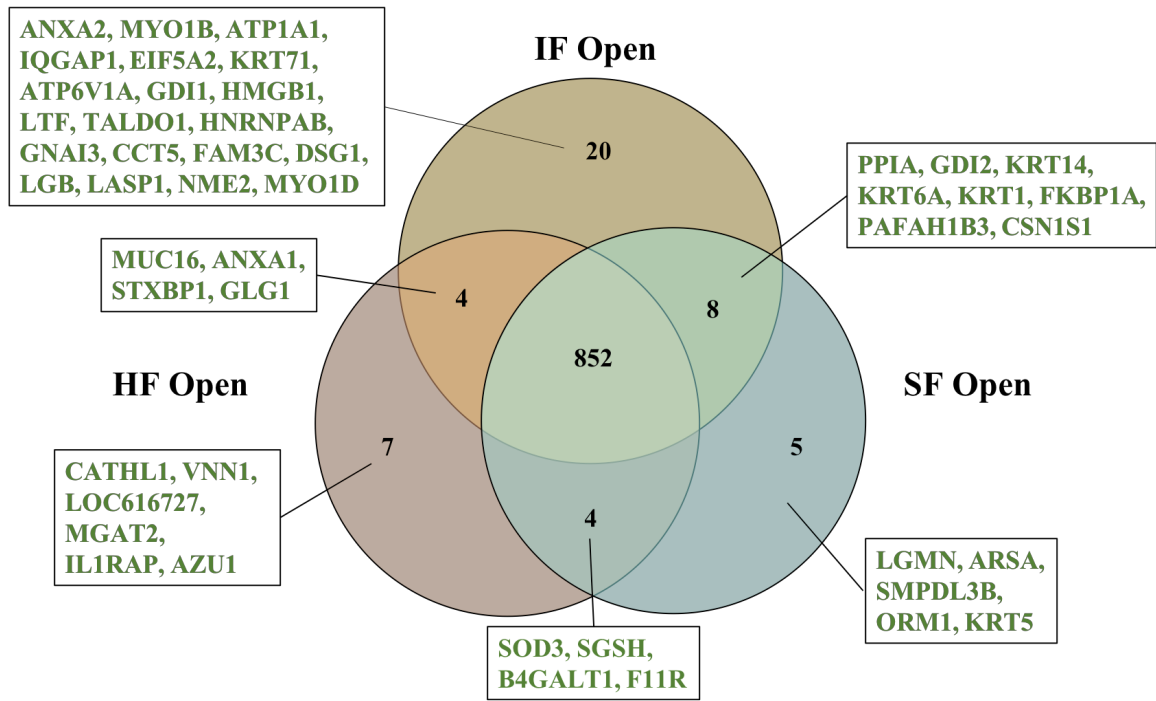


Figure 3. Venn diagram showing the differently abundant proteins among open fertility-classified heifers.

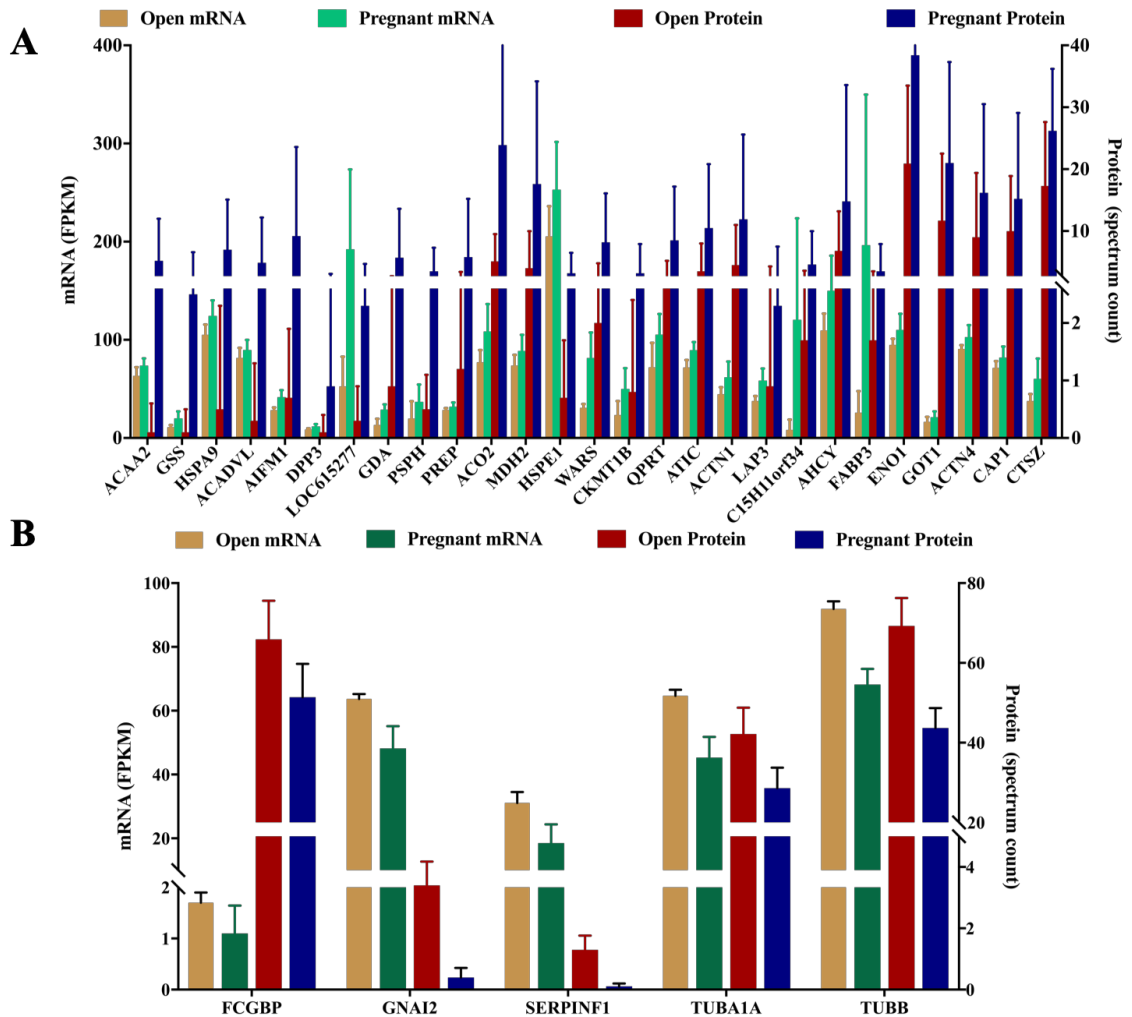


Figure 4. Selected genes/proteins hypothesized to be regulated by pregnancy based on combined data from endometrium transcriptome and ULF proteome.

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**).

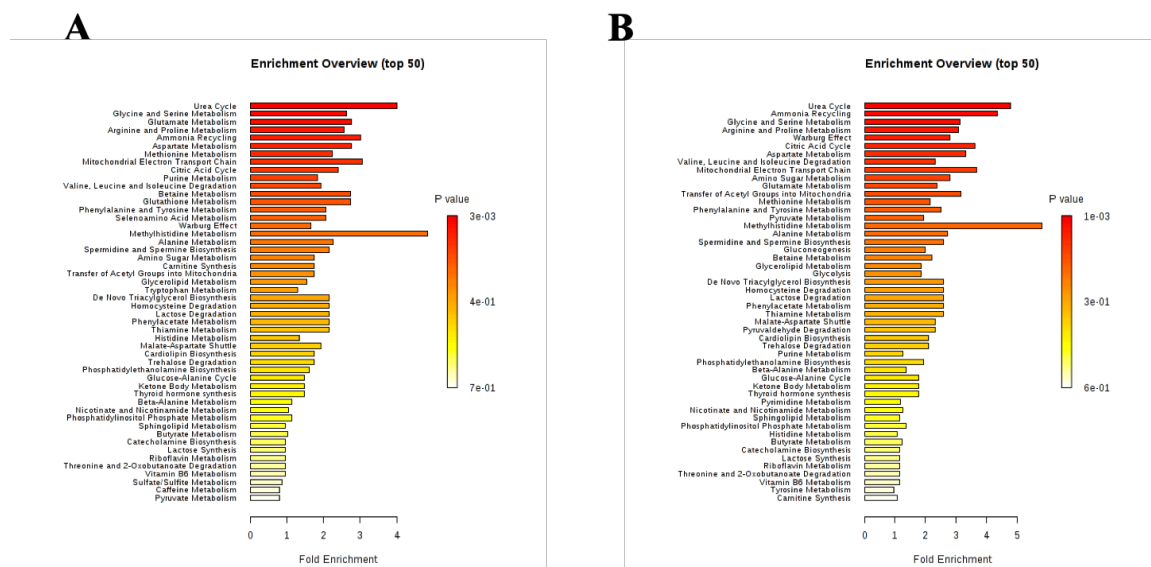


Figure 5. Metabolite set enrichment analysis (MSEA).

Pathways associated with the differential metabolites between pregnant and open ULF (**A**), and pathways associated with the metabolites that increased (fold change > 2) in the ULF of pregnant HF than SF heifers (**B**).

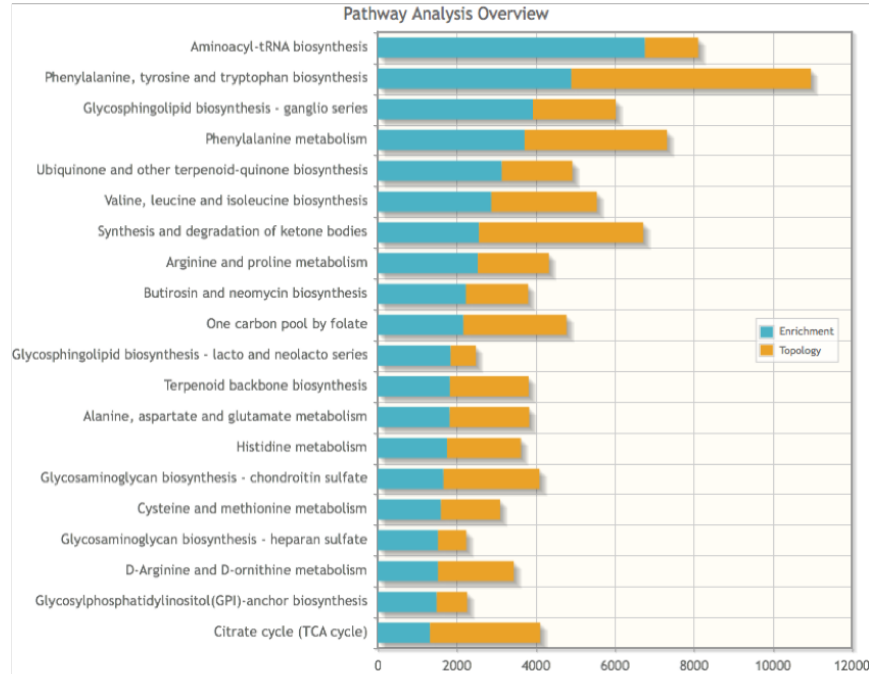
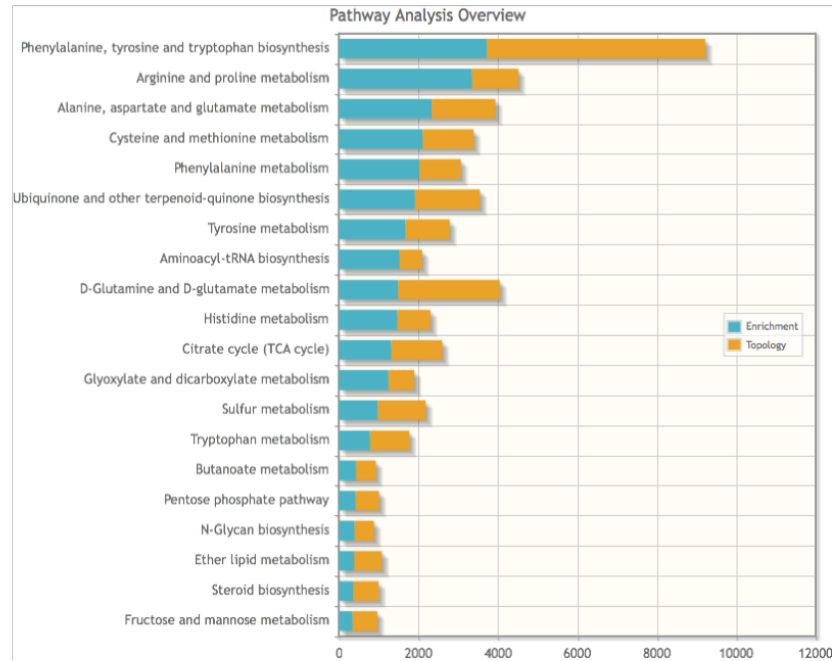
A**B**

Figure 6. Joint pathway analysis for endometrium transcriptome and ULF metabolome.

Joint pathway analysis using differently expressed genes (DEG) in the endometrium and the differential metabolites detected in the 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.

TABLES

Table 1. Expression of interferon tau (IFNT) by conceptuses and of selected classical and nonclassical interferon stimulated genes by the endometrium

Conceptus	Short FPKM ¹	Long FPKM ¹	FDR	HF FPKM ¹	SF FPKM ¹	FDR
IFNT	3 ± 0.9	2 ± 0.4	0.98	2 ± 0.4	4 ± 0.8	0.21
Endometrium	Open FPKM ¹	Preg FPKM ¹	FDR	HF Preg FPKM ¹	SF Preg FPKM ¹	FDR
IFNAR1	24 ± 1	22 ± 1	1.00	22 ± 2	22 ± 1	1.00
IFNAR2	13 ± 1	14 ± 0.5	0.45	13 ± 1	15 ± 0.3	0.80
OXTR	5 ± 2	1 ± 0.1	9.96E-05	1 ± 0.2	1 ± 0.1	0.93
Classical ISGs						
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

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

Table 2. Proteins differentially abundant in ULF by method.

Compariso ns	Significant proteins (P-value < 0.05)		
	In-solution method ^a	SDS-PAGE method ^b	Common different proteins
Preg vs Open	167	446	103
HF Preg vs Open	97	341	50
SF Preg vs Open	95	212	48
Only Preg HF vs SF	37	221	14
Only Open HF, SF, IF	27	48	2

^aA total of 699 proteins were identified in the in-solution procedure

^b A Total of 899 proteins were identified in the in-solution procedure.

Table 3. Top 10 proteins that increased or decreased in pregnant compared to open ULF

Identified Proteins	GenInfo Identifier	Protein	P-value	Open ¹	Preg ¹
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 ULF of open and pregnant heifers.

Table 4. Summary of the differential metabolites identified in the ULF.

Comparisons	Identified Metabolites	Unknown Metabolites	Total Metabolites
Preg vs Open	70	248	315
HF Preg vs Open	67	208	271
SF Preg vs Open	0	0	0
Preg HF vs SF	1	12	13
Open HF, SF, IF	0	1	1

Table 5. Top 20 identified metabolites with the highest differences in fold change in ULF of pregnant than to open heifers

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

Table 6. Top 20 identified metabolites with the highest differences in fold change in ULF of pregnant HF than SF heifers

Metabolites	m/z	Retention time	HF/SF - Fold Change (FC)	log2(FC)
Increased in HF				
L-Methionine	150.1	1.4	17.7	4.1
Isovalerylcarnitine	246.2	8.1	7.3	2.9
3-Hydroxy-3-Methylglutarate	161.0	2.9	6.6	2.7
N-Acetylneuraminate	308.1	0.8	6.2	2.6
N-Methyl-L-Glutamate	160.1	0.9	4.6	2.2
Glutarate	131.0	4.6	3.5	1.8
N-Alpha-Acetyl-L-Lysine	189.1	1.2	3.4	1.8
Fumaric Acid	115.0	2.0	2.6	1.4
4-Imidazoleacetic Acid	127.0	1.0	2.5	1.3
Acyl-Carnitine (5-OH)	262.2	6.7	2.5	1.3
Propionylcarnitine	218.1	6.8	2.4	1.3
2-Hydroxyglutarate	147.0	1.5	2.3	1.2
Malate	133.0	1.0	2.2	1.2
L-Glutamic Acid	146.0	0.8	2.1	1.1
Citrulline	174.1	0.8	2.1	1.1
Isobutyrylcarnitine	232.2	7.4	2.1	1.1
Nepsilon,Nepsilon,Nepsilon-Trimethyllysine	189.2	0.7	2.0	1.0
N-Acetyl-L-Alanine	154.0	2.2	2.0	1.0
Increased SF				
P-Cresol Sulfate	187.0	8.4	0.4	-1.4
Adenosine 5'-Monophosphate	348.1	1.5	0.3	-1.9

Table S1. Expression of selected genes involved with the transcriptional control of IFNT expression

Conceptus						
	Short FPKM ¹	Long FPKM ¹	FDR	SF FPKM ¹	HF FPKM ¹	FDR
IFNT supressors						
POU5F1	5 ± 2	3 ± 1	0.20	6 ± 2	2 ± 0.5	0.11
EOMES	0.3 ± 0.2	0.1 ± 0.0	0.45	0.3 ± 0.2	0.1 ± 0.0	0.37
IFNT transactivators						
ETS2	23 ± 3	17 ± 0.9	0.54	28 ± 2	28 ± 2	0.89
CREBBP	5 ± 0.8	4 ± 0.3	0.85	6 ± 0.5	9 ± 0.5	0.07
DLX3	3 ± 0.6	4 ± 0.5	0.67	3 ± 0.2	7 ± 0.5	7.87E-03
CDX2	26 ± 2	22 ± 2	0.52	42 ± 2	35 ± 2	0.24
GATA2	27 ± 5	28 ± 3	0.58	37 ± 3	54 ± 3	0.08
GATA3	21 ± 5	29 ± 4	0.086	19 ± 1	47 ± 4	2.05E-04
Endometrium						
	Open FPKM ¹	Pregnant FPKM ¹	FDR	HF Preg FPKM ¹	SF Preg FPKM ¹	FDR
Maternal factors						
CSF1	7 ± 0.4	14 ± 0.8	1.63E-14	15 ± 0.9	13 ± 1	0.43
CSF2	0.0 ± 0.0	0.1 ± 0.0	0.23	0.0 ± 0.0	0.1 ± 0.1	0.86
IL3	0.0 ± 0.0	0.0 ± 0.0	0.67	0.0 ± 0.0	0.0 ± 0.0	1.00
FGF2	13 ± 0.6	11 ± 0.7	0.77	10 ± 0.7	12 ± 1	0.77

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

CONCLUSIONS AND FUTURE DIRECTIONS

Collectively, these studies support the central hypothesis that the uterus directly influences conceptus development, and that the experimental model used to identify heifers with superior and inferior uterine capacity to support pregnancy was proven efficient. The studies conducted on day 14 established that: (1) circulating progesterone concentrations are not different among fertility-classified heifers; (2) conceptus development and survival by day 14 is not affected by fertility classification; (3) only minimal differences in endometrium transcriptome are detected among pregnant fertility-classified heifers. These results indicated that the mechanisms underlying subfertility and infertility manifests between days 14 and 28, which encompasses the period of maternal recognition of pregnancy, implantation, and beginning of placentation.

Next, uterine influences on conceptus development were investigated on day 17. Taken together, the available data indicated that the mechanism of pregnancy loss in fertility-classified heifers was manifested around the time of maternal recognition of pregnancy (Day 17), and the cause of pregnancy loss in SF heifers is likely a result of reduced endometrial response to pregnancy. Indeed, only minor differences in the endometrium transcriptome were observed among nonpregnant fertility-classified heifers on day 17, but the response of the endometrium to pregnancy was much reduced in SF (1,095 DEGs) than HF (3,422 DEGs) heifers. Moreover, analysis of ligand-receptor interactions indicated dysregulated conceptus-endometrium interactions in SF animals, reinforcing the theory of reduced endometrial response to pregnancy in SF heifers. Differences in uterine transcriptome may influence histotroph composition and uterine structure, and thus have the potential to affect preimplantation conceptus development.

Conceptus from HF heifers were on average twice as long than SF conceptuses on day 17, and their transcriptomes were significantly different (1,287 DEG). The substantial differences in the transcriptome of HF and SF conceptuses was hypothesized to be mediated by differences in uterine environment rather than differences in conceptus size, as only minor differences in transcriptome were detected between long and short conceptuses from HF heifers. Importantly, many of the genes that decreased in SF compared with HF conceptuses have been implicated with abnormal embryogenesis and with embryonic mortality using knockout mouse models.

Analysis of day 17 ULF revealed new and important information regarding histotroph composition during early pregnancy, as well as provided further insights on the biology of subfertility in cattle. IFNT, the major conceptus secreted factor involved with maternal recognition of pregnancy [1-3], was more abundant in the ULF of pregnant HF than SF heifers, which was likely due to conceptus size, as there were no differences in IFNT mRNA levels between HF and SF conceptuses, or in the expression of uterine-derived factors that stimulates IFNT transcription. Nonetheless, no differences were observed in endometrial expression of selected classical and non-classical ISGs. Although the actions of IFNT in the endometrium to establish uterine receptivity does not appear to be substantially different between pregnant HF and SF heifers, the endocrine effects of IFNT may influence pregnancy success. IFNT and PGE2 are luteotropic [4-6], and their concentrations were increased in the ULF of pregnant HF than SF heifers. Thus, it is reasonable to speculate that concentrations of IFNT and PGE2 that exits the uterus and supports CL function were insufficient in SF heifers, which contributed to the observed reduced reproductive efficiency in these animals.

Glucose is a major nutrient supporting embryonic development [7-10], and although its concentration increased in the uterine lumen by pregnancy in our study, ULF glucose concentrations did not differ among fertility-classified heifers. Furthermore, only minor differences in endometrium and conceptus transcriptomes were observed for genes involved with glucose metabolism. Thus, the availability of glucose in the ULF during early pregnancy does not seem to play a major role influencing subfertility and infertility in the present study.

We found that concentrations of PGs tended to increase in the ULF by pregnancy, and in ULF of pregnant HF than SF heifers. Higher concentrations of PGs in the ULF of pregnant HF heifers is likely because they had longer conceptuses. Although pathways associated with PG signaling (through classical and nuclear receptors) in the endometrium and conceptus remains poorly understood in ruminants, PGs are essential for pregnancy success in sheep, as they stimulate a number of progesterone stimulated genes in the endometrium [11], and inhibition of PTGS2 prevents conceptus elongation [12]. Importantly, PGF2 α receptor (PTGFR) was the highest PG receptor expressed by the day 17 conceptus, and therefore PGF2 α signaling may play important role regulating conceptus development during elongation. Additionally, PPARA which regulates FA catabolism in several tissues [13], was the highest PPAR expressed by day 17 endometrium, and perhaps have critical roles modulating uterine luminal lipid content during early pregnancy.

Surprisingly, day 17 ULF fatty acid content of pregnant and open heifers did not differ, and palmitic acid (16:0) and stearic acid (18:0) accounted for around 90% of the total fatty acid detected in the ULF. However, a number of fatty acids were increased in the ULF of pregnant HF than SF heifers, which might be a result of differences in lipid metabolism between HF and SF conceptuses, as several genes involved with fatty acid (e.g.

ELOVL5, ELOVL7, FADS1, PLA2G15, PLA2G1B) and lipid (e.g. GPAM, CHKA, CERS1, ASAHI, CYP11A1, ACAT1) metabolism were differently expressed between HF and SF conceptuses. Furthermore, acyl-carnitine, composed of carnitine bound to acyl CoA (LCFA bound to coenzyme A), was also increased in pregnant HF than SF ULF, further supporting the results of increased LCFA in the ULF of pregnant HF than SF heifers.

The metabolism of preimplantation embryos have been suggested to be similar to that of cancer cells, which metabolizes glucose primarily through aerobic glycolysis (Warburg effect) [7], and have a high glutamine demand [14, 15]. In our data, several metabolites associated with glutamine metabolism were increased in pregnant than open ULF, and also in ULF of pregnant HF than SF heifers. Two key enzymes (PGD, TKT) that participates in the PPP, an important pathway used by cells undergoing the Warburg effect, were upregulated in the ULF of pregnant HF than SF heifers. PPP plays important role generating ribose sugars for nucleotide synthesis [16], and two precursors of purine biosynthesis (hypoxanthine, inosine), were decreased in pregnant than open ULF, perhaps indicating that these precursors were being used in an accelerated rate during pregnancy to supports conceptus growth. Additionally, 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 the ULF of HF heifers by pregnancy. These results support the theory that the Warburg effect is an important metabolic process during bovine conceptus elongation, and that the rate of aerobic glycolysis was diminished in SF heifers. Moreover, several amino acids (e.g. methionine, malate, phenylalanine, histidine, lysine, arginine, tyrosine, threonine/homoserine) and intermediates of the TCA cycle (fumarate, malate) that are clearly important during early pregnancy were increased in the ULF by pregnancy.

Additionally, several of these amino acids and TCA cycle intermediates were also increased in the ULF of pregnant HF than SF heifers, supporting our theory of dysregulated endometrial response to pregnancy in SF heifers.

Our data also provides further insights on the mechanisms regulating oxidative stress during early pregnancy in cattle. Glutathione (GSH) is a major antioxidant in mammalian cells [17], and in the present study, glutathione synthetase (GSS) was increased by 19 fold in the ULF of pregnant than 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 nonpregnant ULF perhaps indicates that oxidative stress in the uterine lumen is reduced during pregnancy, an event that could be mediated by conceptus derived glutathione reductase (GSR), which is highly expressed by the bovine conceptus during elongation.

Proteomics of the ULF demonstrated that conceptus-endometrium interactions alter the profile of proteins in the uterine lumen, and a number of proteins expressed by the endometrium were hypothesized as being regulated by pregnancy, based on data from endometrium transcriptome and ULF proteomics. Additionally, differences in the ULF proteome between pregnant HF and SF heifers indicated that HF heifers have a superior uterine environment to support conceptus development, as a number of proteins involved with energy metabolism, amino acid metabolism, and cell proliferation and differentiation were upregulated in the ULF of pregnant HF than SF heifers.

Collectively, the observed differences in histotroph composition further supports the theory dysregulated conceptus-endometrium interactions in the SF heifers, as a number of proteins, metabolites, PGs, IFNT, and lipids, all with essential roles during early pregnancy, were increased in the ULF of pregnant HF than SF heifers. However, histotroph

composition is directly influenced by conceptus-secreted factors and conceptus-endometrium interactions. Thus, the observed differences in histotroph composition may simply be because HF heifers had longer conceptuses, and not necessarily the cause of reduced fertility in SF animals. Future studies should aim to understand the mechanisms regulating the diminished endometrial response to pregnancy in SF heifers. These studies may include investigating the genome and endometrial epigenome of fertility-classified heifers. It would be particularly interesting to investigate the transcriptional regulation of genes found to be altered by pregnancy in the endometrium of HF heifers but not in SF animals. Exploring endometrial expression of microRNAs might also provide useful information regarding post-transcriptional regulation of endometrial genes. Additionally, the use of transmission electron microscopy (TEM) to further investigate the phenotype of uterine cells from fertility-classified heifers could provide valuable information related to the dysregulated conceptus endometrium-interactions in SF heifers, as the physical contact between the conceptus and endometrium is thought play an important role during conceptus elongation [18-20].

Additional research opportunities to enhance our current knowledge regarding early pregnancy events in ruminants includes: (1) improve current knowledge concerning PG signaling in the conceptus and endometrium; (2) improve current knowledge concerning lipid metabolism by conceptus and endometrium during pregnancy; (3) investigate metabolic requirements of conceptus during elongation. (4) investigate the gluconeogenic capacity of uterine cells; (4) conduct loss-of-function studies to investigate placental development; (5) investigate the mechanisms regulating conceptus elongation; (6) study the process of epigenetic reprogramming of the bovine conceptus during the preimplantation period.

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VITA

Joao Moraes grew up in Minas Gerais, a state located in the north of Southeastern Brazil, with his parents Adelio and Marcia, and his sister Nayara. Joao became interested in farm animals as a child, following the steps of his grandfather Joaquim, which was a dairy farmer. Due to his love for animals and exposure to farming growing up, Joao decided to pursue a career as a veterinarian, and was accepted as a student at the Universidade Federal de Uberlandia, Brazil in 2005. During veterinary school, Joao became fascinated with animal reproduction, and during his last academic year, he had the opportunity to participate in an externship at the University of Minnesota (UMN), under the supervision of Dr. Ricardo Chebel. Following graduation from Veterinary School in 2010, Dr. Chebel invited Joao to come back to the United States and work as a technician in his laboratory at the UMN. In 2011, Joao had the opportunity to further improve his training by pursuing a master's in Animal Sciences and a residency in Dairy Production Medicine, at the UMN. After completion of the masters in 2013 and residency in 2014, Joao joined the laboratory of Dr. Thomas Spencer at Washington State University (WSU), to work towards a PhD in reproductive biology with focus on uterine-conceptus interactions. In 2015, Dr. Spencer was recruited by the University of Missouri (MU), and Joao moved with him from Pullman to Columbia, where he completed his experiments and requirements to earn a PhD from MU. After completing his doctoral degree, Joao will stay at Mizzou and begin a postdoctoral position in the laboratory of Dr. Matthew Lucy, in the Division of Animal Sciences. In Dr. Lucy's lab, Joao will continue his studies investigating uterine biology, with the overall goal of improving reproductive efficiency in livestock animals and humans.