EFFECTS OF MATERNAL NUTRITION ON FRUCTOSE, GLUCOSE, AND CATIONIC AMINO ACID TRANSPORTER EXPRESSION IN BOVINE UTERO-PLACENTAL TISSUES FROM DAYS 16 TO 50 OF GESTATION

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Poor Maternal nutrition has been implicated to reduce nutrient transport to the conceptus. Therefore, we hypothesized that maternal nutrition and day of gestation would impact mRNA expression of nutrient transporters *GLUT1*, *GLUT3*, *GLUT5*, *GLUT14*, *CAT-1*, *CAT-2*, and *CAT-3* in beef heifers. Crossbred Angus heifers (n = 49) were bred via AI, assigned to nutritional treatment (CON = 100% of requirements for 0.45 kg/d gain and RES = 60% of CON) and ovariohysterectomized on d 16, 34, or 50 of gestation. Expression of *CAT-2* was the only gene in any tissue to demonstrate a day × treatment interaction. Expression of nutrient transporters were not influence by nutritional treatment ($P > 0.05$); however, transporters were differentially expressed by day of gestation ($P \leq 0.05$). We interpret these results to indicate that day, has a greater influence than a 40% global nutrient restriction on mRNA expression of nutrient transporters in bovine utero-placental tissues.

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LIST OF ABBREVIATIONS

ADF.....Acid Detergent Fiber

ADG.....Average Daily Gain

AI .....Artificial Insemination

AKT1 .....Proto-Oncogenic Protein Kinase 1

ALF .....Allantoic Fluid

AMF .....Amniotic Fluid

AMN .....Amnion

AMPK .....Adenosine Monophosphate-Activated Protein Kinase

ART.....Artificial Reproductive Technologies

β-Actin .....Reference Gene

BW .....Body Weight

CAR .....Caruncles

CAT-1 .....Solute Carrier Family 7 Member 1 Facilitated diffusion cationic amino acid transporter

CAT-2 .....Solute Carrier Family 7 Member 2 Facilitated diffusion cationic amino acid transporter

CAT-3 .....Solute Carrier Family 7 Member 3 Facilitated diffusion cationic amino acid transporter

CL .....Corpus Luteum

COOH .....Carboxyl

CON .....Control

COT.....Cotyledon

CP.....Crude Protein

Ct.....Cycle Threshold

d.....Day

DDGS.....Dried Distillers Grains with Solubles

DEPTOR.....Domain-Containing mTOR-Interacting Protein

EIF4E.....Eukaryotic Translation Initiation Factor 4E

EIF4EBP1.....Eukaryotic Translation Initiation Factor 4E-Binding Protein 1

ESR1.....Estradiol-17 β Receptor

FAK.....Focal Adhesion Kinase

FGF10.....Fibroblast Growth Factor-10

FM.....Fetal Membranes [Chorioallantois]

GE.....Glandular Epithelium

GFPT1.....Glutamine-Fructose-6-Phosphate Transaminase 1

GlcN-6-P.....Glucosamine-6-Phosphate

GLM.....Generalized Linear Model

GLUT1.....Solute Carrier 2 Member 1 Facilitate Diffusion Glucose Transporter

GLUT2.....Solute Carrier 2 Member 2 Facilitate Diffusion Hexose Transporter

GLUT3.....Solute Carrier 2 Member 3 Facilitate Diffusion Glucose Transporter

GLUT4.....Solute Carrier 2 Member 4 Facilitate Diffusion Insulin Dependent Glucose Transporter

GLUT5.....Solute Carrier 2 Member 5 Facilitate Diffusion Fructose Transporter

GLUT14.....Solute Carrier 2 Member 14 Facilitate Diffusion Glucose Transporter

ICAR.....Intercaruncular endometrium

ICM .....Inner Cell Mass

ICOT .....Intercotyledonary Placenta

IFN-τ .....Interferon Tau

IGF2 .....Insulin-Like Growth Factor 2

ILK .....Integrin-Linked Kinases

IRF2 .....Interferon Regulatory Factor 2

IRS1 .....Insulin Receptor Substrate 1

ISG .....Interferon Stimulated Genes

IUGR .....Intrauterine Growth Restriction

K_m .....Michaelis Constant

LE .....Luminal Epithelium

LIMS1 .....LIM and Senescent Cell Antigen-Like Domains 1

LSMEANS .....Least Squared Means

MLST8 .....Mammalian Lethal SEC13 Protein 8

mM .....Millimolar

MSIN1 .....Mammalian Stress-Activated MAP Kinase Interaction Protein 1

mRNA .....Messenger Ribonucleic Acid

mTOR .....Mammalian Target of Rapamycin

mTORC1 .....Mammalian Target of Rapamycin Complex 1

mTORC2 .....Mammalian Target of Rapamycin Complex 2

n .....Number

NCK2 .....Noncatalytic Region of Tyrosine Kinase

NDF .....Neutral Detergent Fiber

NH2.....Amino

NO.....Nitric Oxide

NOS.....Nitric Oxide Synthase

NOS3.....Nitric Oxide Synthase 3

NP .....Non-Pregnant

ODC1 .....Ornithine Decarboxylase

OGA.....O-GlcNAcase

OGT .....O-Linked N-Acetylglucosamine Transferase

OXTR.....Oxytocin Receptor

P4 .....Progesterone

PDK1.....Phosphoinositide Dependent Protein Kinase 1

$PGF_{2\alpha}$ .....Prostaglanding $F_{2\alpha}$

PGR.....Progesterone Receptor

PI3K .....Phosphatidylinositol 3-Kinase

PKC.....Protein Kinase C

PRAS40.....Proline Rich Akt Aubstrate 40 kDa

Preg .....Pregnant

PROTOR.....Protein Observed with RICTOR

qPCR.....Real-Time Quantitative Polymerase Chain Reaction

RAPTOR.....Regulatory-Associated Protein of mTOR

RES .....Restricted

RICTOR.....Rapamycin-Insensitive Companion of mTOR

RPS6 .....Ribosomal Protein S6

RPS6K.....Ribosomal Protein S6 Kinase

S6K .....S6 Kinase

SEM .....Standard Error of the Mean

sGE.....Superficial Glandular Epithelium

SGK.....Serum/Glucocorticoid-Regulated Kinase

SGLT-1 .....Solute Carrier Family 5 Member 1 Facilitate Diffusion Sodium Coupled Glucose Transporters

SGLT-11 .....Solute Carrier Family 5 Member 11 Facilitate Diffusion Sodium Coupled Glucose Transporters

SLC2A1 .....Solute Carrier Family 2 Member 1 Facilitate Diffusion Glucose Transporter

SLC2A3 .....Solute Carrier Family 2 Member 3 Facilitate Diffusion Glucose Transporter

SLC2A5 .....Solute Carrier Family 2 Member 5 Facilitate Diffusion Fructose Transporter

SLC2A14 .....Solute Carrier Family 2 Member 14 Facilitate Diffusion Glucose Transporters

SLC7A1 .....Solute Carrier Family 7 Member 1 Facilitated Diffusion Cationic Amino Acid Transporter

SLC7A2 .....Solute Carrier Family 7 Member 2 Facilitated Diffusion Cationic Amino Acid Transporter

SLC7A3 .....Solute Carrier Family 7 Member 3 Facilitated Diffusion Cationic Amino Acid Transporter

SNP .....Single Nucleotide Polymorphism

SPP1.....Secreted Phosphoprotein 1

TSC2 .....Tuberous Sclerosis 2

UDP-GlcNAc.....UDP-N-Acetylglucosamine

V_{max} .....Substrate Concentration at Which the Transporter is saturated.

LIST OF SYMBOLS

Δ .... Delta

CHAPTER 1: LITERATURE REVIEW

Establishment of Pregnancy

The use of artificial reproductive technologies (**ART**) such as artificial insemination (**AI**), estrous synchronization, and fixed-time AI are some of the most effective tools available to cattle producers to improve productivity and profitability of their cattle operation (Cothren, 2012; Dahlen et al., 2014). Benefits associated with using AI are: the ability to use sires of superior genetic merit, improved production traits in offspring, the ability to mate specific sires to individual cows, reducing the number of herd bulls needed in a single operation, increased genetics for replacement heifers, reducing spread of diseases throughout a herd, higher growth performance and calf value, and if combined with estrous synchronization protocols, can create a shorter calving season with a more consistent and uniform calf crop (Cothren, 2012; Comerford, 2013). Although there are proven benefits for the utilization of ART, less than 10% of US-based operations utilize them (Dahlen, 2010; Cothren, 2012; Comerford, 2013).

Fertilization rates for first service AI are approximately 90% in beef heifers (Bridges et al., 2013); however, by d 30 of gestation, only 50 to 60% of heifers maintain a viable pregnancy. Moreover, Thatcher et al., (1994) indicated that up to 80% of all embryonic loss occurs before d 40 of gestation in sheep and cattle. Across all livestock species, as well as humans, it is estimated that 2/3 of all embryonic losses occur during the peri-implantation/peri-attachment period of gestation (Bazer and First, 1983; Nancarrow, 1994). The main cause of this is failed implantation/attachment (humans or ruminants, respectively) of the chorioallantois to the uterine endometrium, which accounts for 50 to 75 % of failures to establish pregnancy (Bazer et al., 2011a).

Multiple events must take place (Figure 1.1) during the first 50 days of gestation for a successful pregnancy to be established and maintained, beginning with ovulation from the ovary and successful fertilization of the ovum by the sperm within the oviduct to form a zygote. The zygote will undergo multiple cleavage events in order to form into a morula (32 to 64 cell embryo) by days 4 to 7 after fertilization (Senger, 2012). During these cleavage events, the developing morula will move from the site of fertilization in the oviduct, and migrate to the uterus. The second stage of development is compaction, in which the cells of the morula continue to grow and divide forming two distinct cell layers of a blastocyst called the inner cell mass (**ICM**) and the trophoblast (formed 7 to 12 days after fertilization; Senger, 2012). Sodium is pumped into the blastocyst by the outer cells of the morula which causes fluid to accumulate within the blastocyst forming the blastocoele. The blastocyst therefore consists of an ICM as well as the fluid filled blastocoele surrounded by a single layer of cells called the trophoblast. The inner cell mass is what will give rise to the embryo/fetus, and the trophectoderm will give rise to the placenta (Rowson and Moor, 1966; Bindon, 1971; Wintenberger-Torres and Flechon, 1974; Barcroft et al., 1998; Senger, 2012). Fluid continues to build up within the blastocyst, cells continue to divide and grow, and pressure builds up within the blastocyst until, with the aid of proteolytic enzymes, the zona pellucida cracks, and the blastocyst "hatches". The hatching of the blastocyst takes place around days 9 to 11 after fertilization (Senger, 2012), yielding a free blastocyst which is floating in the uterus. During this time point, the blastocyst begins to transition from its spherical form, and elongate into a large spherical, tubular, and filamentous conceptus consisting of the embryo and the extra embryonic membranes. At d 13 of gestation, the bovine blastocyst is roughly 3 mm in diameter, but by day 17, the blastocyst will become approximately 250 mm in length. This step of elongation of the conceptus is very important for

the subsequent attachment steps as well as maternal recognition of pregnancy. As the conceptus elongates, it orients itself within the uterine lumen, and begins to adhere to the uterine endometrium. It is at this time, that the mononucleated trophectoderm (formerly described as trophoblast) of the conceptus begins to release interferon-τ (**IFN-τ**; 16 to 17 days post-fertilization; Senger, 2012), which is the maternal recognition of pregnancy signal in ruminants. Successful release of IFN-τ from the conceptus silences expression of genes involved in the pulsatile release of prostaglandin (**$PGF_{2\alpha}$**), which would terminate the pregnancy, by causing regression of the corpus luteum (**CL**) and termination of production of progesterone at sufficient levels to maintain pregnancy (Bazer et al., 1997; Bazer, 2013; Bazer et al., 2015). The developing placenta has been forming during this time from the extraembryonic membranes of the pre-attached embryo. This development is an obligatory step in the acquisition of the embryo's ability to attach to the uterus. As the hatched blastocyst begins to grow and expand, a layer just beneath the ICM called the mesoderm and endoderm, begins to grow. The endoderm grows downward lining the trophoblast forming the first membrane called the yolk sac. The mesoderm continues to grow and expand forming a sac that surrounds the yolk sac and pushes against the trophectoderm forming a double membrane called the chorion. The chorion then expands surrounding the embryo forming the amniotic sac. The allantois is the final membrane compartment which originates form the primitive gut of the embryo. This membrane collects liquid waste from the embryo. As the embryo continues to grow, the allantois grows in size as well eventually fusing with the chorion forming the chorioallantois, which will grow and develop into the placenta (Bryden et al., 1972; Mossman, 1987). By days 18-22 the conceptus would have successfully adhered to the uterine endometrium, and by day 30, complete attachment of the chorioallantois to the endometrium would have taken place.

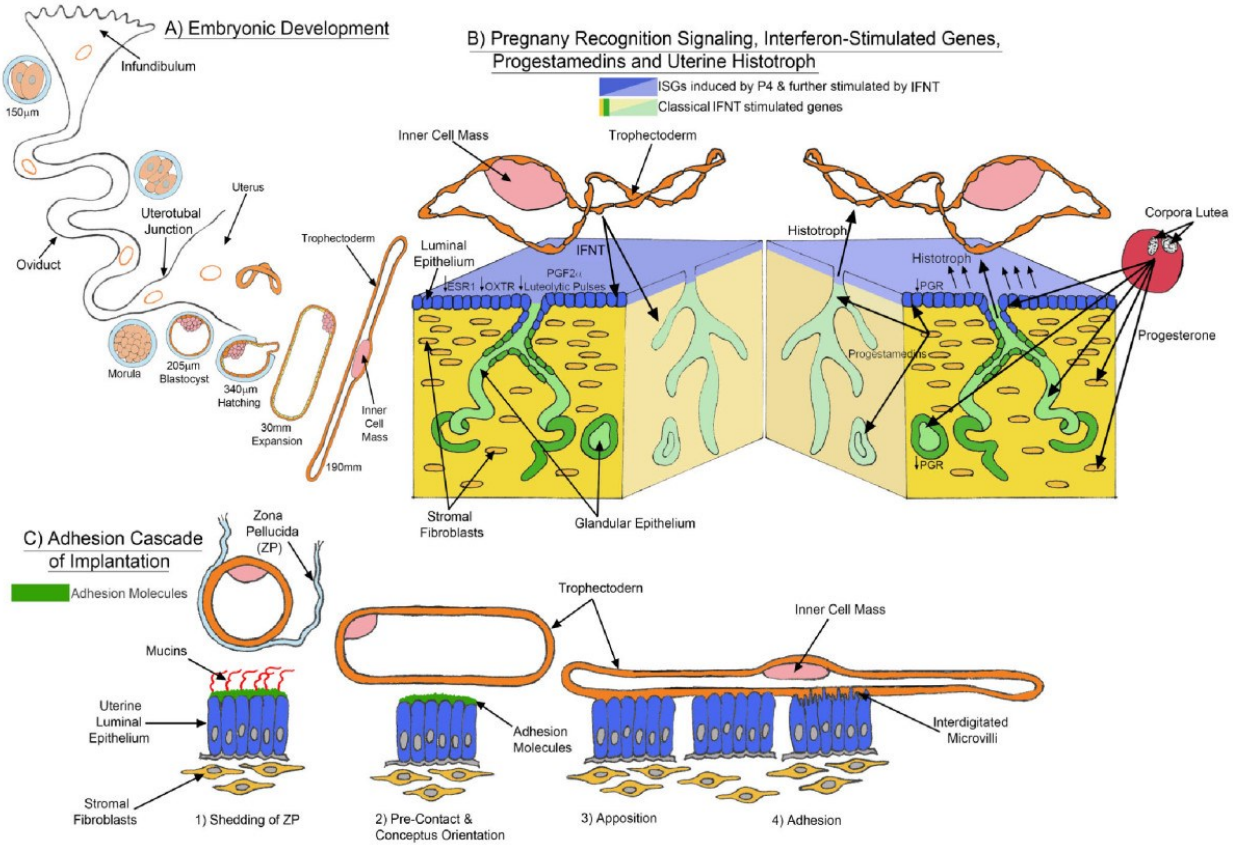

Figure 1.1. Embryonic development and uterine adaptations **A)** Oocytes fertilized in the oviduct enter the uterus at the morula stage and advance developmentally after hatching from the zona pellucida to spherical blastocysts that then transition to the large spherical, tubular, and filamentous conceptus (the embryo and its extraembryonic membranes) with IFN-τ, the pregnancy recognition signal secreted from mononuclear trophectoderm cells between Days 10 and 21 of pregnancy. **B)** Endometrial epithelia cease expressing receptors for progesterone (PGR) due to autoregulation by progesterone, while IFN-τ silences expression of receptors for estradiol-17 β (ESR1) and oxytocin receptor (OXTR) to abrogate development of the mechanism for oxytocin-mediated pulsatile release of Prostaglandin F ($PGF_{2\alpha}$), which would otherwise cause regression of the corpus luteum and cessation of their secretion of progesterone. Endometrial stromal fibroblasts express PGR and secrete progestamedins, particularly fibroblast growth factor-10 (FGF10), that regulate uterine epithelium cell function. With down-regulation of PGR in uterine epithelia, uterine luminal epithelium (LE) and superficial glandular epithelium (sGE) express genes that are either induced by progesterone (P4) or induced by P4 and further stimulated by IFN-τ. Furthermore, IFN-τ induces expression of interferon regulatory factor-2 (IRF2) in uterine LE and sGE to silence expression of classical IFN-stimulated genes and allows expression of a unique set of genes that promotes conceptus growth and development. Endometrial GE cells and stromal fibroblasts do not express IRF2 and, therefore, express classical IFN-stimulated proteins. Collectively, molecules secreted by uterine epithelia or transported into the uterine lumen by uterine epithelia form histotroph required for conceptus development. C) The ovine conceptus undergoes the adhesion cascade for implantation. Taken with permission from Soc. Study Reprod., Bazer et al., 2011.

After d 30 of gestation, the chorioallantois begins to differentiate between the cotyledonary tissue of the placenta, and the intercotyledonary tissue. The cotyledons arise due to increased local force of blood pressure from the endometrium on specific points of the placenta. This causes structural changes in the placenta involving a three step process: 1) cavitation, 2) crowning and extension of anchoring villi, and 3) completion by development of the supplying vascular pattern (Reynolds, 1966). These cotyledons will form the point of attachment between the caruncles on the uterine endometrium and the placenta, allowing for exchange of nutrients through hemotrophic nutrition, and will continue to grow and develop throughout gestation.

Throughout the first 50 day timeframe, the embryo is growing rapidly. By approximately day 45, organogenesis (development of the fetal organs) would be completed for some organs; however, although differentiated, organ systems like the gastrointestinal system (Noah et al., 2011) or lungs are not completely developed and functional. This indicates, that during the first 50 days of gestation, all of the fetal organ systems would be growing, developing, and differentiating from one another (Winters et al., 1942), growing in size after d 50 as well as further developing and differentiating, following the steps of 1) morphogenesis and cell proliferation, 2) cell differentiation, and 3) functional maturation (Colony, 1983).

Histotroph and mTOR

In the early 20th century, the terms hemotroph and histotroph were coined to describe those substances essential for support of conceptus and fetal development supplied via the uterus either: (1) directly from blood (hemotroph); or (2) from the uterine endometrium (histotroph) (Bazer, 1975; Filant and Spencer, 2014). Because hemotrophic nutrition has yet to be established during the first 50 days of gestation, a proper uterine milieu (histotroph) must be present in order

to supply the developing embryo with the necessary nutrients to support the growth and differentiation of tissues (Bazer, 2011a). Histotroph includes nutrient transport proteins, ions, mitogens, cytokines, lymphokines, enzymes, hormones, growth factors, proteases and protease inhibitors, amino acids, glucose, fructose, vitamins and other substances (Bazer, 1975; Bazer et al., 2011a; Spencer and Bazer, 2004; Bazer et al., 2011b). In ruminants, histotroph is essential to the development of the conceptus throughout pregnancy (Bazer et al., 2011a). Histotroph, also called uterine milk (Bonnet, 1882), is secreted from glands within the uterine endometrium (Bazer, 1975; Gray et al., 2001; Spencer et al., 2008). The uterine endometrium of cattle consists of multiple aglandular caruncular areas, which are dense stromal areas covered by a simple luminal epithelium (**LE**), as well as intercaruncular areas (Filant and Spencer, 2014). The intercaruncular areas of the endometrium contain many hundreds of glands in a cross-section of the uterine wall (Filant and Spencer, 2014). These glands begin to develop as invaginations of LE that progressively invade the stroma, resulting in an extensive network of epithelial glands (Filant and Spencer, 2014). If these glands were to not develop, the conceptus would not be able to elongate, IFN-τ would not be produced, and $PGF_{2\alpha}$ would lyse the CL, and pregnancy would be terminated. This was established with the uterine gland knockout model in sheep (Bartol et al., 1988a; Bartol et al., 1988b). Knockdown of uterine glands in sheep resulted in infertility, although blastocysts were normal on d 9 after fertilization, conceptuses were severely growth-retarded by d 12 or 14 (Filant and Spencer, 2014). This lack of elongation emanated from the absence of histotrophic secretions into the uterine lumen from the GE (Gray et al., 2001a; Gray et al., 2002).

Some of the essential constituents of histotroph, which are the main focus of the remainder of this thesis, are glucose, fructose, and cationic amino acids. These components of

histotroph act on a nutrient sensing pathway called the mammalian target of rapamycin (**mTOR**). The mammalian target of rapamycin is a serine/threonine protein kinase that regulates a variety of anabolic and catabolic processes in response to nutrients, growth factors, hormones, as well as some stresses (Chantranupong et al., 2016; Tan and Miyamoto 2016; Wang et al., 2016b). The mammalian target of rapamycin is the mammalian homologue of TOR which was cultured in yeast cells. Rapamycin is a potent antifungal macrolide originally isolated form the soil of Rapa Nui (Easter Island; Vazina et al., 1975). Rapamycin is responsible for anti-proliferative and immunosuppressive activities in both prokaryotes and eukaryotes. The mammalian target of rapamycin is the direct target of rapamycin, and is responsible for regulation of protein, lipid, and nucleotide synthesis, ribosome and lysosome biosynthesis, expression of metabolism-regulated genes, autophagy and cytoskeletal reorganization (Wang et al., 2016b). The mammalian target of rapamycin is made up of two complexes, mTORC1 (proliferation) and mTORC2 (cytoskeletal reorganization, migration and adhesion) with both aiding in the same task of conceptus elongation and attachment for implantation (Wang et al., 2016b).

The mammalian target of rapamycin is a nutrient sensing pathway and behaves differently under nutrient rich and nutrient poor conditions. Under nutrient rich conditions, mTORC1 and mTORC2 support cellular growth, cytoskeletal remodeling, and suppresses autophagy. Under nutrient starved conditions, mTORC1 is inhibited and autophagy is induced to provide an energy source for the cell (Tan and Miyamoto, 2016). Therefore, it is understandable that the lysosome is a critical organelle for mTORC1 activation, and amino acids promote the translocation of mTORC1 to its surface where it is to be activated.

The mTORC1 complex consists of mTOR, Regulatory-associated protein of mTOR (**RAPTOR**), DEP domain-containing mTOR-interacting protein (**DEPTOR**), proline rich Akt

substrate 40 kDa (**PRAS40**), mammalian lethal SEC13 protein 8 (**MLST8**) as well as upstream and downstream kinases responsible for either inducing or suppressing mTOR or autophagy at the lysosome (Tan and Miyamoto, 2016; Wang et al., 2016b). The kinase activator Rheb, resides on the lysosome surface. Additionally, bound to the membrane are the Ragulator, Rag GTPases which are composed of four members which form heterodimers RagA/B, and RagC/D (Chantranupong et al., 2016; Tan and Miyamoto 2016). These Rag complexes mediate the mTORC1 translocation to the lysosome in response to amino acid stimulation (Sancak et al., 2010).

The mTORC2 complex consists of mTOR, rapamycin-insensitive companion of mTOR (**RICTOR**), mammalian stress-activated MAP kinase interaction protein 1 (**MSIN1**), MLST8, DEPTOR, and protein observed with RICTOR (**PROTOR**) (Loewith et al., 2002; Pearce et al., 2007; Laplante and Sabatini, 2009; Wang et al., 2016).

Arginine Activation of mTORC1 and mTORC2

In the presence of amino acids, mTOR translocates to the lysosome in which it binds the the Ragulator/Rag complex as previously stated. Rheb, a direct activator of mTORC1 is located on the lysosomal membrane, and once mTOR is in close proximity to Rheb, allows activation of mTOR. Thus, the docking of mTOR to the lysosome, allows for the necessary proximity of the mTOR/Rheb associated complex to inhibit autophagy related signals. When amino acids become scarce, mTORC1 is removed from the Ragulator/Rag complex, loses its proximity with Rheb, and becomes inactive. Once inactivated, TFEB (transcription factor EB) becomes bound to the lysosome and induces autophagy in the cell (Tan and Miyamoto, 2016).

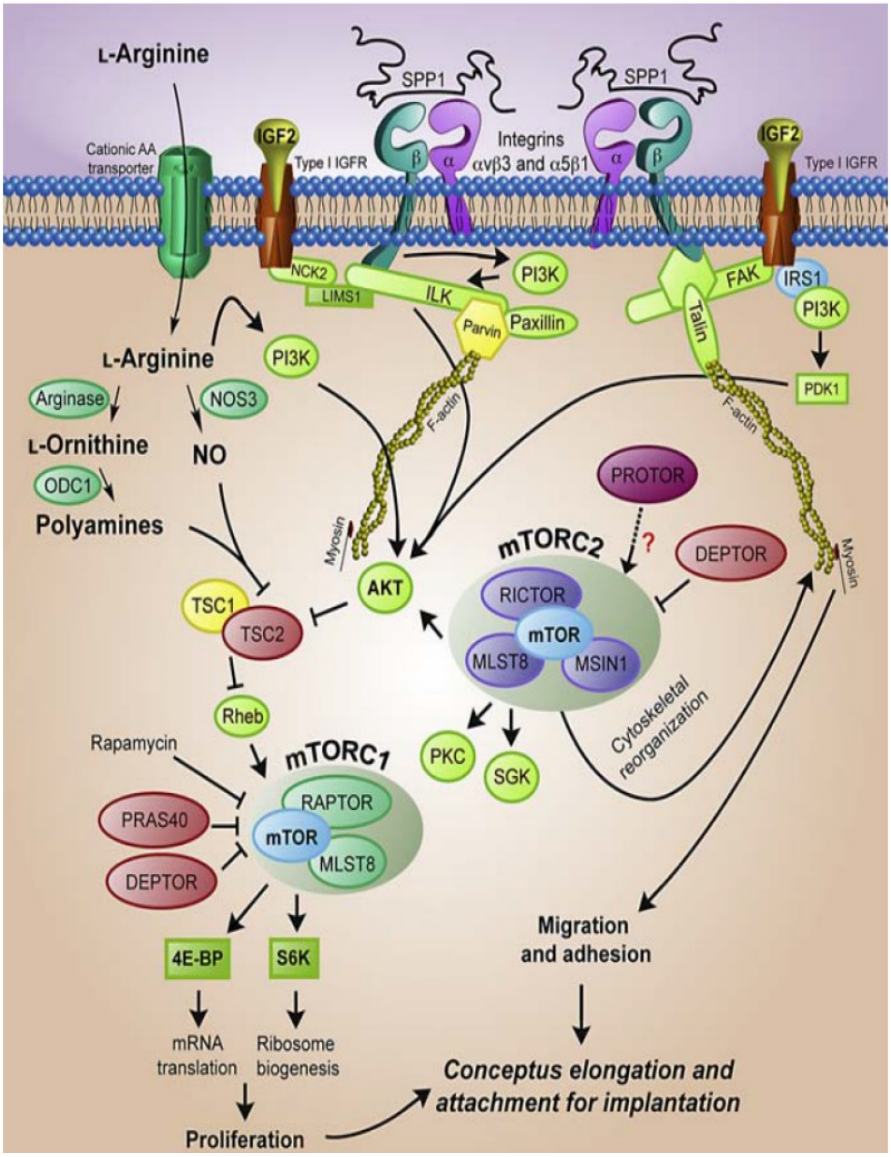

Figure 1.2. Model for induction of cell signaling for proliferation, migration, adhesion, and cytoskeletal remodeling of conceptuses via mTORC1 and mTORC2 signaling cascade. AKT1, proto-oncogenic protein kinase 1; FAK, focal adhesion kinase; PDK1, phosphoinositidedependent protein kinase 1; mTOR, mammalian target of rapamycin; RAPTOR, regulatoryassociated protein of mTOR; RICTOR, rapamycin-insensitive companion of mTOR; IGF2, insulin-like growth factor 2; type I IGF2, type I insulin-like growth factor receptor; ILK, integrin-linked kinases; IRS1, insulin receptor substrate 1; PKC, protein kinase C; SGK, serum/glucocorticoid-regulated kinase; MLST8, mammalian lethal with SEC13 protein 8; PRAS40, proline-rich Akt/PKB substrate 40 kDa; DEPTOR, DEP domain-containing mTORinteracting protein; MSIN1, mammalian stressactivated MAP kinase interacting protein 1; PROTOR, protein observed with RICTOR; NCK2, noncatalytic region of tyrosine kinase, beta; NO, nitric oxide; NOS3, nitric oxide synthase 3; ODC1, ornithine decarboxylase; PI3K, phosphatidylinositol 3-kinase; LIMS1, LIM and senescent cell antigen-like domains 1; S6K, S6 kinase; SPP1, secreted phosphoprotein 1. Taken with permission from Elsevier, from Wang et al., 2016b.

Specific arginine activation of mTORC1 complex comes through its interaction with another protein, Secreted Phosphoprotein 1 (SPP1; Wang et al., 2016b). Secreted Phosphoprotein 1 is responsible for regulation of cell proliferation, migration, adhesion, differentiation, survival, and immune factors, as well as its actions in adhesion of the trophectoderm to the uterine luminal epithelium during implantation (Wang et al., 2016b). However, SPP1 alone does not cause implantation and adhesion. Physiological concentrations of arginine acting in coordination with SPP1 signal the mTOR, PI3K, and MPK3 pathways to signal cell migration and proliferation. Arginine is metabolized to ornithine and subsequently to polyamines, or to nitric oxide (NO) which all activate mTOR through interacting mTOR with Rheb (Wang et al., 2016b). Once activated, mTOR acts on downstream effectors by phosphorylating them, and regulating cell proliferation.

Arginine and SPP1, together work to activate mTORC2 as well. This is the driving force behind the elongation of the blastocysts, causing the cellular reorganization and change from a spherical blastocyst, to the long filamentous tubule which will secrete IFN-τ and implant on the uterine epithelium (Wang et al., 2016a).

Glucose and Fructose Activation of mTORC1

Within particular cell types, glucose and fructose stimulate mTORC1 differently. In cardiac cells, the enzyme hexokinase which catalyzes the phosphorylation of glucose to glucose-6-phosphate, activates mTORC1 through its binding with Raptor. In the absence of glucose, AMPK, inactivates mTORC1 yielding increased autophagy (Tan and Miyamoto, 2016). In allantoic and amniotic fluids during early gestation; however, fructose is the main hexose in fetal fluids, not glucose (Kim et al., 2012). This is because the placenta metabolizes glucose-6-

phosphate to fructose-6-phosphate, making fructose more abundant in allantoic fluid (Wang et al., 2016). Once inside the cell, glutamine-fructo-phosphate transamidase-1 (GFPT-1) catalyzes the change from fructose-6-phsophate to GlcN-6-P. In this mechanism, glutamine is deaminated to glutamate, which will be converted to α-keto-glutarate, also stimulating mTOR, connecting glucose and fructose metabolism to glutamine activation of mTOR (Wang et al., 2016). Thus, UDP-GlcNAc, directly stimulates Akt (the proline rich substrate) which activates mTOR. Once activated, mTOR phosphorylates downstream effectors in a similar mechanism compared with arginine activation of mTOR.

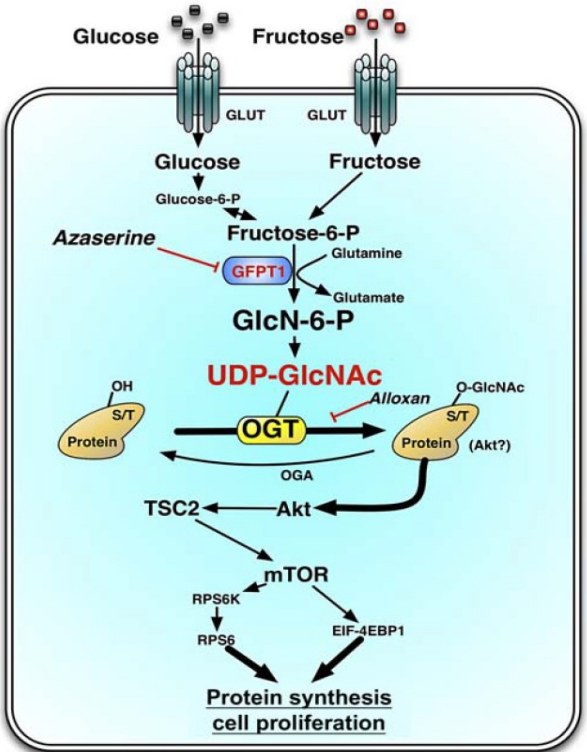

Figure 1.3. Schematic diagram of the GFPT1-OGT-mediated Akt-TSC2-mTOR signaling cascade affected by glucose and fructose in oTr1 cells. Fructose stimulates GFPT1 for hexosamine biosynthesis to provide UDP-GlcNAc for O-GlcNAcylation, thereby activating Akt-TSC2-mTOR signaling cascade for stimulation of oTr1 cell proliferation and growth. Akt, proto-oncogenic protein kinase Akt; TSC2, tuberous sclerosis 2; mTOR, mechanistic target of rapamycin; RPS6, ribosomal protein S6; RPS6K, ribosomal protein S6K; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; GlcN-6-P, glucosamine-6- phosphate; GFPT1, glutamine-fructose-6-phosphate transaminase 1; OGT, O-linked N-acetylglucosamine transferase; OGA, O-GlcNAcase; UDP-GlcNAc, UDP-N-acetylglucosamine. Taken with permission from Elsevier, Wang et al., 2016b.

mTOR Downstream Effectors

The mammalian target of rapamycin's downstream effectors to protein synthesis and cellular proliferation are Ribosomal Protein S6K (RPS6K), Ribosomal Protein S6 (RPS6), and Eukaryotic Translation Initiation Factor 4E-Binding Protein (EIF-4EBP1). Both types of downstream effectors work in different ways and are stimulated differently by mTOR to increase proliferation of cells. 4E-BP aids to increase mRNA translation, and S6K is responsible to increase ribosome biogenesis (Wang et al., 2016).

Initiation factor 4E-Binding Protein is a series of multiple proteins which bind to Eukaryotic Translation Initiation Factor 4E (eIF4E). Eukaryotic Translation Initiation Factor 4E is bound by binding proteins such as 4E-BP1, which once released, allows for eIF4E to bind to the 5' end of mRNA and along with helicase, unwind the mRNA in order to promote translation of the protein for increased proliferation. In order to do this, mTOR must phosphorylate multiple components of 4E-BP1 including Thr37, Thr46, Ser65, and Thr70 cooperatively in order to successfully dissociate 4E-BP1 from EIF4E (Hay and Sonenberg 2004).

The other downstream effect S6 Kinase is made of two kinase proteins S6K1 and S6K2. Both proteins are phosphorylated and regulate cell growth in mammals. S6K1 acts through increased mRNA translation, which encode specifically for components of the translation process, including ribosomal proteins, elongation factors, and poly (A)-binding protein. This action on translation is indirect and is mediated through S6K action on EIF4B in order increase mRNA translation and ribosome biogenesis (Hay and Sonenberg, 2004).

Therefore, the presence of glucose, fructose, and arginine are vital to the development of the conceptus, and are secreted by transporters in the glandular epithelium (**GE**), caruncle,

chorioallantois, cotyledon and amnion in order to sufficiently supply nutrients to the developing conceptus.

Utero-placental Glucose Trans*porters*

There are two types of glucose transporters in the utero-placenta: 1) Facilitated diffusion transporters which transport glucose down their concentration gradients, known at GLUTs or SLC2 family, and 2) sodium-coupled glucose transporters which co-transport one molecule of glucose with two sodium ions, known as SGLTs or SLC5 family (Frolova and Moley, 2011). The main focus of this section of the review will be on sodium-independent glucose transporters **GLUT1**, **GLUT3**, and **GLUT14** (SLC2A1, SLC2A3, and SLC2A14) and sodium-independent fructose transporter **GLUT5** (SLC2A5). Another glucose transporter in utero-placental tissues that is also heavily investigated is GLUT4 (SLC2A4); however, this transporter is insulin-dependent (Cushman and Wardzala, 1980; Suzuki and Kono, 1980); Wardzala and Jeanrenaud, 1981; James et al., 1988) and will not be a topic of discussion for this review.

The SLC2 protein family contains 14 currently known isoforms and each member has a distinct tissue-specific expression and binding affinities for individual hexose substrates (Augustin, 2010). These isoforms are facilitative diffusion transporters that alternate between two conformational states with the substrate-binding site facing extracellularly (Bell et al., 1993). Each transporter contains approximately 500 amino acids and a 12 membrane-spanning α-helical segment with intracellularly located NH2 and COOH terminal. There is only one large intracellular loop connecting the transmembrane segments between helices 6 and 7.

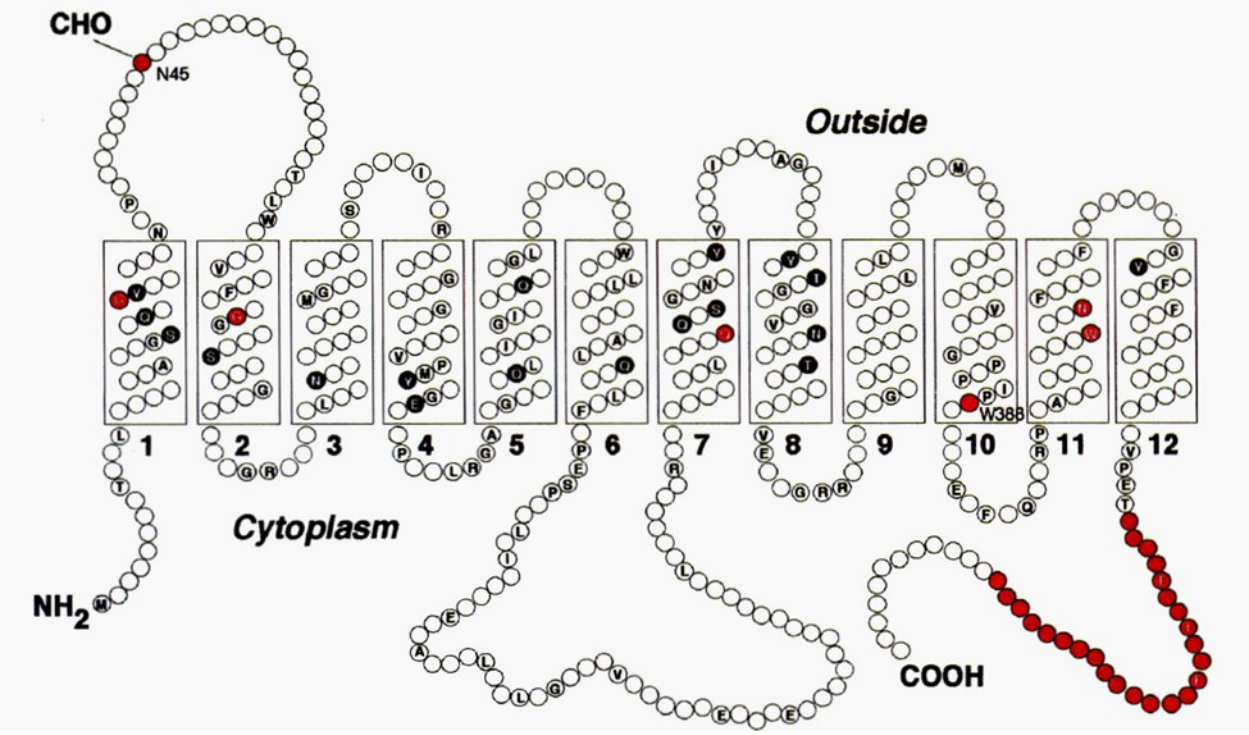

Figure 1.4. Model for the orientation of mammalian facilitative sugar transporters in the membrane. The 12 transmembrane helices are shows as boxes and are numbered 1-12. The potential site for N-glycosylation in the extracellular loop connecting transmembrane segments 1 and 2 is show. Invariant residues are noted using the single-letter abbreviations for the amino acids with polar residues located in the lipid bilayer being show in filled circles. The residues whose mutagenesis or deletion affects transporter function are noted in red. Taken with permission from J. Biol. Chem, Bell et al., 1993.

The remaining loops are very short, consisting of anywhere from 8 to 12 residues. The potential site for N-glycosylation can be found in the extracellular loop connecting transmembrane segments 1 and 2 (Figure 1.4). Moreover, mutation of the N-glycosylation site increases the K_m for glucose transport by 2-fold, indicating that the glycosylation site may contribute to the formation of structure that is capable of high affinity transport (Asano et al., 2001).

In order to fully understand facilitative diffusion transporters, it is vital to understand transport kinetics, and how carrier mediated transport functions. All transporters investigated

within are facilitative diffusion transporters. Indicating that these transporters facilitate the transport of molecules down their concentration gradient in order to equilibrate their concentration gradients and are not coupled with the translocation process of another chemical or vector (Busch and Saier, 2002). The transport process can be described by a four step process: 1) Binding- solute binds to one face of the membrane, 2) Transport- conformational change closes first binding site and exposes binding site on other side of the membrane, 3) Dissociation- solute dissociates, and 4) Recovery- empty transporter reverses to initial conformation (Figure 1.5; Voet and Voet, 2011). As stated previously, individual transporters operate with individual tissue specificities and solute affinities which is determined through their K_m (Michaelis constant). This is determined by indicating the transporters V_{max} (substrate concentrations at which the transporter is saturated), and dividing by 2, such that the K_m is the substrate concentration at which the transport velocity is half-maximal. Therefore, if a transporter has a small K_m value, it achieves maximal transport efficiency at a low substrate concentration (Voet and Voet, 2011).

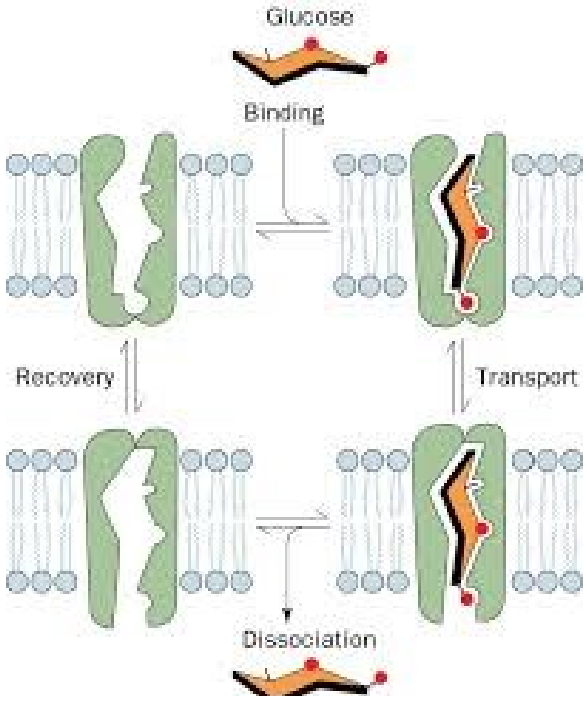

Figure 1.5. Alternating conformation model for glucose transport. Taken with permission from Wiley, Voet and Voet, 2011.

Glucose transporter GLUT1 (Solute carrier 2 member 1, SLC2A1) is ubiquitous in expression across all organs, and is commonly thought to be responsible for basal level of glucose uptake in most cell types (Frolova and Moley, 2011). GLUT1 is found in high levels in all fetal tissues as well as being most abundant in fibroblasts, erythrocytes, and endothelial cells with low expression in muscle, liver and adipose tissue in adults (Olson and Pessin, 1996). It has a high affinity for glucose ranging from 3-5 mM depending on the tissue, with a uterine transport K_m of 3 mM (Mueckler, 1994; Augustin, 2010). In uterine tissue, GLUT1 has been localized in the stroma, LE, and GE (Korgun et al., 2001; Gao et al., 2009; Frolova and Moley, 2011). During gestation, expression of GLUT1 changes dramatically. In sheep, GLUT1 expression was greatest on d 14 of gestation (time of maternal recognition of pregnancy in sheep; Senger, 2012), and greater in pregnant, compared with cyclic ewes (Gao et al., 2009). In humans and rodents, GLUT1 increases in abundance in stromal tissue during decidualization (differentiation in uterine cells in response to progesterone; Frolova and Moley, 2011). Therefore, it is of no surprise to see that GLUT1 expression is increased in response to progesterone and decreased in response to estrogen, which has been noted in human, mouse and ovine endometria (Frolova and Moley, 2011; Gao et al., 2009). Additionally, GLUT1 is classified as an interferon stimulated gene (**ISG**), and expression of GLUT1 is increased in response to IFN-τ in the uterine lumen. In sheep, expression of GLUT1 in the presence of progesterone (**P4**) was 4.2-fold greater than controls, and 2.1-fold greater than controls in the presence of IFN-τ. When P4 and IFN-τ were combined in the media, expression of GLUT1 increased nearly 8-fold greater than controls (Gao et al., 2009). This indicates that across species, GLUT1 behaves similarly, and may serve as the main glucose transporter for the pre-attachment embryo. In placental tissue of sheep, GLUT1 was localized to the trophoblast and baso-lateral membranes, with none found in the microvillar

membrane of the developed chorioallantois (Wooding et al., 2005), and was also localized in the trophectoderm and extra embryonic endoderm of the developing conceptus (Gao et al., 2009). In humans, GLUT1 was isolated in both the microvillous and basal membranes of the placenta, with microvillar membrane transport being 20-fold greater than basal transport (Jansson et al., 1993), indicating that basal glucose transport is the rate limiting step (Illsley, 2000), and that GLUT1 would be primarily established for transport from the histotrophic secretions into the lumen of the uterus (Desoye and Shafrir; 1994).

Glucose transporter GLUT3 (Solute carrier 2 member 3, SLC2A3) has the highest known transport capacity of any glucose transporter at 1.5 mM, and also has the greatest turnover number of the GLUT isoforms ensuring efficient uptake of glucose (Thorens and Mueckler, 2010). GLUT3 is the major neuronal glucose transporter, present in both dendrites and axons, and its level of expression in different regions of the brain correlates with regional cerebral glucose utilization (Simpson et al., 2008, Thorens and Mueckler, 2010). In mouse sperm, *GLUT3* is highly expressed and controls glucose uptake and metabolism necessary for motility and maturation (Thorens and Mueckler, 2010). Glucose transporter GLUT3 is unique in comparison with other glucose transporters in utero-placental tissues in that a mutation of the *GLUT3* gene in mice caused early embryonic mortality, indicating that the amount of glucose being transported by GLUT3 simply could not be made up for by other utero-placental glucose transporters including GLUT1, GLUT4, or GLUT8 (Ganguly et al., 2001). In the mouse uterus, the presence of GLUT3 is very low during the beginning stages of gestation and increasing in presence post decidualization of the uterus and throughout gestation (Yamaguchi et al., 1996). Similarly, GLUT3 protein was reported in the uterine epithelium and stroma starting on gestational day 1 and increasing through day 4 in the rat (Korgun et al., 2001). In sheep, GLUT3 was not localized

from d 10 to 20 of gestation (Gao et al., 2009), however in Holsteins, *GLUT3* was isolated in both caruncular and intercaruncular endometrium, but was not different in expression from days 28 to 42 of gestation (Lucy et al., 2012). GLUT3 is commonly known for being a placental transporter, due to the high energy demands of both the growing placenta as well as the developing conceptus (Simpson et al.,2008). In sheep, GLUT3 was localized to the microvillous membrane of the placenta, but not on the placentome. GLUT3 was exclusively localized to the microvillar junction, with trophoblast and uterine epithelia surfaces remaining unlabeled (Wooding et al., 2005). During early development of the conceptus, *GLUT3* is highly expressed on the trophoblast cell layer indicating that during this time of cell growth and differentiation of the conceptus from tubular to filamentous, a scavenging of energy may be taking place to ensure proper growth and development (Frolova and Moley, 2011).

The glucose transporter gene *GLUT14* (solute carrier family 2 member 14, also known as SLC2A14), is one of the least studied transporters, and encodes a protein that is 497 amino acids in length, and shares 95% homology to *GLUT3* (Wu and Freeze, 2002). The *GLUT14* gene is considered a duplicon, as it may have arisen as a possible result of a duplication of *GLUT3* (Wu and Freeze, 2002). Contrary to *GLUT3,* however, *GLUT14* has previously been specifically expressed in the testis, and was found to have expression levels four-fold greater than *GLUT3* in testis (Wu and Freeze, 2002). No research has been found in the literature determining the transport capacity of GLUT14, to determine if it is comparable to GLUT3. However, like other GLUT transporters, GLUT14 contains 12 putative membrane-spanning helices along with sugar-transporter signature motifs that have previously been shown to be essential for sugar transport activity (Wu and Freeze, 2002). This indicates that GLUT14 should be able to transport glucose,

and establishment of its transport capacity would more greatly determine its role in glucose transport.

Fructose transporter, GLUT5 is the only GLUT protein with a high specificity for fructose (Douard and Ferraris, 2008). GLUT5 is most predominantly found in the apical membrane of the enterocyte, where it provides a major route for the absorption of dietary fructose (Thorens and Mueckler, 2010). In the human oocyte, GLUT5 does not exhibit glucose transport capabilities; however, is able to transport fructose at a K_m of ~ 6mM (Burant et al., 1992). In the bovine, high concentrations of GLUT5 are found on fast-cleaving embryos which are more likely to develop to the blastocyst stage due to their enhanced nucleotide synthesis (Gutierrez et al., 2004). Additionally, expression of *GLUT5* in-vitro, was greater in the low oxygenated environment, compared with the high oxygenated environment (Balasubramanian et al., 2007), indicating that expression of *GLUT5* would be greater in the pre-attachment uterine environment compared with 2nd or 3rd trimester of gestation in which hemotrophic nutrition has been established and exchange of gases is taking place.

Normal serum glucose concentration in 2 year old beef heifers is 3.7 mM (Doornenbal et al., 1988). This indicates that GLUT1 and GLUT3, as well as possibly GLUT14 would be able to transport glucose from the maternal to the fetal system at a greater capacity than what is available in the maternal system. Fructose concentrations in maternal serum are low (< 1 mM) due to metabolism of fructose by the liver (absorbed via GLUT2), where-as glucose may pass through the liver and be metabolized anywhere in the body (Quezada-Calvillo et al., 2006). Therefore, it may be assumed that the ratio of glucose to fructose in fetal fluids (allantoic and amniotic) would be high; however, in early pregnancy this isn't the case. In the uterine lumen of sheep, glucose concentrations increase 6-fold from d 10 to 15 of gestation (500 to 3,000 nmol,

respectively (Gao et al., 2009). However, in allantoic fluid, concentrations of fructose range from 11.1 to 33.30 mM whereas glucose concentrations range from 0.55 to 1.67 mM from d 25 to 120 in sheep (Bazer et al., 2012). This is because fructose, is more abundant sugar in fetal blood, being found in 3-4 times greater amounts than glucose (Bacon and Bell, 1946 and 1948; Barklay et al., 1949, Battaglia and Meshia, 1978; Kim et al., 2012). Several studies outlining fructose and glucose utilization by the fetus have determined several conclusions: 1) injection of glucose into ewes results in a rapid increase in glucose followed by a protracted increase in fructose in fetal blood; (2) injection of glucose into the umbilical vein of the fetus increases glucose in maternal blood and hyperfructosemia in the fetus, indicating that glucose can move from conceptus vasculature to maternal blood, whereas fructose derived from glucose is not transported into maternal blood; 3) the placenta is the site of conversion of glucose to fructose; 4) fructose is produced continuously by the placenta independent of glucose concentration in maternal or fetal blood; and 5) the flux of glucose from the maternal to the fetal circulation can be as much as 70 mg/min in ewes made hyperglycemic (Barklay et al., 1949; Huggett et al., 1951; Alexander et al., 1955a; Alexander et al. 1955b; Kim et al., 2012). Also, during early gestation, the uterine lumen is in a low oxygenated state, due to matured vascular beds between the uterine and fetal circulations not being present (Barry and Anthony, 2008). This environment supports the utilization of fructose by the pre-implantation embryo due to their inability to metabolize reactive oxygen species (Johnson and Nasr-Esfahani, 1994). This was determined with in-vitro culture of bovine embryos, in which embryos cultured in a low oxygen environment (< 0.5% O_2) had greater percent of blastocysts (28.5%) compared with the high oxygen environment (20% O_2; 18.7% blastocysts; Balazubramanian et al., 2007). Additionally, it has been reported that glucose could only supply 49% of energy requirements of the fetus (Battaglia and Meschia,

1978), therefore, additional carbohydrates sources such as fructose or lactate must make up the balance.

Utero-Placental Cationic Amino Acid Transporters

The transport of cationic amino acids is mediated by five transporter systems: y^+, y^{+L}, b^+, $b^{0,+}$, and $B^{0,+}$ (Hyde et al., 2003; Palacin et al., 1998). In the case of amino acid transporters, lower case letters indicate sodium independent transport, and upper case letters indicate sodium-dependent transport. Additionally, the super script indicates the charge of the molecule to be transported: "0" = neutral and "+" = cationic. The main transporters of interest to our research group are the high affinity, sodium-independent facilitative diffusion y^+ transporters of arginine and lysine, CAT-1, CAT-2, and CAT-3 (SLC7A1, SLC7A2, and SLC7A3).

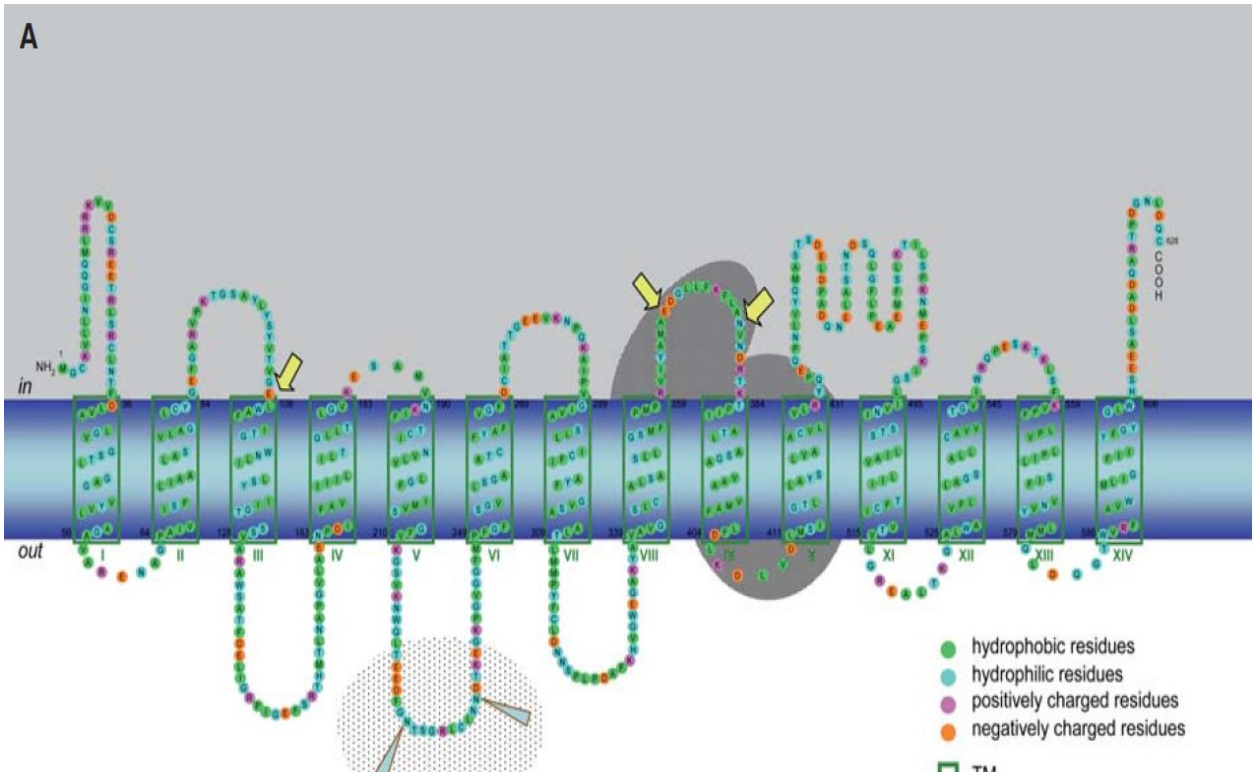

Figure 1.6. SLC7 family members with 14 putative transmembrane domains (TM). (A) Snake model of hCAT-1: the yellow arrows point to residues identified to be important for transport function. The white wedges indicate the glycosylation sites. Taken with permission from Springer, Closs et al., (2006).

The CAT proteins have a 14 putative transmembrane domain with intracellular N- and C-termini (Figure 1.6; Albritton et al., 1989). The third extracellular loop contains a very well conserved glycosylation site across species for CAT-1, and CAT-2 contains two N-linked glycosylation sites in this loop (Wang et al., 1996). Proper function of these proteins does not tolerate many alterations in the protein sequence, therefore only very few polymorphisms can exist in the sequence, and the National Center for Biotechnology Information data base only lists silent single nucleotide polymorphisms (**SNP**: Closs et al., 2006). Cationic amino acid transporters are also, to varying degrees, *trans*-stimulated. In this case, the *trans* side of the membrane would be the intracellular, or transport channel pore facing the fetal system. This indicates that in utero-placental tissues, facilitative diffusion would occur up the concentration gradient, as the concentration of cationic amino acids in fetal fluids, is greater than that of maternal circulation (discussed in detail later). This is contradictory to facilitative diffusion membrane transporters which are known to transport down a concentration gradient in order to balance nutrient concentrations between the two sides of the membrane as discussed previously. However, in high proliferating tissues, such as the developing conceptus, nutrient sensing pathways such as mTOR, which is regulated by intracellular amino acid concentrations, is intrinsically linked to amino acid transport activity as well as intracellular amino acid metabolism (Hyde et al., 2003). This indicates that during fetal growth and development, when mTOR is being activated by cationic amino acids, CAT's are thereby stimulated in order to increase the intracellular amino acid concentration, and continue the growth trajectory of the fetus through activation of nutrient sensing signaling pathways.

Cationic amino acid transporter CAT-1 is capable of transporting arginine, lysine, and at low pH histidine (Closs et al., 2006). Transporter CAT-1 exhibits K_m values for L-arginine and

L-lysine of 100-150μM, and is strongly stimulated by substrate on the *trans*-side of the membrane (Kim et al., 1991; Closs et al., 1997). A homozygous deletion of CAT-1 in mice is lethal, and results in a 25% reduction in size compared to a wild-type littermate, and death the day after birth (Perkins et al., 1997). The normal development of the CAT-1 knockout mouse before birth is hypothesized to be due to the expression of *CAT-3* during embryogenesis and fetal development (Ito and Groudine, 1997; Nicholson et al., 1998). In sheep, knockout of *CAT-1* resulted in decreased arginine transport by 73%, arginine. Additionally arginine catabolites citrulline and ornithine, decreased by 76% and 40% respectively. Furthermore, decreases in arginine transport resulted in decreased ornithine decarboxylase, nitric oxide synthase (**NOS**), and polyamines which resulted in retarded growth development of the conceptus (Wang et al., 2014). In sheep, *CAT-1* expression increased 4-fold between days 10 and 14, and an additional 2-fold between days 16 to 20 (Gao et al., 2009b). Additionally, CAT-1 expression was increased (1.8-fold greater than control) in the presence of P4, and tended to be increased in the presence of IFN-τ. In the peri-implantation bovine uterus, *CAT-1* expression increased to d 16 of gestation (Forde et al., 2014). Additionally, expression of *CAT-1* was greater in intercaruncular endometrium compared with the caruncular endometrium (Forde et al., 2014). Localization of CAT-1 in ovine utero-placental tissues was determined to be in the uterine LE, GE, and stromal cells, as well as in low levels in the trophectoderm and endoderm (Gao et al., 2009b).

The second CAT transporter, CAT-2, has a ten-fold lower substrate affinity compared with CAT-1, and is less dependent on *trans*-stimulation (Closs et al., 1993; Kavanaugh et al., 1994; Closs et al., 1997). In ewes, *CAT-2* mRNA expression is greater in pregnant vs. cyclic ewes. Additionally, expression of CAT-2 increased 3.9-fold from d 10 to 14, and another 2.6-fold and 4.2-fold on d 18 and 20, respectively (Gao et al., 2009b). In swine, mRNA expression of

CAT-2 in uterine endometria was greatest on d 12 of gestation (d of maternal recognition; Senger, 2012), and in placenta, was greatest on d 30 of gestation (nearing the end of the 1st trimester; Shim et al., 2012). In the bovine, expression of *CAT-2* from d 10 to 20 of gestation, decreased with advancing gestation (Forde et al., 2014). Expression of *CAT-2* increased 4.1-fold in sheep in response to progesterone, and an additional 1.7-fold when co cultured with IFN-τ. These effects were only seen in the LE and GE of the uterus, and not in the stromal cells (Gao et al., 2009b). Expression of *CAT-2* in response to P4 or IFN-τ in the bovine has yet to be elucidated. Cationic transporter CAT-2 was localized in the uterine LE and GE between days 16 and 20 of gestation, and in the trophectoderm and extra embryonic endoderm of the conceptus between days 13 and 18 of gestation (Gao et al., 2009b).

The final CAT transporter investigated within is CAT-3, which is widely expressed in embryonic tissues, and total knockout of this gene would most likely result in embryonic mortality due to interference with normal development (Closs et al., 2007). It is also hypothesized, that CAT-3 would be the main transporter during embryogenesis and fetal development, as a knockout of CAT-1 resulted in normal growth throughout gestation, however cause mortality one day post-parturition (Ito and Groudine, 1997; Nicholson et al., 1998). This theory has yet to be confirmed due to no CAT-3 knockout models having been established thus far. In the uterine endometrium of sheep, *CAT-3* expression was not influenced by day of gestation or pregnancy status (Gao et al. 2009b). Expression of CAT-3 in response to progesterone or IFN-τ has yet to be established in the bovine or ovine model (Bazer et al., 2015). Localization of CAT-3 has been established in the trophectoderm and extra embryonic endoderm of the conceptus of sheep; however, no data has been reported as to the location in uterine endometrium (Bazer et al., 2015).

Although most emphasis will be placed on arginine, most amino acids are found in greater concentrations in fetal fluids, compared with maternal circulation (Kwon et al., 2003). Concentrations of arginine in serum (~ 0.13 mM) are less that what is found in uterine luminal fluid (~0.46-0.80 mM; Wu et al., 1996; Bazer et al., 2015). It has been determined that in porcine allantoic fluid, arginine concentrations are ~4.5 mM, and that arginine increases in concentration by 18-fold between days 30 and 40 of gestation with the total nitrogen accounting for ~50% of α-amino acid nitrogen (Wu et al., 1996). In the ovine, total recoverable levels of arginine in uterine flushing's was greater in pregnant vs. cyclic heifers, and increased 8-fold from d 10 to 16 of gestation (Gao et al., 2009c). In the bovine, total recoverable levels of arginine in uterine luminal fluid was nearly 2-fold greater on d 19 of gestation compared with d 10, 13, and 16 (Forde et al., 2014). Additionally, lysine, which is also transported by CAT's, is greater in ovine uterine flushing's of pregnant ewes compared with cyclic ewes. Concentrations of lysine in pregnant ewes were also greater on d 14, 15, and 16 of gestation compared with earlier days (Gao et al., 2009). Following a similar trend as arginine, lysine is also greater on d 19 of gestation in bovine uterine flushings, a nearly 2-fold increase in lysine concentration compared with all previous days (Forde et al., 2014). Interestingly, concentrations of arginine and lysine in bovine uterine flushing's were lower on days 7 to 13 of gestation compared with the same days of the estrous cycle (Forde et al., 2014).

As stated previously, arginine catabolism is an important activator of mTOR. Arginine can be metabolized by NOS directly to citrulline, and by doing so creates nitric oxide (**NO**). Additionally, arginine can be metabolized to ornithine via arginase, which creates urea as a by-product. From here, ornithine decarboxylase 1 metabolizes ornithine to the polyamines: putrescine, spermidine, and spermine. Polyamines and NO are critical for implantation and

development of the conceptus through their activation of mTORC1 (Wu et al., 2009; Bazer et al., 2011a). Feeding arginine-free diets during gestation in rats or inhibiting NO synthesis resulted in increased fetal resorption, intrauterine growth restriction, increased perinatal mortality, and decreased the number of live fetuses (Greenberg et al., 1997). One of the main reasons for these pregnancy failures, is due to the decreased abundance of products of arginine metabolism, NO and polyamines. These products, in addition to activating mTOR, are also essential for placental growth and angiogenesis, and therefore, for increasing uterine and placental-fetal blood flow (Wu et al., 2008). Polyamines and NO synthesis in both porcine and ovine placenta are greatest during early gestation when placental growth is most rapid (Kwon et al., 2004; Wu et al., 2005; Wu et al., 2006). Therefore, it is hypothesized that the use of arginine may prevent and treat IUGR in humans and animals.

Statement of the Problem and Experimental Objectives

The long-term effects of restricted nutrient intake during early gestation include impaired placental development during establishment of the feto-maternal interface, leading to poor fetal growth and IUGR. The prenatal growth trajectory is sensitive to direct and indirect effects of maternal dietary intake from the earliest stages of embryonic life even though nutrient requirements for conceptus growth are negligible (Wallace et al., 2006; Robinson et al., 1999). Data indicates that production efficiency and health complications throughout life are 'programmed' into the offspring due to poor fetal development associated with low birth weight (Wu et al., 2006; Caton and Hess 2010; Funston et al., 2010; Reynolds et al., 2010; Reynolds et al., 2012). In livestock, low birth weight is associated with preweaning death, which ranges from 6.6 to 12.0 % for lambs (USDA, 2007; USDA, 2010) and calves (Azzam et al., 1993) in the U.S.,

and can also influence profitability by reducing weaning weights (Corah et al., 1975) and increasing costs associated with health treatments of unthrifty animals.

Fetal growth is vulnerable to maternal dietary nutrient deficiencies during the 1st trimester of gestation (Wu et al., 2004). During the first 50 days of gestation, organogenesis is taking place. This time period of gestation is a critical developmental window with significant cellular and tissue differentiation. Nutritional influences may alter the mammalian phenotype through affecting gene regulatory mechanisms involved in DNA synthesis and replication, thus "imprinting" potential susceptibilities to chronic disease and metabolic issues into the genome (Waterland and Jirtle, 2004). Currently, fetal undernutrition occurs in grazing livestock worldwide (Wu et al., 2004). Maternal undernutrition has been implicated in fetal growth restriction and altered placental growth, reduced amino acid and glucose transport, and increased apoptosis and autophagy, which overall can yield decreased fetal growth during gestation (Zhang et al., 2015). Before the establishment of hemotrophic nutrition, the placenta is developing and the fetus begins to utilize increasing quantities of glucose and amino acids (Groebner et al., 2011). Thus, the expression of glucose and amino acid transporters in the utero-placenta becomes essential to the viability of the conceptus. Therefore, we studied the utero-placental glucose and fructose transporters *GLUT1, GLUT3, GLUT5,* and *GLUT14* (*SLC2A1. SLC2A3, SLC2A5,* and *SLC2A14*). The amino acid transporters investigated are *CAT-1*, *CAT-2*, and *CAT-3* (*SLC7A1*, *SLC7A2*, and *SLC7A3)* whose substrates are cationic amino acids such as arginine and lysine, which are directly linked to angiogenesis and cell proliferation. Understanding how day of gestation affects gene expression and nutrient concentrations in bovine fetal fluids, as well as the effects of compromised maternal nutrition on the mRNA expression of nutrient transporters and subsequently nutrient concentrations in bovine utero-placental tissues, may lead to novel

approaches to feeding heifers in order to mitigate spontaneous embryonic abortion, and lead to increased production efficiencies.

With this in mind, we tested the hypothesis that mRNA for glucose and cationic amino acid transporters in utero-placental tissues would be differentially expressed due to day of gestation and maternal nutritional status, and that the concentrations of glucose, fructose, and cationic amino acids would be different due to day of gestation and maternal nutritional status.

Therefore the objectives of these studies were to 1) Isolate glucose and cationic amino acid transporters in utero-placental tissues and determine the effect of day of gestation on their respective mRNA expression, while establishing key dates of gestation for further analysis 2) Determine the effects of aberrant maternal nutrition on the mRNA expression of glucose, fructose, and cationic amino acid transporters in bovine utero-placental tissues on days 16, 34, and 50 of gestation, and 3) Determine the effects of aberrant maternal nutrition on the concentrations of nutrients in maternal and fetal fluids from days 16 to 50 of gestation.

The following chapters of this thesis will be divided by these experimental objectives: 1) Establishing the pattern of mRNA expression over the first 50 days of gestation in Chapter 2, 2) Confirming presence of, and isolating novel glucose transporters GLUT3 and GLUT14 in bovine utero-placental tissues while simultaneously elucidating their relative expression as influenced by aberrant maternal nutrition in Chapter 3, 3) Determining the effects of maternal nutritional status on the mRNA expression of *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* as well as concentrations of glucose, fructose, and cationic amino acids in maternal and fetal fluids on days 16, 34, and 50 of gestation in Chapter 4, and 4) Concluding with a general discussion and future directions in Chapter 5.

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CHAPTER 2: NUTRIENT TRANSPORTERS IN BOVINE UTERO-PLACENTAL TISSUES ON DAYS 16 TO 50 OF GESTATION[1]

Abstract

During early gestation, nutrients are transported to the developing embryo via transporters in the uterine endometrium and chorioallantois. In the present study, we examined glucose transporters, *GLUT1* and *GLUT3*, and the cationic amino acid transporters, *SLC7A1*, *SLC7A2,* and *SLC7A3,* in order to test the hypotheses that: 1) relative mRNA expression of transporters would be different among utero-placental tissue type as gestation progresses; and 2) concentrations of glucose and cationic amino acids would be different among target sites (placental compartments, serum, histotrophic) and days of gestation. To test these hypotheses, crossbred Angus heifers (n = 46), were synchronized, bred via AI and then ovariohysterectomized on d 16, 22, 28, 34, 40, or 50 of gestation (n = 5 to 9/d), or were not bred and ovariohysterectomized on d 16 of the synchronized estrous cycle (n = 7) to serve as non-pregnant (NP) controls. Utero-placental tissues (maternal caruncule, CAR; intercaruncular endometrium, ICAR; and fetal membranes, FM [chorioallantois, d 22 and later]) were collected from the uterine horn ipsilateral to the Corpus Luteum immediately following ovariohysterectomy. Relative mRNA expression of the glucose transporters and cationic amino acid transporters was determined for each tissue from d 16 to 50 of gestation and NP controls. Chorioallantoic, amniotic, and plasma fluids were collected from heifers on d 40 and 50 of

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[1]The material in this chapter was co-authored by M. S. Crouse, K. J. McLean, M. R. Crosswhite, L. P. Reynolds, C. R. Dahlen, B. W. Neville, P. P. Borowicz, and J. S. Caton. It has been accepted for publication by the Journal of Animal Science, doi: 10.2527/jas.2016-0857. M. S. Crouse and K. J. McLean had primary responsibility for collecting samples. M. S. Crouse was the primary developer of the conclusions that are advanced here. M. S. Crouse also drafted and revised all versions of this chapter. J. S. Caton served as proofreader and checked the math in the statistical analysis conducted by M. S. Crouse

gestation to determine concentrations of glucose and cationic amino acids. Expression of *GLUT1* and *SLC7A2* showed a tendency ($P < 0.10$) towards being greater on d 16 ICAR, and d 34 ICAR, respectively. Day × tissue interactions ($P < 0.05$) were present for *GLUT3, SLC7A1* and *SLC7A3*. Expression of *GLUT3* was greater in d 50 CAR, *SLC7A1* was greater on d 34 in ICAR, and *SLC7A3* was greater in CAR tissue on d 34 compared with all other tissues and days of gestation. Glucose concentrations tended ($P = 0.10$) to be impacted by a day × fluid interaction. A day × fluid interaction ($P = 0.01$) for arginine concentration was observed with greater concentrations in allantoic fluid on d 40 compared with all other days and fluid types. These data support our hypothesis that glucose and cationic amino acid transporters differ in their level of mRNA expression due to day of gestation and utero-placental tissue type. In addition, concentrations of nutrients were impacted differentially by day, target site, and/or their respective interaction.

Key words: cationic amino acids, early gestation, glucose, nutrient transporters, utero-placental

Introduction

Currently, fertilization rates for first service AI are approximately 90% in beef heifers (Bridges et al., 2013); however, by d 30 of gestation, only 50 to 60% of heifers are gestating viable embryos. Moreover, Thatcher et al. (1994) indicated that up to 80% of all embryonic loss occurs before d 40 of gestation. Nutrient transport across the placenta is the only method of nutrient flux to the embryo during early gestation, and expression of transporters in the interplacentomal region of the placenta provides nutrients via histotroph to the early conceptus (Wooding and Flint, 1994). The presence of nutrient transporters and nutrient flow to the embryo and placenta is crucial for proper development and growth. During the preimplantation period

through the establishment of hemotrophic nutrition, the conceptus utilizes increasing quantities of glucose and amino acids supplied by uterine histotroph (Gardner, 1998; Groebner et al., 2011; Bazer et al., 2014). Histotroph is a mixture of enzymes, growth factors, adhesion proteins, cytokines, hormones, transport proteins, amino acids, and saccharides which are supplied from the luminal, superficial glandular and deep glandular epithelium of the uterine endometrium (Bazer, 1975; Roberts et al., 1988; Wang et al., 2014). Inappropriate composition of uterine histotroph may cause improper development of conceptuses (Wang et al., 2014). Thus, the expression and function of glucose and amino acid transporters in the utero-placenta becomes essential to the viability of the conceptus. In this study, we tested the hypotheses that; 1) relative mRNA expression of transporters would be differentially expressed due to day of advancing gestation, and that transporters would be differentially expressed due to utero-placental tissue type and 2) concentrations of glucose and cationic amino acids would be different due to day of gestation and maternal or fetal fluid type.

Materials and Methods

All animal procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Animals

Crossbred Angus heifers (n = 46, ~15 mo of age; BW = 362.3 ± 34.7 kg ADG = 0.22 kg) were fed once daily, grass hay (DM: 90%, CP: 10.7%, ADF: 38.9%) and supplemented with cracked corn (DM: 90%, CP: 9.8%, ADF: 3.3%) via a Calan Head Gate System (American Calan, Northwood, NH) and were individually fed to gain 0.25kg/d. Heifers were obtained from the Central Grasslands Research Extension Center (Streeter, ND; 2.5 h southwest of Fargo), and

housed at the NDSU Animal Nutrition and Physiology Center in group pens with six heifers in each pen. All heifers were exposed to the 5-d CO-Synch + CIDR estrus synchronization protocol (Bridges et al., 2008). Seven heifers were not inseminated to serve as non-pregnant (NP) controls, but received ovariohysterectomy on d 16 of the synchronized estrous cycle. The remaining heifers were AI bred to a single sire and ovariohysterectomized on d 16 (n = 9), d 22 (n = 6), d 28 (n = 6), d 34 (n = 7), d 40 (n = 6), or d 50 (n = 5) of gestation. Heifer selection for ovariohysterectomy on d 16 of gestation was random selection based on inability to confirm pregnancy via ultrasonography, and upon hysterectomy, heifers without fetal membranes were removed from the study. Heifer selection on d 22 was based upon no observed estrus by d 21 and localization of a Corpus Luteum and fetus/fetal fluids via ultrasonography. Heifers on d 28 through 50 of gestation were selected for ovariohysterectomy after confirmed pregnant via ultrasonography.

Sample Collection and Analysis

Ovariohysterectomy procedures were conducted as described by McLean et al (2016). Briefly, ovariohysterectomy was conducted as a standing procedure, with a left flank incision. Uterine, and ovarian arteries were sutured and ligated, along with sutures being placed along the cervix. The uterus was clamped caudal to the bifurcation, and incised along the clamp, thereby collecting the entire uterine body and horns, along with the attached ovaries. Immediately following ovariohysterectomy, utero-placental tissues (caruncle, **CAR**; intercaruncular endometrium, **ICAR**; and fetal membranes, **FM** [chorioallantois, d 22 and later]) were separated and collected individually from the uterine horn ipsilateral to the Corpus Luteum, as previously described (Grazul-Bilska et al., 2010, 2011). Fetal membranes were collected only from d 22 and

later due to limited tissue volume present on d 16 and absence of FM in NP controls. Once collected, all tissues were snap frozen in liquid nitrogen cooled isopentane, and stored at -80°C.

Real-time quantitative PCR (qPCR) was done on CAR, ICAR, and FM samples to determine mRNA expression of glucose transporter 1 (**GLUT1**-facilitative glucose transporter which is found in most tissues throughout the body and is ubiquitous across mammalian species), glucose transporter 3 (**GLUT3**- Facilitative glucose transporter specifically known for neural and placental glucose transport), and cationic amino acid transporter 1, 2, and 3 (**SLC7A1**, **SLC7A2**, and **SLC7A3**- All of which are facilitated diffusion arginine and lysine transporters). The RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA), total quantity of RNA was determined using Take3 module of a Synergy H1 Microplate Reader (BioTek, Winooski, VT), and cDNA was synthesized using QuantiTect Reverse 2.1 Transcription Kit (Qiagen). Primer sequences (Table 2.1) for glucose and amino acid transporters were obtained from GenBank (GenBank, Bethesda, MD). The cDNA dilutions were determined by primer validation for each gene and tissue type across stages of gestation. For PCR, dilutions of 1:100 were used for *GLUT1* and *SLC7A1*, and 1:10 for *GLUT3*, *SLC7A2*, and *SLC7A3*. Gene expression was quantified using a 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY) with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA); 18µL total for *GLUT1* and *SLC7A1*, and 15 µL total for *GLUT3*, *SLC7A2*, and *SLC7A3*.

Plasma samples were collected via jugular venipuncture at time of ovariohysterectomy using 10 mL heparin vacutainer tubes (Becton Disckinson HealthCare, Franklin Lakes, NJ), and centrifuged at 1,500 × g for 30 minutes. Plasma was separated from blood constituents and stored at -20°C. Allantoic Fluid (ALF) was collected by isolating the embryo within the uterine horn, and extracting 10 mL of fluid from the chorioallantoic sac using a 22 gauge needle

(Medtronic, Minneapolis, MN) to prevent rupture of the sac. Amniotic Fluid (AMF) was collected using a 22 gauge needle (Medtronic) inserted through the amnion with suction applied via syringe after the amniotic sac containing the embryo was visualized; 5 mL of fluid was collected from the d 40 embryo and 10 mL from d 50 embryos. Allantoic and amniotic fluids were only collected on d 40 and 50 of gestation. Once collected, all fluids were snap frozen in liquid nitrogen cooled isopentane and stored in -20°C.

Arginine, ornithine, citrulline, and lysine concentrations were determined using ACQUITY UPLC System (Waters Corporation, Milford, MA). For UPLC, 250 µL of fluid was used for plasma, ALF, and AMF. The MassTrac Amino Acid Analysis System for Waters UPLC was used to determine the full profile of amino acids in physiological fluids. Derivatization chemistry for physiological samples is a precolumn method and is based on a derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), which converts both primary and secondary amino acids to stable chromophores for UPLC detection. Glucose concentrations were determined using Infinity Glucose Hexokinase Liquid Stable Reagent (Fisher Diagnostics, Middletown, VA), and analyzed with a Synergy H1 Microplate Reader (BioTek, Winooski, VT). For glucose determination, 5 µL of fluid was used for plasma, ALF, and AMF with 250µL of reagent (Intraplate CV = 2.99; Interplate CV = 2.91).

Statistical Analysis

Day of Gestation CAR and ICAR.

Data were analyzed for day of gestation effects using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, NY), with individual heifer serving as the experimental unit. Means were separated using the LSMEANS procedure of SAS and *P*-values ≤ 0.05 were considered different. Effect of day of gestation was determined for each individual tissue (CAR, ICAR).

Table 2.1. Primer Sets used for real-time quantitative reverse-transcription PCR

Gene[1]	Product size (bp)	Forward primer (5'-3')	Reverse Primer (5'-3')	GenBank Accession No.
GLUT1	2533	CGGCTGCCCTGGATGTC	GCCTGGGCCCACTTCAAA	NM_174602
GLUT3	1404	CAAGTCACAGTGCTAGAGTCTTTC	GGAGAGCTGGAGCATGATAGAGAT	XM_001256170
SLC7A1	695	CCGATAATCGCCACCTTAACCT	ACCAGGTCCTTCAGGTCGAA	DQ399522
SLC7A2	490	AAGGAAATGTGGCAAACT	TTGAAAAGCAACCCATCCTC	XM_865568.2
SLC7A3	473	TACCAGCCTCTTGGGCTCTA	AAAGCAGTGGAATGGACCAC	BC126655

[1]*GLUT1* and *GLUT3*- Glucose transporter solute carrier family 2 member 1 and 3. *SLC7A1*, *SLC7A2*, and *SLC7A3*- Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.

Day of Gestation FM.

Data were analyzed for day of gestation effects using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, NY), with individual heifer serving as the experimental unit. Means were separated using the LSMEANS procedure of SAS and *P*-values ≤ 0.05 were considered different. Due to no NP control for fetal membranes, data is given as ΔCt values after being normalized to β-actin. Lowest ΔCt would yield highest total expression after second normalization to a control; however, since there is no FM in a non-pregnant animal, there is no second normalization for effect of day of gestation. Therefore in analysis, a lower ΔCt will be described as having the higher level of expression.

Day × Tissue.

In order to evaluate the level of expression of glucose and cationic amino acid transporters between CAR, ICAR, and FM from d 16 to 50 of gestation, data were analyzed for an interaction between day of gestation (d 16, 22, 28, 34, 40, or 50) and utero-placental tissue type (CAR, ICAR, and FM) using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, NY), with individual heifer serving as the experimental unit. Means were separated using the LSMEANS procedure of SAS and *P*-values ≤ 0.05 were considered different. Interactions were determined across utero-placental tissue type and day of gestation utilizing NP endometrium sample as the baseline. If no significant interaction was present, main effect of tissue was analyzed within gene of interest. Effect of tissue was determined using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, NY), with individual heifer serving as the experimental unit. Means were separated using the LSMEANS procedure of SAS and *P*-values ≤ 0.05 were considered different. The effect of day of gestation given is not an accurate representation of significance within gene due to a magnitude change compared with NP endometrium, and was

therefore conducted as discussed previously to provide an effect of d of gestation without an increased magnitude yielding significance.

Day × Fluid.

Data were analyzed for an interaction between day of gestation and maternal or fetal fluid type using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, NY), with individual heifer serving as the experimental unit. Means were separated using the LSMEANS procedure of SAS and *P*-values ≤ 0.05 were considered different.

Results

Effects of Day of Gestation

CAR.

Expression of *GLUT1* was greater ($P < 0.01$) at d 16 (7.8-fold increase over NP) compared with d 22 (4.3 fold increase over NP), which was greater ($P < 0.05$) than the remaining days of early pregnancy and NP (SEM = 1.0; Table 2.2). Expression of *GLUT3* was greater ($P = 0.01$) on d 50 (13.68-fold increase over NP) of gestation compared with NP and d 16, 22, 28, and 40 of gestation (SEM = 2.3; Table 2.2). Additionally, d 34 was greater (9.13-fold increase over NP; $P \leq 0.05$) compared with NP and d 16 (Table 2.2). Relative expression of *SLC7A1* was greater ($P \leq 0.05$) on d 28, 34, and 40 (5.12, 6.82, and 5.10-fold increase over NP) compared with NP, d 16, 22, and 50 of gestation (SEM = 0.9; Table 2.2). Day of gestation did not influence *SLC7A2* expression ($P = 0.20$). Expression of *SLC7A3* was greater ($P = 0.01$) on d 16 (6.9-fold greater than NP) compared with NP and all other days of gestation evaluated (SEM = 1.7; Table 2.2).

ICAR.

Expression of *GLUT1* in ICAR followed a similar pattern as in CAR, with d 16 (13.24-fold greater than NP) being greater ($P < 0.01$) than all other days measured (SEM = 2.2; Table 2.2). Additionally, d 22 *GLUT1* expression was greater (6.07-fold greater than NP; $P \leq 0.05$) than NP on d 34, 40, and 50 of gestation. Relative expression of *GLUT3* tended ($P = 0.06$) to be greater on d 28 and 34 compared with NP. On d 34 of gestation, relative expression of *SLC7A1* was 16-fold greater ($P < 0.01$) than NP, and on d 40 was still greater (12.02-fold greater than NP; $P \leq 0.05$) than NP or d 16, 22, 28, and 50 of gestation (SEM = 2.0; Table 2.2). Expression of *SLC7A2* was greater ($P = 0.02$) on d 34 (10.25-fold greater than NP) compared with NP and all other days except d 28 (7.84-fold greater than NP) which was greater compared with NP and d 16, 22, and 50 (SEM = 3.1; $P \leq 0.05$; Table 2.2). Expression of *SLC7A3* was greater ($P \leq 0.05$) on d 28 (1.87-fold greater than NP) compared with d 16, 22, and 50 (SEM = 0.5; Table 2.2). Additionally *SLC7A3* in NP, d 34, and 40 heifers was greater ($P \leq 0.05$) compared with d 16 and 22 (Table 2.2).

Table 2.2 Level of expression of nutrient transporters *GLUT1*, *GLUT3*, *SLC7A1*, *SLC7A2*, and *SLC7A3* in CAR and ICAR tissue in non-pregnant controls and from d 16 to 50 of gestation

Tissue[3]	Gene of Interest	Day of Gestation[2]							SEM[4]	*P* - value[5]
		NP	16	22	28	34	40	50		
CAR	*GLUT1*	1.00[a]	7.77[c]	4.34[b]	2.06[a]	1.38[a]	1.20[a]	1.70[a]	1.0	< 0.0001
	GLUT3	1.00[a]	3.89[a]	5.62[ab]	4.80[ab]	9.13[bc]	4.93[ab]	13.68[c]	2.3	0.01
	SLC7A1	1.00[a]	1.54[a]	1.99[a]	5.12[b]	6.82[b]	5.10[b]	0.98[a]	0.9	< 0.0001
	SLC7A2	1.00	2.38	1.37	0.68	3.24	2.95	0.35	1.0	0.2
	SLC7A3	1.00[a]	6.89[b]	0.69[a]	0.42[a]	0.52[a]	0.54[a]	0.14[a]	1.7	0.01
ICAR	*GLUT1*	1.00[a]	13.24[c]	6.07[b]	2.48[ab]	1.65[a]	1.04[a]	0.66[a]	2.2	< 0.0001
	GLUT3	1.00	0.93	1.67	4.32	4.90	3.51	0.73	1.7	0.06
	SLC7A1	1.00[a]	2.03[a]	1.63[a]	6.30[b]	16.03[d]	12.02[c]	5.73[b]	2.0	< 0.0001
	SLC7A2	1.00[a]	1.25[a]	1.23[a]	7.84[bc]	10.25[c]	4.08[ab]	2.15[a]	3.1	0.02
	SLC7A3	1.00[abc]	0.50[a]	0.20[a]	1.87[c]	1.49[bc]	1.46[bc]	0.61[ab]	0.5	0.03

[1]Gene of Interest = *GLUT1* and *GLUT3* - Glucose transporter solute carrier family 2 member 1 and 3. *SLC7A1*, *SLC7A2*, and *SLC7A3* - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.
[2]Day of Gestation = number of days after insemination. Day 0 is non-bred non-pregnant control and serves as the baseline of expression for that gene. Each gene expression is given as a fold change in relation to NP level of expression.
[3]CAR - caruncular tissue (caruncles taken from the uterine horn ipsilateral to the CL), ICAR - inter-caruncular tissue (endometrial tissue not including caruncles; taken from uterine horn ipsilateral to the CL)
[4]The average SEM was used within gene. NP n = 7, d 16 n = 9, d 22 n = 6, d 28 n = 6, d 34 n = 7, d 40 n = 6, d 50 n = 5.
[5]Probability values for effect of day on level of expression of individual genes.
[a-c]Means within a row without a common superscript differ ($P < 0.05$).

FM.

The expression of *GLUT1* was greater ($P < 0.01$; Table 2.3) on d 34 and 50 (5.58 and 5.32 ΔCt) compared with d 22 and 28. Additionally, d 40 (5.84 ΔCt) was intermediate and greater ($P \leq 0.05$) compared with d 22 (SEM = 0.3; Table 2.3). Expression of *GLUT3* in FM was similar among all days evaluated ($P = 0.72$; Table 2.3). The ΔCt of *SLC7A1* was greater ($P < 0.01$) on d 34 and 50 (6.99 and 7.05 ΔCt respectively; Table 2.3) compared with d 22, 28, and

40. Additionally, d 28 (8.16 ΔCt) was greater ($P \leq 0.05$) compared with d 22, and d 40 being equivalent to both d 22 and 28 (SEM = 0.4; Table 2.3). The expression of *SLC7A2* was similar throughout early gestation (~5.5 ΔCt; $P = 0.14$; Table 2.3). The cationic amino acid transporter *SLC7A3* tended ($P = 0.08$) to be greater in expression on d 28 (9.64 ΔCt) of gestation (Table 2.3).

Day × Tissue Interaction

When using NP endometrium as a baseline level of expression of 1.0, a tendency ($P = 0.10$) for a day × tissue interaction for *GLUT1* was observed. In addition, a main effect of tissue was observed with mean expression of *GLUT1* in CAR and ICAR (5.90 and 6.53 fold greater than NP endometrium, respectively; SEM = 0.75) being greater ($P < 0.01$) than FM (Table 2.4). Expression of *GLUT3* was impacted by a day × tissue interaction ($P = 0.01$) with d 50 CAR (15.04-fold greater than NP endometrium), which was greater than CAR on all other days or ICAR and FM on all days (Table 2.4). Cationic amino acid transporter *SLC7A1* expression was greater in day × tissue interaction ($P < 0.01$) on d 34 in ICAR (11.03-fold greater than NP endometrium) compared with d 40 ICAR (8.27-fold greater than NP endometrium), which was greater ($P \leq 0.05$) than ICAR on all other days or CAR and FM on all days (Table 2.5). Expression of *SLC7A2* tended ($P = 0.07$) to be affected by a day × tissue interaction.

In addition, a main effect of tissue was observed in which expression of CAR and ICAR was greater (11.8 and 7.7-fold greater than NP endometrium, respectively; $P < 0.01$) compared with FM (2.39 fold greater than NP endometrium; SEM = 1.69; Table 2.5). Relative mRNA expression of *SLC7A3* was impacted by day × tissue interaction, being greater ($P = 0.02$) in CAR on d 16 (28.05-fold greater than NP endometrium) compared to CAR on all other days or ICAR or FM on all days (SEM = 4.5; Table 2.5).

Table 2.3 Level of expression of nutrient transporters *GLUT1*, *GLUT3*, *SLC7A1*, *SLC7A2*, and *SLC7A3* in fetal membranes from d 22 to 50 of gestation.[1] Due to no second normalization, data is presented as a ΔCt value.[2]

Gene of Interest	Day of Gestation[3]					SEM[4]	*P* - value[5]
	22	28	34	40	50		
GLUT1	6.72[a]	6.35[ab]	5.58[c]	5.84[bc]	5.32[c]	0.3	< 0.01
GLUT3	6.38	6.06	6.29	6.35	5.66	0.4	0.72
SLC7A1	9.93[a]	8.16[b]	7.05[c]	8.86[ab]	6.99[c]	0.4	< 0.01
SLC7A2	6.76	6.05	6.09	4.77	5.57	0.6	0.14
SLC7A3	12.25	9.64	10.4	11.61	13.08	0.9	0.08

[1]*GLUT1* and *GLUT3* - Glucose transporter solute carrier family 2 member 1 and 3. *SLC7A1*, *SLC7A2*, and *SLC7A3* - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.
[2]Lower ΔCt value indicated higher level of expression.
[3]Day of Gestation = number of days after insemination. Values for expression of genes are provided as ΔCt values for that gene after being normalized to β-actin.
[4]The average SEM was used within gene. d 22 n = 6, d 28 n = 6, d 34 n = 7, d 40 n = 6, d 50 n = 5.
[5]Probability values for effect of day on level of expression of individual genes.
[a-c]Means within a row without a common superscript differ ($P < 0.05$).

Table 2.4 Level of expression of nutrient transporters *GLUT1* and *GLUT3* in CAR, ICAR, and FM tissues in non-pregnant (NP) controls and from d 16 to 50 of gestation compared with non-pregnant endometrium tissue.[1]

Item	Day of Gestation[2]							Tissue Means[4]	SEM[5]	*P*- value[3]		
	NP	16	22	28	34	40	50			Day	Tissue	Day × Tissue
GLUT1									1.9	< 0.01	0.02	0.10
CAR	0.719	18.929	9.494	3.108	3.009	2.343	3.715	5.90[h]				
ICAR	1.711	24.597	10.391	3.297	2.823	1.781	1.131	6.53[h]				
FM	-	-	0.590	0.642	1.106	0.936	1.307	0.92[g]				
GLUT3									2.7	< 0.01	< 0.01	0.01
CAR	1.308[abc]	6.412[abcd]	9.254[d]	7.911[bcd]	15.044[e]	8.127[cd]	24.475[f]	10.36				
ICAR	0.258[a]	0.892[ab]	1.598[abc]	4.126[abcd]	5.339[abcd]	3.353[abcd]	1.183[abc]	2.39				
FM	-	-	1.407[abc]	2.105[abc]	1.956[abc]	1.774[abc]	2.494[abcd]	1.95				

[1]*GLUT1* and *GLUT3* - Glucose transporter solute carrier family 2 member 1 and 3. CAR - caruncular tissue (caruncles taken from the uterine horn ipsilateral to the CL), ICAR - inter-caruncular tissue (endometrial tissue not including caruncles; taken from uterine horn ipsilateral to the CL), and FM - chorioallantois d 22 and later.

[2]Day of Gestation = number of days after insemination. The NP equals a non-bred, non-pregnant control d 0 and serves as the baseline of expression for that gene. Each gene expression is given as a fold change in relation to NP endometrium level of expression.

[3]Probability values for the effect of day, tissue, and day × tissue on level of expression of individual genes.

[4]The mean gene expression for the tissue across all days of gestation.

[5]The average SEM was used within gene. NP- n = 7, d 16 n = 9, d 22 n = 6, d 28 n = 6, d 34 n = 7, d 40 n = 6, d 50 n = 5.

[a-f]Means within a gene without a common superscript differ in day × tissue ($P < 0.05$).

[g-h] Means different across tissue ($P < 0.05$).

Day × Fluid Interaction

When comparing concentrations of metabolites on d 40 and 50 across maternal plasma, allantoic, and amniotic fluids, glucose tended ($P = 0.10$) to be affected by a day × fluid interaction. A main effect of day determined glucose concentrations to be greater ($P = 0.05$) on d 50 when compared to d 40 of gestation (2.76 vs. 2.35mM, respectively; SEM = 0.14; Table 2.6). Concentration of glucose in plasma was greater ($P < 0.01$) than both allantoic and amniotic fluid (4.6 vs. 1.6, and 1.50, respectively; SEM = 0.17; Table 2.6). The concentration of arginine showed a significant day × fluid interaction ($P < 0.01$), with allantoic fluid having greater ($P < 0.01$) concentrations of arginine on d 40 compared with amniotic and plasma fluids on all other days (Table 2.6). Concentrations of ornithine, the first catabolite of arginine, were not affected by a day × fluid interaction ($P = 0.51$), but were greater ($P < 0.01$) in allantoic fluid compared with plasma and amniotic fluids (153.58 vs. 70.43 and 69.43 μmol/L; SEM = 7.76; Table 2.6). Citrulline, which is an additional catabolite of arginine, was not affected by a day × fluid interaction ($P = 0.13$). There was a main effect of fluid, in which citrulline was greater ($P < 0.0001$) in maternal plasma, intermediate in allantoic fluid, and least in amniotic fluid as it was undetected by the UPLC (69.36, 22.58 μmol/L, and undetected, respectively; SEM = 3.24; Table 2.6).

Table 2.5 Level of expression of nutrient transporters *SLC7A1*, *SLC7A2*, and *SLC7A3* in CAR, ICAR, and FM tissues in non-pregnant (NP) controls and from d 16 to 50 of gestation compared with non-pregnant endometrium tissue.[1]

	Day of Gestation[2]								*P*-value[3]		
Item	NP	16	22	28	34	40	50	Tissue Means[4]	SEM[5]	Day	Tissue	Day × Tissue
SLC7A1									0.8	< 0.01	< 0.01	< 0.01
CAR	0.671^{a}	1.291^{a}	1.661^{ab}	4.276^{def}	5.699^{f}	4.263^{cdef}	0.687^{a}	2.65				
ICAR	0.688^{a}	1.393^{a}	1.118^{a}	4.330^{ef}	11.026^{h}	8.267^{g}	3.944^{bcdef}	4.40				
FM	-	-	0.086^{a}	0.324^{a}	0.624^{a}	0.233^{a}	0.624^{a}	1.89				
SLC7A2									4.3	< 0.01	< 0.01	0.07
CAR	5.718	7.625	7.143	4.25	16.904	10.189	1.841	7.67^{h}				
ICAR	1.098	4.856	4.777	19.137	28.267	15.914	8.388	11.78^{h}				
FM	-	-	1.077	2.761	1.657	3.861	2.605	2.39^{g}				
SLC7A3									4.5	< 0.01	0.40	0.02
CAR	2.39^{a}	28.049^{b}	2.816^{a}	1.69^{a}	2.132^{a}	2.191^{a}	0.697^{a}	5.71				
ICAR	1.55^{a}	0.771^{a}	0.311^{a}	2.9^{a}	2.306^{a}	2.267^{a}	0.203^{a}	1.58				
FM	-	-	0.61^{a}	1.603^{a}	1.949^{a}	0.634^{a}	0.212^{a}	1.00				

[1]*SLC7A1*, *SLC7A2*, and *SLC7A3* - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3. CAR - caruncular tissue (caruncles taken from the uterine horn ipsilateral to the CL), ICAR - inter-caruncular tissue (endometrial tissue not including caruncles; taken from uterine horn ipsilateral to the CL), and FM - chorioallantois d 22 and later.

[2]Day of Gestation = number of days after insemination. The NP equals a non-bred, non-pregnant control d 0 and serves as the baseline of expression for that gene. Each gene expression is given as a fold change in relation to NP endometrium level of expression.

[3]Probability values for the effect of d, tissue, and d × tissue on level of expression of individual genes.

[4]The mean gene expression for the tissue across all days of gestation.

[5]The average SEM was used within gene. NP- n=7, d16 n = 9, d22 n = 6, d28 n = 6, d34 n = 7, d40 n = 6, d50 n = 5.

[a-f]Means within a gene without a common superscript differ in d × tissue ($P < 0.05$).

[g-h] Means different across tissue ($P < 0.05$).

Lysine did not show a significant day × fluid interaction ($P = 0.31$), but showed a main effect of fluid, being greater ($P < 0.01$) in allantoic fluid, intermediate in amniotic fluid, and least in plasma (634.50, 236.95, and 99.86 μmol/L, respectively; SEM = 28.37; Table 2.6).

Discussion

This study is the first to report changes in the mRNA expression of key glucose and cationic amino acid transporters in bovine utero-placental tissues from before maternal recognition of pregnancy and implantation through the embryonic stage of development. Results of this study indicate that the expression of glucose and cationic amino acid transporters in bovine utero-placental tissues changes dramatically during the first 50 days of gestation in beef heifers. In the current report, facilitative glucose transporters (*GLUT1* and *GLUT3*) and cationic amino acid transporters (*SLC7A1*, *SLC7A2*, and *SLC7A3*) were all present in uterine tissue, CAR and ICAR, and developing placenta during the first 50 d of gestation as well as in NP uterine tissues. Additionally, these data also demonstrate an effect of day of gestation on the mRNA expression of these genes in bovine utero-placental tissues, as well as maternal and fetal fluid type on the concentration of glucose and arginine.

In sheep endometrium, temporal changes of *GLUT1* expression were similar during early gestation in which *GLUT1* was greater during maternal recognition of pregnancy (Gao et al., 2009a) as reported herein. In the Holstein however, no effects of day of gestation were seen in the endometrium from d 28 to 42 of gestation (Lucy et al., 2012). Localization of GLUT1 in the ovine endometrium has been determined to be in the luminal (LE) and superficial glandular epithelium (sGE) of the ovine uterus (Gao et al., 2009a). In the ovine, *GLUT1* expression was increased 4.2-fold with progesterone (P4) treatment, and 2.1-fold with interferon tau (IFN-τ)

infusion (Gao et al., 2009a). In-situ hybridization and immunohistochemistry revealed that the effects of P4 and IFN-τ on GLUT1 were mainly localized to the LE and sGE (Gao et al., 2009a). It would be of interest in future studies to determine GLUT1 location in bovine uterine tissues and determine if GLUT1 is a progesterone induced and interferon stimulated gene as it is in the ovine. Expression of *GLUT1* in bovine FM increased in level of expression from d 22 to 50 following a differential pattern of expression as seen in maternal uterine tissues. This pattern of expression is also different than what was seen in Holstein cows, in which mRNA concentration of *GLUT1* decreased from d 28 to 42 of gestation (Lucy et al., 2012).

In contrast to the pattern of expression for *GLUT1* in bovine endometrium, *GLUT3* in CAR increased throughout gestation reaching peak level of expression on d 50. In the Holstein however, *GLUT3* mRNA was similar from d 28 to 42 of gestation in caruncular tissue (Lucy et al., 2012). Localization of GLUT3 in uterine tissue was established in the rat, in which GLUT3 was localized in both uterine stroma (dense connective tissue), and uterine epithelium, increasing in abundance as gestational day increased (Korgun et al., 2001). Expression of *GLUT3* in FM remained consistent as day of gestation increased. Similar results were reported with the ovine model, in which peri-implantation conceptus *GLUT3* mRNA was abundant and consistent from d 12 to 20 of gestation (Gao et al., 2009a), and in the Holstein model, in which placental *GLUT3* mRNA was abundant and consistent from d 28 to 42 of gestation (Lucy et al., 2012). The known localization of GLUT3 in the ruminant placenta has been determined to be along the microvillous membrane in sheep and cattle (Wooding et al, 2005), and in the peri-implantation trophectoderm and extraembryonic endoderm of the ovine conceptus (Gao et al., 2009).

Table 2.6 Glucose, arginine, ornithine, citrulline, and lysine concentrations in allantoic fluid, amniotic fluid and maternal plasma on d 40 and 50 of gestation.

			Fluid[1]					*P* - value[2]		
Nutrient[3]	Day of Gestation[4]		Plasma	Allantoic	Amniotic	Day Mean[5]	SEM[6]	Day	Fluid	Day × Fluid
Glucose							0.2	0.05	< 0.01	0.10
	40		5.08	1.33	1.06	2.35[g]				
	50		4.54	1.81	1.94	2.76[h]				
		Fluid								
	Mean	Mean[7]	4.59[y]	1.57[x]	1.50[x]					
Arginine							58.4	0.03	< 0.01	< 0.01
	40		118.13[a]	578.41[b]	164.12[a]	286.89				
	50		108.64[a]	237.97[a]	186.34[a]	177.64				
		Fluid								
		Mean	113.39	408.19	175.23					
Ornithine							36.4	0.95	< 0.01	0.51
	40		76.15	154.05	62.35	97.52				
	50		64.71	153.11	76.51	98.11				
		Fluid								
		Mean	70.43$_b$	153.58[a]	69.43[b]					
Citrulline										
	40		73.71	12.54	Undetected	28.75	4.6	0.91	< 0.01	0.13
	50		65.01	22.58	Undetected	29.19				
		Fluid								
		Mean	69.36[a]	17.56[b]	Undetected[c]					
Lysine							39.9	0.80	< 0.01	0.31
	40		103.07	671.64	209.01	327.91				
	50		96.65	597.35	264.88	319.63				
		Fluid								
		Mean	99.86[x]	634.50[z]	236.95[y]					

Table 2.6 Glucose, arginine, ornithine, citrulline, and lysine concentrations in allantoic fluid, amniotic fluid and maternal plasma on d 40 and 50 of gestation (continued).

[1]Fluid = Concentrations of glucose (mM), arginine (µmol/L), ornithine (µmol/L), citrulline (µmol/L), and lysine (µmol/L) in maternal plasma, allantoic fluid, and amniotic fluid.
[2]Probability values for the effect of day, fluid, and day × fluid on the concentration of glucose, arginine, ornithine, citrulline, and lysine in fluids.
[3]Nutrient = Glucose, arginine, ornithine, citrulline, and lysine.
[4]Day of Gestation = number of days after insemination.
[5]Mean of nutrient concentration across fluid type within a given day of gestation.
[6]SEM = The average SEM for day × tissue interaction was used for each item. d 40 n = 6, d 50 n = 5.
[7]Mean of nutrient concentration across day of gestation within a given fluid type.
[a-b]Means within nutrient without a common superscript differ in day × fluid ($P < 0.05$).
[g-h]Means across fluid within day without a common superscript differ ($P < 0.05$).
[x-z]Means across day within fluid without a common superscript differ ($P < 0.05$).

Although GLUT1 and GLUT3 are both high affinity glucose transporters, (~5 mM and ~1.5mM respectively) GLUT3 transport capacity is the highest calculated of all the known GLUT isoforms, thus facilitating proportionally greater glucose uptake (Thorens and Mueckler, 2010). This is supported by Ganguly et al. (2007) who reported that a null mutation of GLUT3 resulted in early embryonic mortality in mice due to increased apoptosis in blastocysts. Data from Crouse et al. (2016) in bovine chorioallantois, found that *GLUT3* expression was greater in pre-implantation compared with post-implantation fetal membranes. Additionally, GLUT3 null mice failed to survive post implantation, even in nutrient rich growth mediums, due to failed neurulation (d ~ 8.5; Total gestation ~ 20 d; d 18-22 in bovine, Winters et al., 1942), or late-gestation fetal growth restriction characterized by decreased GLUT3- mediated transplacental glucose transport (Ganguly et al., 2007).

Glucose is not the most abundant hexose sugar to be utilized by the fetus; rather it is fructose (Wang et al., 2016). Glucose is metabolized to fructose, however, by the placenta and stored in allantoic fluid (White et al 1979; Wu et al., 2004; Wang et al., 2016). Glucose concentrations are at maximum 1.1 mM in ovine allantoic fluid whereas fructose can range from 11.1 to 33.3 mM during gestation (Kim et al 2012). Even so, glucose is required for stimulation of the mammalian target of rapamycin (mTOR) by decreasing adenosine monophosphate-activated protein kinase (AMPK; Tan and Miyamoto, 2016), as well as being coupled with fructose and glutamine activation of mTOR (Wang et al., 2016), and stimulating the abundance of the phosphorylated forms of the mTOR cell signaling pathway proteins, thereby decreasing autophagy and increasing protein synthesis.

Cationic amino acid transporters *SLC7A1*, *SLC7A2*, and *SLC7A3* also known as the CAT transporters (CAT-1, CAT-2, and CAT-3) are part of the y^+ system of transporters which are a

facilitated diffusion sodium-independent group of transporters known for transporting cationic amino acids such as arginine and lysine and at low pH, histidine (Closs et al.,2007). The three transporters studied vary in their stimulation and affinity; *SLC7A1* exhibits K_M values for L-arginine, L-lysine of 100 – 150 μM, and is strongly stimulated by substrate on the *trans*-side of the membrane (Kim et al., 1991; Closs et al., 1997); *SLC7A2* and *SLC7A3* exhibit lower substrate affinity and are less dependent on *trans*-stimulation (Closs et al., 1997; Vekony et al., 2001), with *SLC7A2* having about a tenfold lower substrate affinity compared with *SLC7A1* (Closs et al., 1993; Kavanaugh et al., 1994; Closs et al., 1997). Knockout of *SLC7A1* resulted in decreased arginine transport by 73%, arginine and its catabolites, citrulline and ornithine, by 76% and 40% respectively, as well as decreased ODC1, NOS, and polyamines which resulted in retarded growth development of the conceptus (Wang et al., 2014).

In all three cationic amino acid transporters investigated (*SLC7A1*, *SLC7A2*, and *SLC7A3*), greater levels of mRNA expression were observed during key days of gestation; d 16 at maternal recognition of pregnancy and implantation, and d 28 to 40 the critical window of placental development beyond which little embryonic/fetal loss occurs (Thatcher et al.,1994; Bridges et al., 2013). In ewes, mRNA of *SLC7A1* and *SLC7A2* increased through d 20 of gestation (Gao et al., 2009b). This is similar to what is reported in the current study in which *SLC7A1* and *SLC7A2* increased in expression through d 28 to 42. mRNA of *SLC7A1* was determined to be most abundant in ovine uterine LE, sGE, and GE, and also in low abundance in conceptuses. mRNA of *SLC7A2* was weakly expressed in trophectoderm and endoderm of conceptuses during peri-implantation in the ovine (Gao et al., 2009b). Additionally, P4 stimulates expression of *SLC7A1*, but not IFN-τ, and that *SLC7A2* is P4 induced as well as being IFN-τ stimulated (Gao et al., 2009b). Establishing the cellular localization of these transporters

as well as their influences by P4 and IFN-τ in cattle would provide additional insight into the function of these transporters in early gestation. In FM, *SLC7A1* peaked in expression on d 34 and 50, coinciding with increased expression on d 34 in maternal tissues. Concentration of arginine was greater on d 40 compared with d 50 of gestation, following a similar pattern as seen in maternal and fetal transporter expression, being greater d 34 of gestation and decreasing to d 50.

Arginine is well known for its roles in angiogenesis and cellular proliferation as well as its actions on mTOR for protein synthesis and decreased protein degradation (Wang et al., 2016). When supplemented in diets of sheep, arginine increases embryonic and conceptus survival and growth rate (Wang et al., 2016), therefore suggesting that the amount of arginine transported into the uterine lumen and allantoic and amniotic fluids by nutrient transporters is vital to conceptus survival. Nitric Oxide (NO) is an important product of arginine catabolism, and plays a vital role in placental angiogenesis and exchange of nutrients and oxygen from maternal to fetal systems (Gouge et al 1998; Bird et al., 2003; Wang et al., 2014). Additionally, polyamines (produced from arginine catabolism) contribute to embryogenesis and placental development through their actions in DNA and protein synthesis, scavenging reactive oxygen species, cell proliferation, and differentiation of tissues (Wang et al., 2014). Supplemental lysine in the diet of rats reduced proteolysis and autophagy as well as upregulating the mTOR signaling pathway (Sato et al., 2015). Additional information regarding the significance of lysine in fetal mTOR pathways was not found in ruminants; however, mean concentrations of lysine, were greater than that of arginine in our heifers, suggesting a role for lysine in conceptus viability.

Genes were greater in mean expression across all days in either CAR (*GLUT3* and *SLC7A3*), ICAR (*SLC7A1*), or both CAR and ICAR (*GLUT1* and *SLC7A2*) compared with FM

during the first 50 days of gestation. Although there was a general numerical increase in the expression of all transporters in FM; the overall expression of these transporters were never greater than the expression seen in either CAR or ICAR. Investigating specific cellular locations of these transporters using immunohistochemistry in future studies would provide greater insight into the functions of these transporters. Knowing temporal changes of transporters in endometrial and fetal tissues using immunohistochemistry, along with mRNA expression in utero-placental tissues, and nutrient concentrations in fluids would provide a more complete picture of establishment and function of transporters in utero-placental tissues. Ultimately, new knowledge in this area will facilitate the development of strategies to increase efficiencies associated with beef cattle production and contribute to meeting projected world food demands.

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CHAPTER 3: RAPID COMMUNICATION: ISOLATION OF GLUCOSE TRANSPORTERS *GLUT3* AND *GLUT14* IN BOVINE UTERO-PLACENTAL TISSUES FROM DAYS 16 TO 50 OF GESTATION[1]

Abstract

Glucose transporter *GLUT14* is a duplicon of *GLUT3* with a 95% shared homology to *GLUT3,* and has not previously been isolated in ruminant utero-placental tissues. The *GLUT3* transporter has been previously isolated in Holstein heifer uterine epithelium, but not in ovine epithelium. We hypothesized that *GLUT3* and its duplicon *GLUT14* would be found in bovine utero-placental tissues, and that maternal nutrition and day of gestation would impact mRNA expression of *GLUT3* and *GLUT14*. Crossbred Angus heifers (n = 49) were estrus synchronized, bred via AI, and assigned to nutritional treatment (CON = 100% of requirements to gain 0.45 kg/d; RES = 60% of CON) at breeding. Ovariohysterectomy was performed on d 16, 34, or 50 of gestation (n = 6 to 9/d); non-pregnant (NP) controls were not bred and ovariohysterectomized on d 16 of the synchronized estrous cycle (n = 6). The resulting treatment arrangement was a 2 × 3 factorial + 1. Utero-placental tissues (caruncle, CAR; intercaruncular endometrium, ICAR; and fetal membrane [chorioallantois], FM) were obtained from the pregnant uterine horn immediately after ovariohysterectomy. For NP controls, only CAR and ICAR were obtained. There were no day × treatment interactions for *GLUT3* or *GLUT14* gene expression in CAR, ICAR, or FM. Expression of *GLUT3* in CAR was greater ($P = 0.03$) on d 50 compared with d 16.

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In ICAR, *GLUT3* was greatest ($P = 0.02$) on d 50 compared with d 16 and 34 of gestation. In FM, *GLUT3* was greater ($P = 0.04$) on d 16 compared with d 50. Expression of *GLUT3* was greater ($P = 0.05$) in pregnant compared with non-pregnant heifers. Additionally expression of *GLUT3* was greater ($P = 0.01$) on d 34 and 50 compared with d 16. Expression of *GLUT14* in CAR was greater ($P = 0.03$) on d 50 compared to d 16 and 34. In CAR, *GLUT14* tended ($P = 0.07$) to be greater on d 34 and 50 compared with d 16, and was greater ($P = 0.02$) on d 50 compared with d 34. There was no effect of treatment for either *GLUT3* or *GLUT14* in CAR, ICAR, or FM. These data demonstrate that glucose transporters *GLUT3* and *GLUT14* are expressed in beef heifer utero-placental tissues, and that they are expressed differentially by day of gestation in bovine utero-placental tissues.

Key words: early gestation, facilitated transporters, glucose

Introduction

Glucose is a major source of energy for the conceptus during development, and acts as a regulator for cell proliferation and function through activation of the mammalian target of rapamycin (**mTOR**; Gao et al., 2009; Tan and Miyamoto, 2016). Glucose must be transported to the conceptus from the maternal system because neither conceptuses, nor uterine endometrium carry out gluconeogenesis (Gao et al., 2009). The most efficient known isoform of glucose transporter is **GLUT3** (glucose transporter solute carrier family 2 member 3, also known as SLC2A3), which has highest transport capacity of any GLUT transporter, and is found in specific subcellular locations to ensure glucose transport (Ganguly et al., 2001). The known localization of GLUT3 in the ruminant placenta has been determined to be along the microvillous membrane (Wooding et al, 2005). Known localization of GLUT3 in uterine tissue was established in the rat, in which GLUT3 was localized in both uterine stroma (dense

connective tissue), and uterine epithelium (Korgun et al., 2001). Specific uterine mRNA of *GLUT3* in Holstein heifers was determined to be in the caruncular and intercaruncular epithelium (Lucy et al., 2012). The glucose transporter gene ***GLUT14*** (glucose transporter solute carrier family 2 member 14, also known as SLC2A14), encodes a protein that is 497 amino acids in length, and shares 95% homology to *GLUT3* (Wu and Freeze, 2002). The *GLUT14* gene is considered a duplicon, as it may have arisen as a possible result of a duplication of *GLUT3* (Wu and Freeze, 2002). Contrary to *GLUT3* however, *GLUT14* has previously been specifically expressed in the testis, and was found to have expression levels four-fold greater than *GLUT3* in testis. (Wu and Freeze, 2002). Therefore, we tested the hypothesis that the mRNA for *GLUT3* and *GLUT14* would be present in beef heifer utero-placental tissues, and would be differentially expressed due to day of gestation and maternal nutritional status.

Materials and Methods

Protocols described herein were approved by the North Dakota State University Institutional animal care and use committee.

Animals

Crossbred Angus heifers (n = 49, ~15 mo of age; average initial BW = 324.9 kg) were exposed to the 5-d CO-Synch + CIDR (Eazi-Breed CIDR Cattle Insert, Nasko, Fort Atkinson, Wisconsin) estrus synchronization protocol. Six heifers were not inseminated to serve as non-pregnant (**NP**) controls, but underwent ovariohysterectomy for tissue collections on d 16 of the synchronized estrous cycle. The remaining heifers (n = 6 to 9/d of gestation/treatment) were bred by AI bred to a common sire at 12 h after observed estrus and ovariohysterectomized at d 16, 34, or 50 of gestation (McLean et al., 2016).

Diet and Housing

Heifers were housed at the North Dakota State University Animal Nutrition and Physiology Center. Heifers were acclimated to individual bunk feeding (American Calan, Northwood, NH) for two weeks before the beginning of the trial. Immediately following AI, heifers were randomly assigned to one of two treatment groups. Control heifers (**CON**) received 100% of NRC (2000) requirements for 0.45 kg/d gain to reach 80% of mature BW at first calving. Restricted heifers (**RES**) were placed on a 40% global nutrient restriction which was accomplished by reducing total diet delivery to 60% of CON. The diet was delivered as a TMR (48.37% DM, 5.28% CP, 6.77% ash, and 29.43% NDF on a DM basis) of grass hay, corn silage, alfalfa haylage, and grain supplement. Dried distillers grains (87.5% DM, 8.2% ash, 31.3% CP, and 53.4% NDF on a DM basis) were supplemented on an individual basis to meet CON ADG, and restricted 40% for RES heifers.

Sample Collection

Immediately following ovariohysterectomy, utero-placental tissues (caruncle, **CAR**; intercaruncular endometrium, **ICAR**; fetal membrane [chorioallantois], **FM**) were obtained from the uterine horn containing the conceptus, as previously described (Grazul-Bilska et al., 2010, 2011). Fetal membranes were collected only from pregnant heifers due to a lack of FM in NP controls. Once collected, all tissues were flash frozen in liquid nitrogen-cooled isopentane and stored at -80°C.

Real-Time Quantitative PCR (qPCR)

Total RNA was extracted and purified using Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA), and cDNA was synthesized utilizing iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The RNA concentration was determined using Take3 module of a Synergy H1

Microplate Reader (BioTek, Winooski, VT). Gene expression was quantified using a 7500 Fast, Real-Time PCR System (Applied Biosystems, Grand Island, NY) with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) in duplicate. Relative expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) with *β-Actin* as a reference gene due to consistency of expression across day of gestation and utero-placental tissue type, compared with other reference genes investigated such as *GAPDH*, *YWHAZ*, *TBP*, and *SDHA*. The average of NP expression was used as the control within each tissue, with the exception of FM, in which NP endometrium sample was used as the control.

Primer selection and validation

Coding sequences for *GLUT3* (Genbank accession number NM_174603.3) and *GLUT14* (Genbank accession number NC_007316.5) were aligned using CLUSTALW (EBI, Cambridgeshire, UK). The *GLUT3* primer sequences were: forward-CAAGTCACAGTGCTAGAGTCTTTC, and reverse- GGAGAGCTGGAGCATGATAGAGAT (Mattmiller et al., 2011). The primers for *GLUT14* were designed after alignment with *GLUT3*, and chosen based upon the area of greatest single nucleotide polymorphisms (SNP; bolded bases), forward- TA**T**G**G**CTTTGGAA**A**AG**T**GGTCAGGAACC, reverse-GA**T**GGAGAAGGA**A**CC**G**ATCATA. Each primer pair was validated using 7500 Fast Real-Time PCR system in CAR, ICAR, and FM from heifers on d 16, 34, and 50 of gestation to ensure product specificity. To confirm assay specificity, the end products of the qPCR reaction were sequenced. PCR products were purified using the Purelink Quick PCR Purification Kit (Invitrogen, Carlsbad, CA) and then sequenced using SimpleSeq (Eurofins MWG Operon LLC, Louisville, KY). Sequences from each tissue were aligned using MacVector version 14.5

(MacVector, Inc., Apex, NC) to ensure products were *GLUT3* and *GLUT14* respectively (Fig. 3.1).

Statistical Analysis

Data were analyzed using the GLM procedure of SAS 9.3 (SAS Inst. Inc., Cary, NY) with day, treatment and day × treatment in the model. If no significant interactions were present, main effects of maternal nutrition and day of gestation were reported. Means were separated using LSMEANS, and *P*-values ≤ 0.05 were considered significant. Additionally, contrasts were conducted comparing the gene expression for NP vs. pregnant heifer, d 16 vs. d 34 and 50 (pre-implantation vs. post-implantation), and d 34 vs. d 50 using the GLM procedure of SAS, with *P*-values ≤ 0.05 being considered significant.

Results

GLUT3

There was no day × treatment interaction for *GLUT3* in CAR, ICAR, or FM. Expression of *GLUT3* in CAR was greater ($P = 0.03$) on d 50 compared with d 16 (10.38 vs. 2.59 respectively, SEM = 2.86; Table 3.1). In ICAR, *GLUT3* was greatest ($P = 0.02$) on d 50 compared with d 16 and 34 of gestation (3.20 vs. 1.96 and 1.14 respectively, SEM = 0.70; Table 3.1). Conversely in FM, *GLUT3* was greater ($P = 0.04$) on d 16 compared with d 50. Expression of *GLUT3* in CAR was greater ($P = 0.05$) in pregnant heifers compared with NP controls.

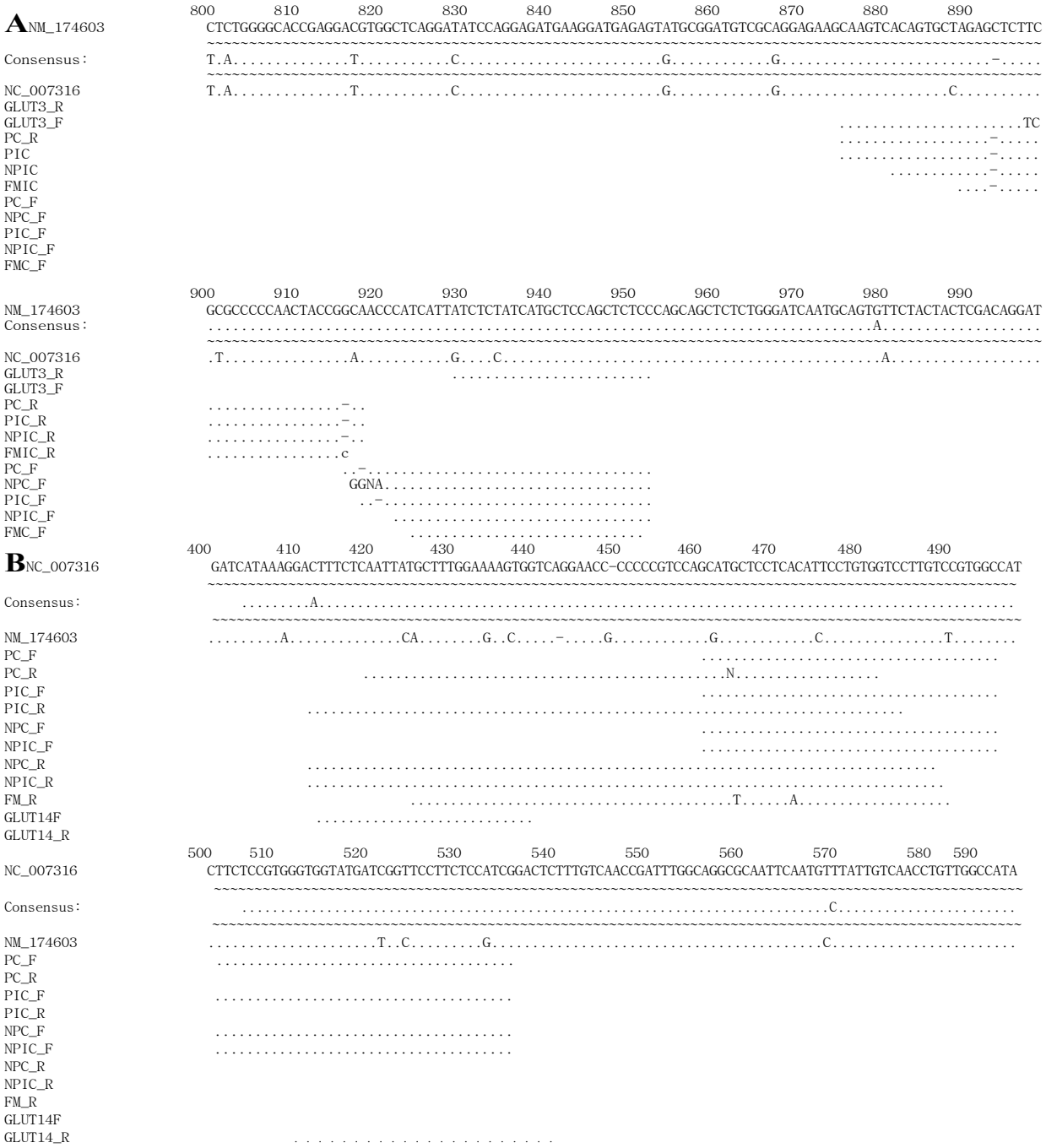

Figure 3.1 Primer validation with Sanger sequencing results. **A.** *GLUT3* forward and reverse primers, along with validation in bovine utero-placental tissues. **B.** *GLUT14* forward and reverse primers, along with validation in bovine utero-placental tissues. NM_174603- GLUT3 coding sequence, NC_007316- GLUT14 coding sequence, PC- caruncles from the uterine horn containing the conceptus, PIC- intercaruncular tissue from the uterine horn containing the conceptus, NPC- caruncles from the uterine horn not containing the conceptus, NPIC- intercaruncular tissue from the uterine horn not containing the conceptus, FM- fetal membranes [chorioallantois], FMC- cotyledons from fetal membranes, FMIC- intercotyledonary fetal membrane.

Additionally expression of *GLUT3* in CAR was greater ($P = 0.01$) on d 34 and 50 compared with d 16. Glucose transporter *GLUT3* tended ($P = 0.08$) to be greater in CON heifers in ICAR compared with RES heifers.

GLUT14

There was no day × treatment interaction for *GLUT14* in CAR, ICAR, or FM. Expression of *GLUT14* in CAR was greater ($P < 0.05$) on d 50 compared to d 16 and 34 (9.27 vs. 1.53 and 2.65 respectively, SEM = 2.93; Table 3.1). In CAR, *GLUT14* was greater ($P = 0.02$) on d 50 compared with d 34 and tended ($P = 0.07$) to be greater on d 34 and 50 compared with d 16 (Table 3.1). In ICAR, *GLUT14* tended ($P = 0.09$) to be greater on d 16 compared with d 34 (Table 3.1). There were no main effects of day of gestation or treatment in FM.

Discussion

This study is the first to confirm the presence of *GLUT14* in bovine utero-placental tissues (using both qPCR and Sanger sequencing to ensure product specificity) on days 16, 34, and 50 of gestation. This study also validates previous data produced by Crouse et al. (2016) isolating *GLUT3* expression in beef heifer utero-placental tissues on days 16, 22, 28, 34, 40, and 50 of gestation.

Glucose is a major source of energy for the conceptus and placenta during early gestation and is required for the maintenance of normal fetal metabolism and growth (Hay, 2006; Dunlap et al., 2015). Glucose must be transported into the uterine lumen and conceptuses via nutrient transporters in the endometrium and developing placenta. The main glucose transporters previously isolated in the ruminant uterus are GLUT1 (SLC2A1), GLUT4 (SLC2A4), SGLT1 (SLC5A1), and SGLT11 (SLC5A11), of which GLUT1 and GLUT4 are sodium independent

glucose transporters and SGLT1 and SGLT11 are sodium dependent (Gao et al., 2009). In sheep, *GLUT1* mRNA was most abundant in luminal epithelium and superficial glandular epithelium; In rats, GLUT3 was found to be in the uterine stoma and dense connective tissue; *GLUT4* mRNA was most abundant in luminal epithelium and glandular epithelium; *SGLT1* mRNA was most abundant in luminal and glandular epithelium; and *SGLT11* mRNA was most abundant in uterine glandular epithelium (Gao et al., 2009). The main glucose transporters of the ruminant placenta are GLUT1 and GLUT3 with GLUT4 (insulin dependent) and GLUT8 being less abundant (Zhang et al., 2015). The abundance of mRNA expressed for *GLUT1* is found in the basal trophoblast membrane of the placenta (Hay, 2006): *GLUT3* mRNA is more abundant in the microvillous membrane (Korgun et al., 2001); *GLUT4* mRNA was determined to be more abundant in fibroblasts from amnion and chorion: and *GLUT8* mRNA was found to be most abundant in the chorionic epithelial later (Zhang et al., 2015).

The glucose transporters of interest in this study are the high affinity, high capacity GLUT3 and its duplicon, GLUT14. Glucose transporter GLUT3 is unique in comparison with other glucose transporters in utero-placental tissues in that a mutation of the *GLUT3* gene in mice caused early embryonic mortality, indicating that the amount of glucose being transported by GLUT3 simply could not be made up for by other utero-placental glucose transporters including GLUT1, GLUT4, or GLUT8 (Ganguly et al., 2001). This is due to the transport capacity of GLUT3 at 1.5mM (Thorens and Mueckler, 2010) in comparison to GLUT1 at 6.9 mM (Burant and Bell, 1992) and GLUT4 at 4mM (Nishimura et al., 1993) with the lower k_m equating to greater transport capacity. Additionally, isolating the presence of *GLUT14* expression in bovine utero-placental tissues indicates the uterine and placental need for another potentially high capacity glucose transporter. No research has been found determining the

transport capacity of GLUT14 to determine if it is comparable to GLUT3. However, like other GLUT transporters, GLUT14 contains 12 putative membrane-spanning helices along with sugar-transporter signature motifs that have previously been shown to be essential for sugar transport activity (Wu and Freeze, 2002). This indicates that GLUT14 should be able to transport glucose, and establishment of its transport capacity would more greatly determine its role in glucose transport in the bovine utero-placenta. Additional work localizing the transporters using immunohistochemistry or RNA-fluorescent *in situ* hybridization would allow for determination of transporter location (e.g. caruncular or glandular).

Table 3.1 Fold change in gene expression of nutrient transporter *GLUT3* and *GLUT14* in caruncular and intercaruncular tissues due to dietary treatments from d 16 to 50 of gestation (Preg) relative to non-pregnant (NP) controls.

				Day of Gestation[1]					*P* – values[2]					
Gene[3]	Tissue[4]	Trt[5]		16	34	50	Trt[6]	SEM[7]	NP vs. Preg	16 vs. 34 and 50	34 vs. 50	Day	Trt	Day × Trt
GLUT3	CAR	CON		3.60	6.40	10.07	6.69	2.86	0.05	0.01	0.13	0.03	0.85	0.87
		RES		1.57	6.55	10.68	6.27							
			Day[8]	2.59[b]	6.47[ab]	10.38[a]								
GLUT3	ICAR	CON		2.80	0.90	4.13	2.61	0.70	0.27	0.71	0.01	0.02	0.08	0.19
		RES		1.13	1.39	2.27	1.60							
			Day	1.96[b]	1.14[b]	3.20[a]								
GLUT3	FM	CON		0.63	0.30	0.36	0.42	0.15	-	0.02	0.36	0.04	0.12	0.33
		RES		0.90	0.64	0.34	0.64							
			Day	0.78[a]	0.47[ab]	0.35[b]								
GLUT14	CAR	CON		2.62	2.76	11.95	5.78	2.93	0.32	0.07	0.02	0.03	0.30	0.69
		RES		0.44	2.55	6.60	3.20							
			Day	1.53[b]	2.65[b]	9.27[a]								
GLUT14	ICAR	CON		6.74	2.48	4.78	4.66	1.50	0.27	0.36	0.29	0.09	0.33	0.55
		RES		5.14	3.04	2.19	3.46							
			Day	5.94	2.76	3.48								
GLUT14	FM	CON		0.60	0.79	1.51	0.97	0.83	-	0.74	0.38	0.62	0.76	0.94
		RES		1.17	0.93	1.44	1.18							
			Day	0.88	1.86	1.48								

[1] Number of days after insemination.
[2] Probability values for the effect of day, treatment, and day × treatment, as well as contrast statements comparing NP vs. Pregnant heifers, d 16 vs. d 34 and 50, and d 34 vs. 50 of gestation.
[3] *GLUT3* = glucose transporter solute carrier family 2 member 3 (*SLC2A3*). *GLUT14* = glucose transporter solute carrier family 2 member 14 (*SLC2A14*).
[4] CAR = caruncles, ICAR = endometrium not including caruncles, FM = chorioallantois.
[5]CON = heifers fed 100% of NRC requirements to gain 0.45 kg daily. RES = heifers fed 60% of CON diets achieved by reducing intake.
[6] Mean expression across day of gestation within gene and treatment.
[7] Average SEM was used within gene.
[8] Mean expression across treatment within gene and day of gestation.

It is of great interest that, even though *GLUT14* arose as a duplication of *GLUT3*, the expression pattern between the two transporters differs. In CAR, both *GLUT3* and *GLUT14* shared similar patterns of expression, increasing from d 16 to 50 of gestation. In ICAR, *GLUT3* transporter expression increased to d 50, while *GLUT14* tended to be greater on d 16 of gestation. In FM, *GLUT3* expression decreased to d 50 of gestation, while there was no effect of day of gestation for *GLUT14*. This is possibly indicative of differential transport capacity or preferential use of one transporter isoform over another. Specific expression of *GLUT3* and *GLUT14* in CAR demonstrates the establishment of a known high capacity transporter (GLUT3), and its duplicon, on the site of the placentome. Comparison of the overall expression of both transporters will need to be conducted in order to determine preferential use, and therefore further elucidate the functions of *GLUT14* and *GLUT3* in bovine utero-placental tissues.

Glucose transporters are essential in tissues requiring high concentrations of glucose for energy due to their ability to transport glucose at a rate 10,000 times higher than passive diffusion across the lipid bilayer of the cell (Elbrink and Bihler, 1975). Although fructose, not glucose, is the main hexose found in fetal fluids (Kim et al., 2012), glucose is essential for use by trophoblast as an energy source for energy-dependent active amino acid transporters (Hay, 2006). Additionally, the placenta metabolizes glucose to fructose via the aldose reductase pathway thereby supplying fructose as the main hexose sugar to the developing embryo (Meznarich et al., 1987).

The protein kinase mTOR is responsible for regulating cell growth and proliferation or autophagy and is directly activated by nutrient (glucose) presence, and inactivated when nutrients are not present (Tan and Miyamoto, 2016; Wang et al., 2016). In the conceptus, glucose activates mTOR through the conversion of glucose-6-phosphate to fructose-6-phosphate, then

metabolism by glutamine-fructophosphate transamidase 1 (**GFPT-1**). Metabolism by GFPT-1 activates the mTOR signaling cascade, which increases cellular proliferation and autophagy during critical time windows such as implantation and maternal recognition of pregnancy (Wang et al., 2016). Therefore, it is of utmost importance for glucose to be present in the uterine environment and readily available for the conceptus to utilize for activation of mTOR in a concerted effort to increase conceptus proliferation and growth.

In conclusion, this data supports our hypothesis that *GLUT3* and *GLUT14* are present in beef heifer utero-placental tissues from days 16 to 50. Additionally, *GLUT3* was differentially expressed in all tissues and *GLUT14* in CAR from d 16 to 50 of gestation. No effect of nutritional treatment was found on *GLUT3* or *GLUT14* expression. These data have established the presence of *GLUT3* in beef heifer utero-placental tissues, and are the first to isolate *GLUT14* in ruminant utero-placental tissue. Further research should be conducted to determine the exact cellular location of GLUT14 in utero-placental tissues, as well as determine its substrate preferences and affinities. This would better define the role that GLUT14 plays in supplying glucose for the establishment and maintenance of pregnancy.

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CHAPTER 4: MATERNAL NUTRITION AND STAGE OF EARLY PREGNANCY IN BEEF HEIFERS: IMPACTS ON EXPRESSION OF GLUCOSE, FRUCTOSE, AND CATIONIC AMINO ACID TRANSPORTERS

Abstract

We hypothesized that maternal nutrition and day of gestation would impact mRNA expression of nutrient transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in beef heifers. Crossbred Angus heifers (n = 49; 16 mo of age; BW=325 kg) were estrus synchronized, bred via AI, assigned to nutritional treatment (CON = 100% of requirements for 0.45 kg/d gain and RES = 60% of CON) and ovariohysterectomized on d 16, 34, or 50 of gestation (n = 6 to 9/d); non-pregnant (NP) controls were not bred and ovariohysterectomized on d 16 of the synchronized estrous cycle (n = 6). Caruncle (CAR), intercaruncular endometrium (ICAR), and fetal membranes (FM), were obtained from the pregnant uterine horn. For NP controls, only CAR and ICAR were obtained. Relative expression of the glucose transporter *GLUT1*, fructose transporter *GLUT5* and cationic amino acid transporters *CAT-1*, *CAT-2*, and *CAT-3* was determined for each tissue utilizing NP-CAR and NP-ICAR tissue as the baseline. For FM, average of NP CAR and ICAR served as the baseline. There was no day × treatment interaction in CAR for any genes ($P \geq 0.05$). Expression of *GLUT1* was greater ($P < 0.01$) on d 16 compared with d 34 and 50, and *GLUT5*, *CAT-1*, and *CAT-2* were greater ($P \leq 0.05$) on d 34 compared with d 16 and 50. Expression of *CAT-2* in ICAR was the only gene in any tissue to be influenced by a day × treatment interaction ($P = 0.01$) being greater in d 50 CON, compared with d 16 and 50 RES, and d 34 CON. Expression of *GLUT1* in ICAR was greater ($P < 0.01$) on d 16 compared with d 34, and *CAT-1* was greater ($P < 0.01$) on d 34 and 50 compared with d 16 in ICAR. There was no day × treatment interaction in FM for any genes ($P \geq 0.05$). Expression of *GLUT5* was greater (*P*

= 0.04) on d 16 compared with d 50 of gestation, and *CAT-1* in FM was greater ($P < 0.01$) on d 34 and 50 compared with d 16. There was no treatment × tissue interaction for *GLUT1*, *GLUT3*, *GLUT5*, *GLUT14*, *CAT-1*, *CAT-2*, or *CAT-3*. In the days and tissues investigated, *GLUT1* was influenced by a day × tissue interaction, being greater ($P < 0.01$) in d 16 CAR, *GLUT3* was greater ($P = 0.02$) in d 50 CAR, *GLUT5* was greater ($P < 0.01$) in d 34 CAR and 16 FM, and *CAT-1* was greater ($P < 0.01$) in d 34 ICAR. These results partially support our hypothesis that nutrition and day of gestation would alter gene expression, and indicate that day was a more influential factor for mRNA expression of utero-placental glucose and cationic amino acid transporters than maternal nutritional status in heifers during early pregnancy.

Key Words: beef heifers, gestation, nutrient transporters, maternal nutrition

Introduction

Fetal growth is vulnerable to maternal dietary nutrient deficiencies during the 1st trimester of gestation (Wu et al., 2004). The first 50 days of gestation is a critical developmental window in which significant cellular and tissue differentiation and major organogenesis occurs. Nutritional influences may alter the mammalian phenotype through affecting gene regulatory mechanisms involved in DNA synthesis and replication, thus "imprinting" potential susceptibilities to chronic disease and metabolic issues into the genome (Waterland and Jirtle, 2004). Currently, fetal undernutrition occurs in grazing livestock worldwide (Wu et al., 2004). Maternal undernutrition has been implicated in fetal growth restriction and altered placental growth, reduced amino acid and glucose transport, and increased apoptosis and autophagy, which overall can yield decreased fetal growth during gestation (Zhang et al., 2015). Before the establishment of hemotrophic nutrition, the placenta is developing and the conceptus begins to

utilize increasing quantities of glucose and amino acids supplied by histotroph (Groebner et al., 2011). Thus, the expression of glucose and amino acid transporters in the utero-placenta becomes essential to the viability of the conceptus. Therefore, we studied the utero-placental glucose transporters GLUT1, GLUT3, and GLUT14 (SLC2A1, 3, and 14), which are all sodium-independent facilitated diffusion glucose transporters, and GLUT5 (SLC2A5) which is a sodium-independent facilitated diffusion fructose transporter. We also investigated the cationic amino acid transporters CAT-1, CAT-2 and CAT-3 (SLC7A1, 2, and 3), which are all sodium-independent facilitated diffusion arginine and lysine transporters. In this experiment, we tested the hypothesis that mRNA for glucose and cationic amino acid transporters in utero-placental tissues would be differentially expressed due to day of gestation and maternal nutritional status.

Materials and Methods

All animal procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (#A14053 and Al6049).

Animals, Housing and Diet

Crossbred Angus heifers were obtained from the Central Grasslands Research and Extension Center (Streeter, ND; 229 km southwest of Fargo; n = 49, ~16 mo of age; average initial BW = 325 ± 12.5 kg) and housed at the North Dakota State University Animal Nutrition and Physiology Center. Heifers were acclimated to individual bunk feeding (American Calan, Northwood, NH) for two weeks before the beginning of the trial. All heifers were exposed to the 5-d CO-Synch + CIDR estrus synchronization protocol (Bridges et al., 2008). Six heifers were not inseminated to serve as non-pregnant (**NP**) controls, but received ovariohysterectomy for tissue collections on d 16 of the synchronized estrous cycle. The remaining heifers were bred by

AI bred to a common sire at 12 h after observed estrus, and were randomly assigned to one of two treatment groups. Control heifers (**CON)**, received 100% of NRC (2000) requirements for 0.45 kg/d gain to reach 80% of mature BW at first calving. Restricted heifers (**RES)**, were placed on a 40% global nutrient restriction which was accomplished by reducing total diet delivery to 60% of the control delivery. The diet was delivered via TMR (48.37% DM, 5.28% CP, 6.77% ash, and 29.43% NDF on a DM basis) and consisted of grass hay, corn silage, alfalfa haylage, grain and mineral mix, and supplemented dried distillers grains with solubles (**DDG**; 53.4% NDF, 31.3% CP) to meet individual heifers protein requirements. All treated heifers were ovariohysterectomized on either d 16 (CON, n = 7; RES, n = 7), 34 (CON, n = 6; RES, n = 9), or 50 (CON, n = 7; RES, n = 7) of gestation. Thus, experimental design for the pregnancy analysis was a 2 × 3 factorial +1 design.

Sample Collection and Analysis

Ovariohysterectomy procedures were conducted as described by McLean et al. (2016). Immediately following ovariohysterectomy, utero-placental tissues (caruncle, **CAR**; intercaruncular endometrium, **ICAR**; fetal membranes, **FM** [chorioallantois, d 16 and 34]; cotyledonary placenta **COT** [d 50 only]; intercotyledonary placenta, **ICOT** [d 50 only]; and amnion, **AMN** [d 50 only]) were obtained from the uterine horn ipsilateral to the Corpus Luteum, which was also the horn containing the conceptus when observable, as previously describe (Grazul-Bilska et al., 2010, 2011). Once collected, all tissues were snap-frozen in liquid nitrogen cooled isopentane (2-Methylbutane; J.T.Baker, center Valley, PA) and stored at -80°C.

Table 4.1. Primer sets used for real-time quantitative reverse-transcription PCR[1]

Gene[1]	Forward primer (5'-3')	Reverse Primer (5'-3')	Accession No.
GLUT1	CGGCTGCCCTGGATGTC	GCCTGGGCCCACTTCAAA	NM_174602
GLUT3	CAAGTCACAGTGCTAGAGTCTTTC	GGAGAGCTGGAGCATGATAGAGAT	NM_174603
GLUT5	AGTCTCCTGGCAAACGAAGA	AAGAAGGGCAGGAAGAGGAG	NM_001101042.2
GLUT14	TATGCTTTGGAAAAGTGGTCAGGAACC	GATGGAGAAGGAACCGATCATA	NC_007316.5
CAT-1	CCGATAATCGCCACCTTAACCT	ACCAGGTCCTTCAGGTCGAA	DQ399522
CAT-2	CTGCAAGTGCCAGGGACCCAC	GGTTGCAGCCCCAGCCAAAGT	XM_865568.3
CAT-3	GTAGCCCCAACCCAACTCGGC	TGCTAGGAAGGATCGAGGAGCTGT	NM_001078019.1
β-Actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA	AB098974.1

[1]*GLUT1, GLUT3,* and *GLUT14*- Glucose transporter solute carrier family 2 member 1. *GLUT5*- Fructose transporter solute carrier family 2 member 5. *CAT-1*, *CAT-2*, and *CAT-3*- Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3. B-Actin- Reference gene used in all tissues to complete ΔΔ Ct method.

Real-time quantitative PCR (qPCR) was done on CAR, ICAR, and FM samples to determine mRNA expression of *glucose* trans*porter 1* (***GLUT1***- facilitative diffusion glucose transporter which is found in most tissues throughout the body and is ubiquitous across mammalian species), *glucose* trans*porter 3* (***GLUT3***- facilitative diffusion high capacity glucose transporter known for neural and placental glucose transport), *fructose* trans*porter* (***GLUT5***- facilitative diffusion fructose transporter) *glucose* trans*porter 14* (***GLUT14***- duplicon of GLUT3 bearing 95% homology previously isolated in testis and bovine utero-placental tissues), and *cationic amino acid* trans*port 1, 2*, and *3* (***CAT-1, CAT-2***, and ***CAT-3***- all of which are facilitated diffusion arginine and lysine transporters). The RNA was extracted and purified using Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA), and cDNA was synthesized utilizing iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Concentration of RNA was determined using Take3 module of a Synergy H1 Microplate Reader (BioTek, Winooski, VT). Optimal cDNA dilutions were determined by primer validation for each gene and tissue type across days of gestation. Primers (Table 4.1) were obtained from Integrated DNA Technologies (Coralville, IA) and used at a final concentration of 500 nM per reaction. Gene expression was quantified in duplicate using a 7500 Fast, Real-Time PCR System (Applied Biosystems, Grand Island, NY) with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Relative expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001) with *β-Actin* as a reference gene due to consistency of expression across day of gestation and utero-placental tissue type, compared with other reference genes investigated such as *GAPDH*, *YWHAZ*, *TBP*, and *SDHA*.

Statistical Analysis

Day × Treatment.

On d 50, COT and ICOT mRNA level of expression were averaged within heifer to yield the FM value to complete the 2 × 3 factorial arrangement. For CAR, ICAR, and FM, data were analyzed using the GLM procedure of SAS (SAS Inst. Inc.) with day, treatment and day × treatment in the model, with individual heifer serving as the experimental unit. NP CAR, ICAR, and an average NP CAR and ICAR samples served as the baseline of expression set to 1 for CAR, ICAR, and FM level of expression, respectively. If no significant interactions were present, main effects of maternal nutrition and day of gestation were reported. Means were separated using the LSMEANS procedure of SAS, and *P*-values ≤ 0.05 were considered significant. Additionally, if no significant interactions were present, contrasts were conducted incorporating the NP heifers comparing the mRNA expression for NP vs. pregnant heifers, as well as d 16 vs. d 34 and 50 (pre-attachment vs. post-attachment), and day post-attachment comparison (d 34 vs. 50) for each individual gene using the GLM procedure of SAS, with *P*-values ≤ 0.05 being considered different.

Day × Tissue and Tissue × Treatment.

In order to evaluate the level of expression of glucose, fructose, and cationic amino acid transporters between CAR, ICAR, and FM from d 16 to 50 of gestation, data were analyzed for an interaction between day of gestation and utero-placental tissue type, as well as utero-placental tissue type and maternal nutritional treatment using the GLM procedure of SAS 9.3 (SAS Inst. Inc.) with individual heifer serving as the experimental unit. The NP endometrium served as the baseline of expression of 1 within each gene. If no significant interaction was present, main

effect of tissue was analyzed within each individual gene of interest using the GLM procedure of SAS 9.3 (SAS Inst. Inc.).

Day 50 Fetal Tissue Comparison.

For COT, ICOT, and AMN on d 50, a tissue × treatment analysis was conducted using the GLM procedure of SAS 9.3 (SAS Inst. Inc. Cary, NY) with tissue, treatment, and tissue × treatment in the model, and individual heifer serving as the experimental unit. In the absence of an interaction, main effects of tissue and treatment were analyzed within each individual gene of interest using the GLM procedure of SAS 9.3) (SAS Inst. Inc.), with the NP endometrium serving as the baseline of expression set to 1 for each gene. Means for all analysis discussed previously were separated with the LSMEANS procedure of SAS 9.3 (SAS Inst. Inc.) and *P* – values ≤ 0.05 were considered different.

Results

Day × Treatment

CAR

Expression of glucose transporter *GLUT1* was not influence by a day × treatment interaction ($P = 0.23$; Table 4.2) and was greater ($P < 0.01$) on d 16 (2.89-fold) compared with d 34 and 50 (0.85 and 1.14-fold; SEM 0.31; Table 4.2). There was no day × treatment interaction for *GLUT3* in CAR ($P = 0.87$) and was greater ($P = 0.03$) on d 50 compared with d 16 (10.38 vs. 2.59 respectively, SEM = 2.86; Table 4.2). Expression of *GLUT3* in CAR (data previously published and presented here for completeness of data presentation; Crouse et al., 2016) was greater ($P = 0.05$) in pregnant heifers compared with NP controls.

Table 4.2 mRNA level of expression of nutrient transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in caruncular (CAR) tissue due to CON and RES dietary treatments from d 16 to 50 of gestation and in non-pregnant (NP) controls set to 1.

			Day of Gestation[2]					*P* - values[3]					
Gene[1]	Trt[4]		16	34	50	Trt[5]	SEM[6]	NP vs. Preg	16 vs. 34 + 50	34 vs. 50	Day	Trt	Day × Trt
GLUT1	CON		2.40	0.93	1.38	1.57	0.44	0.21	< 0.01	0.47	< 0.01	0.77	0.23
	RES		3.38	0.76	0.89	1.67							
		Day[7]	2.89[g]	0.85[h]	1.14[h]								
GLUT3[8]	CON		3.60	6.40	10.07	6.69	2.86	0.05	0.01	0.13	0.03	0.85	0.87
	RES		1.57	6.55	10.68	6.27							
		Day	2.59[b]	6.47[ab]	10.38[a]								
GLUT5	CON		6.61	22.05	9.07	12.58	15.33	0.24	0.06	< 0.01	0.02	0.35	0.20
	RES		2.97	66.74	3.91	24.54							
		Day	4.79[h]	44.40[g]	6.49[h]								
GLUT14[9]	CON		2.62	2.76	11.95	5.78	2.93	0.32	0.07	0.02	0.03	0.30	0.69
	RES		0.44	2.55	6.60	3.20							
		Day	1.53[b]	2.65[b]	9.27[a]								
CAT-1	CON		1.08	5.24	0.53	2.28	1.03	0.21	0.09	< 0.01	< 0.01	0.78	0.90
	RES		1.85	5.20	0.49	2.51							
		Day	1.47[h]	5.22[g]	0.51[h]								
CAT-2	CON		5.76	14.37	7.98	9.37	3.63	0.06	0.02	0.04	0.02	0.77	0.89
	RES		2.95	14.97	7.58	8.50							
		Day	4.36[h]	14.67[g]	7.78[gh]								
CAT-3	CON		1.29	2.23	4.28	2.60	1.09	0.44	0.24	0.11	0.20	0.06	0.90
	RES		0.45	0.99	2.05	1.16							
		Day	0.87	1.61	3.16								

Table 4.2 Level of expression of nutrient transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in caruncular (CAR) tissue due to CON and RES dietary treatments from d 16 to 50 of gestation and in non-pregnant (NP) controls set to 1 (continued).

[1]Gene = *GLUT1*- Glucose transporter solute carrier family 2 member 1.*GLUT5*- Fructose transporter solute carrier family 2 member 5. *CAT-1*, *CAT-2*, and
CAT-3 - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.
[2]Day of Gestation = number of days after insemination. Each gene expression is given as a fold change in relation to NP level of expression set to 1.
[3]Probability values for effect of d, treatment, and day × treatment on level of expression of individual genes. Probability values for the contrast of mRNA
level of expression of NP vs. Preg (all days of gestation), d 16 of gestation vs. d 34 and 50 of gestation, and d 34 vs. d 50 of gestation.
[4]CON = Heifers fed a diet that meets 100% of NRC requirements to gain 0.45 kg daily. RES = Heifers restricted to 60% of CON diet
[5]Mean level of expression of treatment group across day of gestation within tissue and gene of interest.
[7]Mean level of expression across treatment within day and gene of interest.
[8]Previously published data (Crouse et al., 2016a).
[9]Previously published data (Crouse et al., 2016a).
[a-c]Means within gene and tissue without a common superscript differ in day × treatment ($P \leq 0.05$).
[g-h]Means within row without a common superscript differ in main effect of day ($P \leq 0.05$).

Additionally, *GLUT3* was greater ($P = 0.01$) on d 34 and 50 compared with d 16. Fructose transporter *GLUT5* expression was not influenced by a day × treatment interaction ($P = 0.20$) and was greater ($P = 0.02$) on d 34 (44.4-fold) of gestation compared with d 16 and 50 (4.79 and 6.49-fold; SEM = 10.9; Table 4.2). Additionally, *GLUT5* tended ($P = 0.06$) to be greater in expression on d post-attachment (34 + 50) compared with d pre-attachment (d 16; Table 4.2). Expression of *GLUT14* (Crouse et al., 2016) was not influenced by a day × treatment interaction in CAR ($P = 0.69$) and was greater ($P < 0.05$) on d 50 compared to d 16 and 34 (9.27 vs. 1.53 and 2.65 respectively, SEM = 2.93; Table 4.6). As well, *GLUT14* was greater ($P = 0.02$) on d 50 compared with d 34 and tended ($P = 0.07$) to be greater on d 34 and 50 compared with d 16 (Table 6). A day × treatment interaction did not influence the mRNA expression of *CAT-1* in CAR ($P = 0.90$). However, *CAT-1* was greater ($P < 0.01$) on d 34 (5.22-fold) compared with d 16 and 50 (1.47 and 0.51-fold; SEM = 0.73; Table 4.2). Additionally, expression of *CAT-1* tended ($P = 0.09$) to be greater in expression on days post-attachment compared with day pre-attachment (Table 4.3). Expression of *CAT-2* in CAR was not influenced by a day × treatment interaction ($P = 0.89$), but was greater ($P = 0.02$) on d 34 (14.67-fold) compared with d 16 (4.36-fold; SEM = 2.55) with d 50 (7.78-fold) being not different from both d 16 and 34 of gestation (Table 4.2). Expression of *CAT-2* tended ($P = 0.06$) to be greater in pregnant heifers compared with non-pregnant heifers. Additionally, *CAT-2* expression was greater ($P = 0.02$) post-attachment compared with pre-attachment (Table 4.2). Expression of cationic amino acid transporter *CAT-3* was not influenced by a day × treatment interaction ($P = 0.90$) and*CAT-3* tended ($P = 0.06$) to be greater in expression in CON treated heifers compared to the RES treated heifers across all days of gestation (2.60 and 1.16-fold greater than NP; SEM = 0.64; Table 4.2).

Table 4.3 Level of expression of nutrient transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in intercaruncular ICAR tissue due to CON and RES dietary treatments from d 16 to 50 of gestation and in non-pregnant (NP) controls set to 1.

			Day of Gestation[2]					*P* - values[3]					
Gene[1]	Trt[4]		16	34	50	Trt[5]	SEM[6]	NP vs. Preg	16 vs. 34 + 50	34 vs. 50	Day	Trt	Day × Trt
GLUT1	CON		2.44	0.63	1.77	1.61	0.39	0.43	< 0.01	0.10	< 0.01	0.26	0.42
	RES		1.77	0.87	1.08	1.24							
		Day[7]	2.11^{g}	0.75^{h}	1.43^{gh}								
GLUT3[8]	CON		2.80	0.90	4.13	2.61	0.70	0.27	0.71	0.01	0.02	0.08	0.19
	RES		1.13	1.39	2.27	1.60							
		Day	1.96^{b}	1.14^{b}	3.20^{a}								
GLUT5	CON		1.45	3.60	6.11	3.72	3.30	0.36	0.58	0.97	0.85	0.66	0.31
	RES		3.59	3.82	1.20	2.87							
		Day	2.52	3.71	3.66								
GLUT14[9]	CON		6.74	2.48	4.78	4.66	1.50	0.27	0.36	0.29	0.09	0.33	0.55
	RES		5.14	3.04	2.19	3.46							
		Day	5.94	2.76	3.48								
CAT-1	CON		0.65	13.86	9.94	8.15	2.59	< 0.01	< 0.01	0.13	< 0.01	0.56	0.89
	RES		0.51	15.37	12.33	9.41							
		Day	0.58^{h}	14.62^{g}	11.13^{g}								
CAT-2	CON		6.83^{ab}	2.31^{c}	7.78^{a}	5.64	1.43	-	-	-	0.22	0.68	0.01
	RES		3.10^{bc}	6.08^{abc}	3.17^{bc}	4.11							
		Day	4.97	4.19	5.48								
CAT-3	CON		9.69	1.55	5.53	5.59	1.89	0.09	0.12	0.27	0.13	0.45	0.09
	RES		3.87	4.25	5.05	4.39							
		Day	6.78	2.90	5.29								

Table 4.3 Level of expression of nutrient transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in intercaruncular ICAR tissue due to CON and RES dietary treatments from d 16 to 50 of gestation and in non-pregnant (NP) controls set to 1 (continued).

[1]Gene = *GLUT1*- Glucose transporter solute carrier family 2 member 1. *GLUT5*- Fructose transporter solute carrier family 2 member 5. *CAT-1*, *CAT-2*, and *CAT-3* - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.
[2]Day of Gestation = number of days after insemination. Each gene expression is given as a fold change in relation to NP level of expression set to 1.
[3]Probability values for effect of day, treatment, and day × treatment on level of expression of individual genes. Probability values for the contrast of mRNA level of expression of NP vs. Preg (all days of gestation), d 16 of gestation vs. d 34 and 50 of gestation, and d 34 vs. d 50 of gestation.
[4]CON = Heifers fed a diet that meets 100% of NRC requirements to gain 0.45 kg daily. RES = Heifers restricted to 60% of CON diet
[5]Mean level of expression of treatment group across day of gestation within tissue and gene of interest.
[6]Average SEM was used within gene. NP n = 6, d 16 CON n = 7, d 16 RES n = 7, d 34 CON n = 6, d 34 RES n = 9, d 50 CON n = 7, d 50 RES n = 7
[7]Mean level of expression across treatment within day and gene of interest.
[8]Previously published data (Crouse et al., 2016a).
[9]Previously published data (Crouse et al., 2016a).
[a-c]Means within gene and tissue without a common superscript differ in day × treatment ($P \leq 0.05$).
[g-h]Means within row without a common superscript differ in main effect of day ($P \leq 0.05$).

ICAR

Expression of *GLUT1* in ICAR was not influenced by a day × treatment interaction (P = 0.42) and was greater on d 16 (2.11-fold) compared with d 34 (0.75-fold greater than NP; SEM = 0.28; Table 4.3). Additionally, *GLUT1* expression was greater ($P < 0.01$) in expression pre-attachment compared with post-attachment, as well as tending ($P = 0.10$) to be greater on d 50 compared with d 34 (Table 4.3). Expression of *GLUT3* in ICAR (Crouse et al., 2016) was not influenced by a day × treatment interaction ($P = 0.19$) and was greatest ($P = 0.02$) on d 50 compared with d 16 and 34 of gestation (3.20 vs. 1.96 and 1.14 respectively, SEM = 0.70; Table 4.3). Glucose transporter *GLUT3* tended ($P = 0.08$) to be greater in CON heifers in ICAR compared with RES heifers. Fructose transporter *GLUT5* was not influenced by a day × treatment interaction, or a main effect of day or treatment ($P \geq 0.05$; Table 4.3). In ICAR, *GLUT14* (Crouse et al., 2016) was not influenced by a day × treatment interaction ($P = 0.55$) and tended ($P = 0.09$) to be greater on d 16 compared with d 34 (Table 4.3). Expression of *CAT-1* was not influenced by a day × treatment ($P = 0.89$) and was greater ($P < 0.01$) on d 34 and 50 (14.62 and 11.13-fold, respectively) compared with d 16 (0.58-fold, respectively; SEM = 1.84; Table 4.3). Additionally, average expression of pregnant heifers across all days of gestation was greater ($P < 0.01$) than non-pregnant. Expression of *CAT-2* was influenced by a day × treatment interaction, being greater ($P = 0.01$) on d 50 CON (7.78-fold) compared with d 16 and 50 RES and d 34 CON (3.10, 3.17, and 2.31-fold, respectively; SEM = 1.43; Table 4.3). Additionally, d 16 CON (6.83-fold) was intermediate and greater ($P < 0.05$) compared with d 34 CON (2.31-fold, respectively; SEM = 1.43; Table 4.3). Expression of *CAT-3* tended ($P = 0.09$) to be influenced by a day × treatment interaction (Table 4.3).

Table 4.4 Level of expression of nutrient transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in fetal membrane FM tissue due to CON and RES dietary treatments from d 16 to 50 of gestation and in non-pregnant (NP) controls set to 1.

			Day of Gestation[2]					*P* - values[3]					
Gene[1]	Trt[4]		16	34	50	Trt[5]	SEM[6]	NP vs. Preg	16 vs. 34 + 50	34 vs. 50	Day	Trt	Day × Trt
GLUT1	CON		0.11	0.26	0.26	0.21	0.08	-	0.14	0.94	0.35	0.61	0.90
	RES		0.19	0.27	0.27	0.24							
		Day[7]	0.15	0.26	0.27								
GLUT3[8]	CON		0.63	0.30	0.36	0.42	0.15	-	0.02	0.36	0.04	0.12	0.33
	RES		0.90	0.64	0.34	0.64							
		Day	0.78[a]	0.47[ab]	0.35[b]								
GLUT5	CON		59.78	43.59	33.40	45.59	19.54	-	0.01	0.12	0.04	0.52	0.44
	RES		100.57	46.63	21.38	56.19							
		Day	80.17[g]	45.11[gh]	27.39[h]								
GLUT14[9]	CON		0.60	0.79	1.51	0.97	0.83	-	0.74	0.38	0.62	0.76	0.94
	RES		1.17	0.93	1.44	1.18							
		Day	0.88	1.86	1.48								
CAT-1	CON		0.04	0.22	0.19	0.15	0.04	-	< 0.01	0.24	< 0.01	0.67	0.99
	RES		0.05	0.24	0.20	0.16							
		Day	0.05[h]	0.23[g]	0.19[g]								
CAT-2	CON		0.42	0.84	1.01	0.76	0.28	-	0.04	0.55	0.10	0.82	0.47
	RES		0.24	1.16	0.72	0.70							
		Day	0.33	1.00	0.86								
CAT-3	CON		0.08	1.33	1.32	0.91	1.37	-	0.84	0.47	0.76	0.30	0.52
	RES		2.38	0.93	2.20	1.84							
		Day	1.23	1.13	1.76								

Table 4.4 Level of expression of nutrient transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in fetal membrane FM tissue due to CON and RES dietary treatments from d 16 to 50 of gestation and in non-pregnant (NP) controls set to 1 (continued).

[1]Gene = *GLUT1*- Glucose transporter solute carrier family 2 member 1. *GLUT5*- Fructose transporter solute carrier family 2 member 5. *CAT-1*, *CAT-2*, and *CAT-3* - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.
[2]Day of Gestation = number of days after insemination. Each gene expression is given as a fold change in relation to NP level of expression set to 1.
[3]Probability values for effect of d, treatment, and day × treatment on level of expression of individual genes. Probability values for the contrast of mRNA level of expression of NP vs. Preg (all days of gestation), d 16 of gestation vs. d 34 and 50 of gestation, and d 34 vs. d 50 of gestation.
[4]CON = Heifers fed a diet that meets 100% of NRC requirements to gain 0.45 kg daily. RES = Heifers restricted to 60% of CON diet
[5]Mean level of expression of treatment group across day of gestation within tissue and gene of interest.
[6]Average SEM was used within gene. NP n = 6, d 16 CON n = 7, d 16 RES n = 7, d 34 CON n = 6, d 34 RES n = 9, d 50 CON n = 7, d 50 RES n = 7
[7]Mean level of expression across treatment within day and gene of interest.
[8]Previously published data (Crouse et al., 2016a).
[9]Previously published data (Crouse et al., 2016a).
[a-c]Means within gene and tissue without a common superscript differ in day × treatment ($P \leq 0.05$).
[g-h]Means within row without a common superscript differ in main effect of day ($P \leq 0.05$).

FM

Glucose transporter *GLUT1* was not influenced by a day × treatment interaction (P = 0.90), nor a day or treatment main effect (P = 0.35 and P = 0.61, respectively). Expression of *GLUT3* in FM (data previously published; Crouse et al., 2016) was not influence by a day × treatment interaction (P = 0.33) and was greater (P = 0.04) on d 16 compared with d 50. Expression of *GLUT5* was not influenced by a day × treatment interaction (P = 0.44), however was greater (P = 0.04) on d 16 (80.17-fold) compared with d 50 (27.39-fold; SEM = 13.8; Table 4.4). Additionally, d 34 (45.11-fold) was intermediate and equal (P = 0.12) to both d 16 and 34 (80.17 and 27.39-fold, respectively; SEM = 13.8; Table 4.4). Contrast statements for *GLUT5* revealed that days post-attachment was greater in expression than d pre-attachment (P = 0.01). Expression of *GLUT14* (data previously published; Crouse et al., 2016) in FM was not influence by a day × treatment interaction, or main effects of day of gestation or treatment (P > 0.55). Arginine and lysine transporter *CAT-1* was not influenced by a day × treatment interaction (P = 0.99) and was greater (P < 0.01) on d 34 and 50 (0.23 and 0.19-fold) compared with d 16 (0.05-fold; SEM = 0.03; Table 4.4). Expression of *CAT-2* was not influenced by a day × treatment interaction (P = 0.47) and tended (P = 0.10) to be greater on d 34 (1.00-fold) compared with d 16 of gestation (0.33-fold; SEM = 0.20; Table 4.4). Cationic amino acid transporter *CAT-3* was not influenced by a day × treatment interaction (P = 0.52) nor a main effect of day or treatment (P = 0.76 and P = 0.30, respectively; Table 4.4).

Table 4.5 Relative expression of glucose transporters *GLUT1*, *GLUT3*, and *GLUT14* as well as fructose transporter *GLUT5* across utero-placental tissue type, day of gestation, and maternal nutritional treatment in relation to NP endometrium set to 1.

		Day of Gestation[1]				Treatment[2]			*P* - values[3]		
Gene[4]	Tissue[5]	16	34	50	Tissue Mean[6]	CON	RES	SEM[7]	Tissue	Trt × Tissue	Day × Tissue
GLUT1	CAR	1.81^{a}	0.51^{c}	0.71^{c}	1.01	0.98	1.04	0.26	< 0.01	0.36	< 0.01
	ICAR	1.72^{ab}	0.93^{c}	1.75^{ab}	1.47	1.74	1.19				
	FM	0.17^{c}	0.30^{c}	0.30^{c}	0.26	0.25	0.27				
GLUT3	CAR	1.57^{c}	8.65^{ab}	11.38^{a}	7.20	9.19	5.21	1.93	0.01	0.31	0.02
	ICAR	3.12^{c}	2.05^{c}	5.08^{bc}	3.42	4.21	2.62				
	FM	4.39^{bc}	2.64^{c}	1.53^{c}	2.85	2.44	3.26				
GLUT5	CAR	4.35^{c}	44.56^{a}	5.31^{c}	18.07	12.86	23.28	8.15	0.32	0.71	< 0.01
	ICAR	11.97^{c}	17.20^{bc}	16.58^{bc}	15.28	17.38	13.12				
	FM	39.12^{ab}	22.00^{bc}	13.36^{c}	24.83	24.29	25.38				
GLUT14	CAR	1.21	2.24	4.31	2.59^{h}	3.69	1.49	1.48	< 0.01	0.48	0.34
	ICAR	6.23	4.44	5.65	5.44^{g}	6.05	4.83				
	FM	4.13	0.39	0.09	1.53^{h}	1.04	2.03				

[1]Day of Gestation = number of days after insemination.

[2]Treatment= Mean value of expression within tissue and treatment across all days of gestation. CON = Heifers fed a diet that meets 100% of NRC requirements to gain 0.45 kg daily. RES = Heifers restricted to 60% of CON diet

[3]Probability values for effect of tissue, treatment × tissue and day × tissue on the mRNA expression of *GLUT1*, *GLUT3*, *GLUT5*, and *GLUT14* in CAR, ICAR, and FM tissues.

[4]Gene = *GLUT1, GLUT3,* and *GLUT14*- Glucose transporter solute carrier family 2 member 1, 3, and 14.*GLUT5*- Fructose transporter solute carrier family 2 member 5.

[5]CAR- caruncles collected from the uterine horn ipsilateral to the corpus luteum. ICAR- intercaruncular endometrium collected from the uterine horn ipsilateral to the corpus luteum. FM- chorioallantois.

[6]Mean expression within gene across day of gestation and nutritional treatment.

[7]Average SEM for the day × tissue interaction was used.

[a-c]Means within gene without a common superscript differ in day × tissue ($P \leq 0.05$).

[g-h]Means within tissue mean column without a common superscript differ in main effect of tissue ($P \leq 0.05$).

Day × Tissue and Tissue × Treatment.

There was no treatment × tissue interaction for *GLUT1*, *GLUT3*, *GLUT5*, *GLUT14*, *CAT-1*, *CAT-2*, or *CAT-3* ($P \geq 0.25$; Tables 4.5 and 4.6). Expression of *GLUT1* was influenced by a day × tissue interaction being greater ($P < 0.01$) on d 16 in CAR (1.81-fold) intermediate on d 16 and 50ICAR (1.72 and 1.75-fold) and greater ($P \leq 0.05$) compared with d 16 FM (0.17-fold), d 34 CAR, ICAR, and FM (0.51, 0.93, and 0.30-fold) and d 50 CAR and FM (0.71 and 0.30-fold; SEM = 0.26; Table 4.5). High capacity glucose transporter *GLUT3* was influenced by a day × tissue interaction ($P = 0.02$), being greater on d 50 CAR (11.38-fold) than all other days and tissues except d 34 CAR (8.65-fold) which was intermediate and greater ($P \leq 0.05$) than d 16 CAR and ICAR (1.57 and 3.12-fold, respectively), d 34 ICAR and FM (2.05 and 2.64-fold, respectively), and d 50 FM (1.53-fold; SEM = 1.93; Table 4.5). Fructose transporter *GLUT5* was influenced by a day × tissue interaction, being greater ($P < 0.01$) on d 34 in CAR (44.56-fold) than all other days and tissues except d 16 FM (39.12-fold) which was intermediate and greater ($P \leq 0.05$) than d 16 CAR and ICAR (4.35 and 11.97-fold, respectively), d 34 ICAR and FM (17.20 and 22.00-fold, respectively), and d 50 CAR and FM (5.31 and 13.36-fold; SEM = 8.15; Table 4.5). Additionally, d 50 ICAR (16.58-fold) was equal ($P \geq 0.08$) all days and tissues except d 34 CAR (44.56-fold; SEM = 8.15; Table 5). Duplicon *GLUT14*, was not influenced by a day × tissue interaction ($P = 0.34$) and was greater ($P < 0.01$) in ICAR (5.44-fold) compared with CAR and FM (2.59 and 1.53-fold; SEM = 0.86; Table 4.5). Cationic amino acid transporter *CAT-1* was influenced by a day × tissue interaction ($P < 0.01$), being greater on d 34 ICAR (15.41-fold) than d 50 ICAR (11.72-fold) which was greater ($P \leq 0.05$) than d 34 CAR (4.91-fold), which was greater ($P \leq 0.05$) than all other days and tissues (SEM = 1.08; Table 4.6).

Table 4.6 Relative expression of glucose transporters *CAT-1*, *CAT-2,* and *CAT-3* across utero-placental tissue type, day of gestation, and maternal nutritional treatment in relation to NP endometrium set to 1.

		Day of Gestation[1]				Treatment[2]			*P* - values[3]		
Gene[4]	Tissue[5]	16	34	50	Tissue Mean[6]	CON	RES	SEM[7]	Tissue	Trt × Tissue	Day × Tissue
CAT-1	CAR	1.39[d]	4.91[c]	0.48[d]	2.26	2.15	2.37	1.08	< 0.01	0.77	< 0.01
	ICAR	0.61[d]	15.41[a]	11.72[b]	9.25	8.85	9.91				
	FM	0.07[d]	0.37[d]	0.31[d]	0.25	0.2	0.26				
CAT-2	CAR	1.78	6.06	3.18	3.67[h]	3.85	3.50	0.93	< 0.01	0.57	0.08
	ICAR	6.01	5.79	6.76	6.18[g]	6.96	5.42				
	FM	0.18	0.55	0.47	0.40[i]	0.42	0.38				
CAT-3	CAR	1.30	1.63	3.11	2.01[h]	12.86	23.28	8.15	< 0.01	0.25	0.10
	ICAR	6.94	3.36	5.44	5.24[g]	17.38	13.12				
	FM	0.08	0.07	0.12	0.09[i]	24.29	25.38				

[1]Day of Gestation = number of days after insemination.
[2]Treatment= Mean value of expression within tissue and treatment across all days of gestation. CON = Heifers fed a diet that meets 100% of NRC requirements to gain 0.45 kg daily. RES = Heifers restricted to 60% of CON diet
[3]Probability values for effect of tissue, treatment × tissue and day × tissue on the mRNA expression of *GLUT1*, *GLUT3*, *GLUT5*, and *GLUT14* in CAR, ICAR, and FM tissues.
[4]Gene = *CAT-1*, *CAT-2*, and *CAT-3* - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.
[5]CAR- caruncles collected from the uterine horn ipsilateral to the corpus luteum. ICAR- intercaruncular endometrium collected from the uterine horn ipsilateral to the corpus luteum. FM- chorioallantois.
[6]Mean expression within gene across day of gestation and nutritional treatment.
[7]Average SEM for the day × tissue interaction was used.
[a-c]Means within gene without a common superscript differ in day × tissue ($P \leq 0.05$).
[g-i]Means within tissue mean column without a common superscript differ in main effect of tissue ($P \leq 0.05$).

Expression of *CAT-2* tended ($P = 0.08$) to be influenced by a day × tissue interaction and was greater ($P < 0.01$) in ICAR (6.18-fold) intermediate in (3.67-fold) and least in FM (0.40-fold; SEM = 0.54; Table 4.6). Expression of *CAT-3* tended ($P = 0.10$) to be influenced by a day × tissue interaction and was greater ($P < 0.01$) in ICAR (5.24-fold) intermediate in CAR (2.01-fold) and least in FM (0.09-fold; SEM = 0.51; Table 4.6).

Day 50 COT, ICOT, and AMN comparison.

None of the nutrient transporters investigated were influenced by a tissue × treatment interaction, or a main effect of treatment ($P > 0.05$). Expression of *GLUT1* was greater ($P < 0.01$) in AMN (0.67-fold) compared with both COT and ICOT (0.24 and 0.29-fold; SEM = 0.07; Table 4.7). Expression of *GLUT3* was greater ($P < 0.01$) in AMN (0.53-fold) compared with COT and ICOT (0.26 and 0.19-fold, respectively; SEM = 0.05; Table 4.7). Expression of *GLUT5* was greater ($P < 0.01$) in ICOT (30.49- fold) compared with AMN and COT (3.47 and 6.89-fold, respectively; SEM = 12.89; Table 7). Expression of *GLUT14* was greater ($P < 0.01$) in AMN (4.48-fold) compared with both COT and ICOT (0.35 and 0.25-fold, respectively; SEM = 0.69; Table 7). Expression of *CAT-1* was greater ($P = 0.02$) in AMN (0.30-fold) compared with COT and ICOT (0.22 and 0.17-fold; SEM = 0.03; Table 4.7). Expression of *CAT-2* was greater ($P = 0.05$) in AMN (3.27-fold) compared with ICOT (0.82-fold; SEM = 0.66; Table 7). Expression of *CAT-3* was greater ($P < 0.01$) in AMN (7.64-fold) compared with both COT and ICOT (0.73 and 2.75-fold; SEM = 1.38; Table 4.7).

Table 4.7 Relative expression of nutrient transporters *GLUT1*, *GLUT3*, *GLUT5*, *GLUT14*, *CAT-1*, *CAT-2*, and *CAT-3* in AMN, COT, and ICOT on d 50 of gestation using NP endometrium as a baseline value set to 1.

Gene[1]	AMN[2]	COT[3]	ICOT[4]	SEM[5]	*P*-value[6]
GLUT1	0.67[a]	0.24[b]	0.29[b]	0.07	< 0.01
GLUT3	0.53[a]	0.26[b]	0.19[b]	0.05	< 0.01
GLUT5	3.47[b]	6.89[b]	30.49[a]	12.89	< 0.01
GLUT14	4.48[a]	0.35[b]	0.25[b]	0.69	< 0.01
CAT-1	0.30[a]	0.22[b]	0.17[b]	0.03	0.02
CAT-2	3.27[a]	1.42[ab]	0.82[b]	0.66	0.05
CAT-3	7.64[a]	0.73[b]	2.75[b]	1.38	< 0.01

[1]Gene = *GLUT1*- Glucose transporter solute carrier family 2 member 1. *CAT-1*, *CAT-2*, and *CAT-3* - Cationic amino acid transporters of arginine and lysine, solute carrier family 7 member 1, 2, and 3.
[2]Amnion collected on d 50 of gestation.
[3]Cotyledons collected from fetal membranes on d 50 of gestation.
[4]Intercotyledonary tissue (fetal membrane tissue not including cotyledons; collected from fetal membranes on d 50 of gestation).
[5]Average SEM was used within gene; AMN n = 11, COT n = 14, ICOT n =14
[6]Probability values for the effect of tissue on level of expression of individual genes. There was no significant tissue × treatment interaction or main effect of treatment, therefore they were omitted from the table.
[a-b]Means within gene without a common superscript differ by tissue ($P \leq 0.05$).

Discussion

These data are the first report on the impacts of maternal nutritional treatment and day of gestation on the mRNA expression of glucose and cationic amino acid transporters *GLUT1*, *GLUT5*, *CAT-1*, *CAT-2*, and *CAT-3* in bovine utero-placental tissues from d 16 to 50 of gestation. The effect of maternal nutrition and day of gestation on expression of glucose transporters *GLUT3* and *GLUT14* reported within have been previously published by Crouse et al. (2016a) and are presented here (with permission) to provide a more complete representation of nutrient transporters during early pregnancy in beef heifers. As stated previously, we hypothesized that glucose transporter *GLUT1*, fructose transporter *GLUT5*, and cationic amino

acid transporters *CAT-1*, *CAT-2*, and *CAT-3* would be differentially expressed due to an interaction of day of gestation and maternal nutritional status. In keeping with our hypothesis, we determined that *CAT-2* in ICAR was the only gene in any tissue to be influenced by a day × treatment interaction, and that the expression of glucose, fructose, and cationic amino acid transporters in the bovine utero-placenta are affected more so by day of gestation than a 40% global maternal nutritional restriction up to d 50 of gestation.

Glucose transporter GLUT1 (SLC2A1) is a highly conserved glucose transporter which is found in high levels in all fetal tissues as well as being most abundant in fibroblasts, erythrocytes, and endothelial cells with low expression in muscle, liver, and adipose tissue in adults (Olson and Pessin, 1996). Glucose Transporter GLUT3 (SLC2A3) is the major neuronal glucose transporter, present in both dendrites and axons, and its level of expression in different regions of the brain correlates with regional cerebral glucose utilization (Simpson et al., 2008, Thorens and Mueckler, 2010). In mouse sperm, *GLUT3* is highly expressed and controls glucose uptake and metabolism necessary for motility and maturation (Thorens and Mueckler, 2010). During embryonic development, GLUT3 is present in the trophectoderm at the blastocyst stage and, after implantation, in extra-embryonic tissues (Gao et al., 2009; Thorens and Mueckler, 2010). Although GLUT1 and GLUT3 are both high affinity glucose transporters, (~5 mM and ~1.5mM respectively) GLUT3 transport capacity is the highest calculated of all the known GLUT isoforms, thus facilitating proportionally greater glucose uptake (Thorens and Mueckler, 2010). Fructose transporter GLUT5 (SLC2A5) is a facilitative diffusion fructose transporter (Corpe et al., 2002) known to be expressed in fast-cleaving embryos to enhance fructose uptake for nucleotide synthesis (Gutierrez-Adan et al., 2004; Forde et al., 2011). Most notably, GLUT5 is known for fructose transport in the small intestine, being located on the

apical membrane and facilitating transport of fructose from the lumen of the intestine into the enterocyte (Thorens, 2004). The glucose transporter gene *GLUT14* (glucose transporter solute carrier family 2 member 14, also known as SLC2A14), encodes a protein that is 497 amino acids in length, and shares 95% homology to *GLUT3* (Wu and Freeze, 2002). The *GLUT14* gene is considered a duplicon, as it may have arisen as a possible result of a duplication of *GLUT3* (Wu and Freeze, 2002). Contrary to *GLUT3,* however, *GLUT14* has previously been observed as specifically expressed in the testis, and was found to have expression levels four-fold greater than *GLUT3* in testis (Wu and Freeze, 2002), and only recently, has been isolated in bovine utero-placental tissues (Crouse et al., 2016a).

Cationic amino acid transporters CAT-1, CAT-2, and CAT-3 (SLC7A1, SLC7A2, and SLC7A3) are part of the y^+ system of transporters which are a facilitated diffusion sodium-independent group of transporters known for transporting cationic amino acids such as arginine and lysine and at low pH, histidine (Closs et al., 2006). The three transporters studied vary in their stimulation and affinity; CAT-1 exhibits K_M values for L-arginine, L-lysine of 100 to 150 μM, and is strongly stimulated by substrate on the *trans*-side of the membrane (Kim et al., 1991; Closs et al., 1997); CAT-2 and CAT-3 exhibit lower substrate affinity and are less dependent on *trans*-stimulation (Closs et al., 1997; Vekony et al., 2001), with CAT-2 having about a tenfold lower substrate affinity compared with CAT-1 (Closs et al., 1993; Kavanaugh et al., 1994; Closs et al., 1997). Knockout of CAT-1 resulted in decreased arginine transport by 73%, and its catabolites, citrulline and ornithine, by 76% and 40% respectively, as well as decreased ornithine decarboxylase, NOS, and polyamines which resulted in retarded growth development of the conceptus (Wang et al., 2014).

During the first 50 d of gestation, nutrition to the fetus from the maternal system are provided through secretions from the uterine glands collectively known as histotroph (Bazer et al., 2011). Histotroph, also known as uterine milk (Bonnet, 1882), is comprised of nutrient transport proteins, ions, mitogens, cytokines, lymphokines, enzymes, hormones, growth factors, proteases and protease inhibitors, amino acids, glucose, fructose, vitamins, and other substances (Bazer et al., 2011). Due to the absence of shared blood supply between the maternal and fetal systems during the first 50 days, it is hypothesized that amniotic fluid contains the nutrient reserve from which the conceptus draws to meet its energetic and growth requirements before the establishment of hemotrophic nutrition. Even during late gestation, the importance of transport of key nutrients into the amniotic fluid such as amino acids (glutamine and arginine), proteins, vitamins, minerals, hormones and growth factors become increasingly important in the fetal swallowing mechanism in amniotic fluid which prepares the gastrointestinal tract for postnatal nutrition (Underwood and Sherman, 2006). These data show that most all transporters investigated, with the exception of fructose transporter *GLUT5,* were more greatly expressed in AMN compared with COT and/or ICOT. The reported data further demonstrate the increased emphasis on the establishment of transporters in the amniotic sac to facilitate the transport of nutrients across the amniotic sac to provide sufficient nutrients for conceptus growth and utilization. The placenta serves as the site of conversion of glucose to fructose (Jinyoung et al., 2012). The expression of *GLUT5* on d 50 being greater in ICOT compared with COT and AMN supports this conversion, because as glucose is converted by the chorioallantois, increased transport of fructose into the allantoic fluid cavity would need to be facilitated by *GLUT5* in order to transport fructose from the chorioallantois to the fetal fluids.

Arginine/lysine co-transporter *CAT-2* in ICAR was the only transporter in any tissue to be influenced by a day × treatment interaction. As previously stated, CAT-2 and CAT-3 have lower affinities for arginine and lysine compared with CAT-1, indicating preferential transport of arginine and lysine through CAT-1. These data shown with *CAT-2* in ICAR, as well as the tendency of *CAT-3* in ICAR to be influenced by a day × treatment interaction and *CAT-3* in CAR tending to be greater in CON compared with RES heifers may support the theory that at certain time points and nutritional treatments, the amount of arginine and lysine to be transported to the developing conceptus exceeds the transport capacity of CAT-1, therefore stimulating the expression of *CAT-2* and *CAT-3* as seen in these tissues to increase transporter number and transport capacity to the fetus. The other transporters investigated within, GLUT1, GLUT3, GLUT5, and CAT-1 are all high capacity high affinity glucose, fructose, or arginine/lysine transporters, and due to their transport capabilities, may be less likely to be affected in expression due to nutritional treatment.

Previous data from Crouse et al. (2016b) evaluating the mRNA expression of glucose transporters *GLUT1* and *GLUT3* as well as cationic amino acid transporters *SLC7A1*, *SLC7A2*, and *SLC7A3* (*CAT-1*, *CAT-2*, and *CAT-3*) on d 16, 22, 28, 34, 40, and 50 of gestation in CAR, ICAR, and FM found similar results across day of gestation as reported here.

Expression of *GLUT1* in CAR was greater on d 16 of gestation, in the current study, as well as Crouse et al. (2016b). Additionally, these results present a similar time frame of expression in bovine as they do in ovine, in which *GLUT1* was greater in expression in endometrial tissue from d 10 to 16 of gestation reaching a peak level of expression on d 14 of gestation (~d 16-17 in bovine; Sanger, 2012; Gao et al., 2009a) and decreasing to d 20 (~ d 22 in bovine). Interestingly, expression of *GLUT5* was greater on d 34 in CAR, which corresponds to

the time of completed adhesion and differentiation of the chorioallantois into the cotyledonary and intercotyledonary placenta. These data are very interesting due to the low concentration of fructose in the maternal serum during this time frame (0.80 mM; data not shown), as well as the low level of expression of the cotyledon (site of attachment to the caruncle forming the placentome) compared with the intercotyledonary placenta seen on d 50. Fructose transporter GLUT5, in addition to transporting fructose, also transports glucose, however has a lower affinity for glucose compared with fructose (Kayano, et al., 1990). The increased expression of *GLUT5* may be partially explained by an increased necessity of glucose during this time point in gestation. Similar results across year were found in *CAT-1*, which was greater in expression on d 28, 34, and 40 of gestation (Crouse et al., 2016b), as well as greater in CAR on d 34 compared with d 16 and 50 in the current study. Arginine/lysine cotransporter *CAT-2* and *CAT-3* were expressed differently across day from this study compared with the previous study (Crouse et al., 2016b). In the previous study, *CAT-2* showed no effect of day of gestation in CAR, and *CAT-3* was greater on d 16 and returned to levels similar to NP by d 22 through 50 of gestation. These data may once again be explained by the theory of maximizing transport capacity of CAT-1 thereby increasing transport of arginine and lysine mediated through CAT-2 and CAT-3 during critical time points in gestation. Another hypothesis may be that the diets from this study compared with the previous were greater in energy and protein content per unit dry matter. Crouse et al. (2016b) targeted gains of 0.3 kg/heifer/d whereas the CON diet in the current study targeted 0.45kg/heifer/d with the RES heifers targeted to maintain weight. Additionally, composition of the diets differed, in which the current heifers were supplemented with DDGS increasing the availability of arginine and lysine to be transported to the conceptus, therefore increasing the need of additional cationic amino acid transporters.

The intercaruncular tissue of the uterus is the glandular tissue which transports histotroph into the uterine lumen for use by the conceptus (Bazer et al., 2012). Glucose transporter *GLUT1* showed similar patterns of expression in ICAR as in CAR, being greater on d 16 of gestation, which is also expression seen in previous studies by Crouse et al., (2016). The expression of *CAT-1* in ICAR being greater in pregnant heifers across all days of gestation compared with non-pregnant heifers on d 16 of the luteal cycle indicated the importance of arginine transport into the uterine lumen to be used by the developing conceptus. Arginine, coupled with secreted phosphoprotein1 within the uterine lumen, stimulated the growth, migration, cytoskeletal remodeling, and adhesion of the trophectoderm (Bazer et al., 2011), indicating the high degree of importance of arginine from the glandular epithelium of the uterus as supported by our results.

Expression of *GLUT5* in FM supports previous research from Gutierrez-Adan (2004), in which GLUT5 is established on the fast-cleaving bovine embryo to enhance nucleotide synthesis. Expression decreased from 80-fold greater than NP on d 16 to 27-fold greater than NP by d 50. In addition to the embryo hatching, prior to the establishment of hemotrophic nutrition and gas exchange, the uterus is a hypoxic environment, and it is in this environment in which fructose is more readily used compared with glucose (Jinyoung et al., 2012). As vascularization and development of the chorioallantois increases, expression of *GLUT5* decreases, signaling a potential shift in utilization of fructose and glucose. This data is supported by Greseth et al., (2016) in which concentrations of fructose decrease from d 40 to 50 in amniotic fluid (3.43 to 2.06mM, respectively). Simultaneously, glucose concentration on d 40 and 50 increased in amniotic fluid (1.46 to 1.68 mM, respectively; M. S. Crouse et al., unpublished data).

When comparing *GLUT1* across tissue and day of gestation, expression patterns indicated that *GLUT1* may be the main pre-attachment glucose transporter in order to ensure sufficient

hexose availability to the developing conceptus. Additionally, I hypothesize that GLUT3 would be the main glucose transporter to be established on the placentome due to increased expression levels of *GLUT3* on the caruncle by d 50 of gestation compared with all other tissues and days. Independent analysis of *GLUT3* expression between COT and ICOT on d 50 determined that *GLUT3* tended ($P = 0.10$; data not shown) to be greater in COT compared with ICOT, indicating that in fact, GLUT3 may be the main high capacity glucose transporter to be established on the site of the placentome as gestation progresses. Confirmation of these would need to be obtained by localizing GLUT3 to the placentome and measuring the abundance of GLUT3 in comparison to uterine glands, or inter-cotyledonary placenta. The expression patterns of *GLUT5* between tissues are incredibly interesting. Although concentrations of fructose in maternal serum are nearly 4 to 10-fold lower than what is seen in fetal fluids, expression of *GLUT5* in d 34 CAR is equal to that of d 16 FM. Current literature reviewed does not provide an answer to this, as it would be expected to see a greater expression of *GLUT5* in FM compared with both CAR and ICAR simply on the grounds of fructose concentrations in fetal fluids being greater than that of maternal serum, and site of conversion of glucose to fructose within the chorioallantois (Jinyoung et al., 2012). Although transport capacity of GLUT14 has yet to be established, the expression of *GLUT14* being greater in ICAR across all days of gestation may be beneficial for the conceptus in terms of localization of a known high capacity transporter on the placentome (GLUT3), and a potential high capacity transporter in the glandular tissue of the uterus (GLUT14).

Expression of all three cationic amino acid transporters may indicate a specific transport of arginine by the glandular tissue of the uterus into the uterine lumen. All genes investigated were greater in ICAR, intermediate in CAR, and least in FM, suggesting the increased

prevalence of arginine transport into the uterine lumen compared with across the developing placentome or chorioallantois. Arginine is a major component of histotroph, and along with secreted phosphoprotein1 (**SPP1**), promotes cellular proliferation, migration, adhesion, differentiation, and survival (Wang et al., 2016). The combination of arginine and SPP1 causes the conformational change and cytoskeletal remodeling of the spherical blastocyst to the elongated, tubular and filamentous form before adhesion (d 12-17 of gestation) through the stimulus of mammalian target of rapamycin complex 2 (**mTORC2**; Wang et al., 2016). Although not previously determined, may be possible that additional arginine is to be transported into the uterine lumen around d 34 of gestation, which coincides with completion of attachment and the beginning of differentiation of the cotyledonary and intercotyledonary placenta. This additional arginine may be necessary for activation of mTORC2, which we hypothesize may be involved in the cytoskeletal restructuring of the chorioallantois into the cotyledonary tissue of the placenta. Although previous research by Crouse et al. (2016) and Wu et al., (2004) has shown that concentrations of arginine in the fetal fluids of the bovine and ovine are nearly 3 to 6 times greater than what is found in maternal serum, it is interesting to find that the mRNA expression of fetal tissues is lower than endometrial tissues. Therefore, analysis of concentrations of nutrients in maternal serum, histotroph, allantoic, and amniotic fluids would further delineate the effects of restricted maternal intake on whole nutrient transporter function and flux of nutrients from the maternal to the fetal system.

Conclusion

In conclusion these data emphasize that nutrient transporters in bovine utero-placental tissues are influenced more so by day of gestation, than a 40% global nutrient restriction.

Additional data evaluating concentrations of glucose, fructose, and cationic amino acids in utero-placental tissues should be conducted to determine how a 40% global nutrient restriction affects flux of nutrients to the developing conceptus. Additionally, high concentrations of non-essential amino acids such as glycine, glutamine, and serine have been reported in fetal fluids (Kwon et al., 2003). Investigating the effects of maternal nutritional status on neutral amino acid transporter expression and neutral amino acid flux to the conceptus may provide further insight into the effects of nutrient restriction of the dam on conceptus development.

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CHAPTER 5: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General Discussion and Speculation

The results of this thesis have demonstrated that there is an effect of day of gestation on the mRNA expression of glucose and cationic amino acid transporters in bovine utero-placental tissues from days 16 to 50 of gestation. It has also been established that glucose and arginine concentrations in maternal and fetal fluids are affected by day of gestation. Only one gene transcript, C*AT-2*, was influenced by a day × treatment interaction, and one gene, *CAT-3*, tended to be influenced by maternal nutritional treatment. We interpret these results to imply that day of gestation, more so than maternal nutritional status, affect the expression of glucose and cationic amino acid transporters in bovine utero-placental tissues from days 16 to 50 of gestation.

Additionally, these data are the first to elucidate expression of *GLUT14* in bovine utero-placental tissues, as well as confirm the presence of *GLUT3* in these tissues. Glucose transporter GLUT3 has the highest known transport capacity of any glucose transporter in the mammalian body. Therefore, establishment of GLUT3 as well as its duplicon, GLUT14 in bovine utero-placental tissues supports previous data highlighting the necessity of glucose flux to the developing fetus.

Although GLUT1 and GLUT3 are both high affinity glucose transporters, (~5 mM and ~1.5mM respectively) GLUT3 transport capacity is the highest calculated of all the known GLUT isoforms, thus facilitating proportionally greater glucose uptake (Thorens and Mueckler, 2010). Increased expression of *GLUT3* as gestation progresses (as seen in caruncular tissue increasing to d 50) may indicate the preferential utilization of a high capacity glucose transporter as energetic demands of the conceptus increase in order to ensure the success of pregnancy and

adequate development. This rationale is also supported by Ganguly et al. (2007) who reported that a null mutation of GLUT3 resulted in early embryonic mortality in mice due to increased apoptosis in blastocysts. Data from Crouse et al., (2016) in bovine chorioallantois, found that *GLUT3* expression was greater in pre-implantation fetal membranes compared with post-implantation fetal membranes. Additionally, GLUT3 null mice failed to survive post implantation, even in nutrient rich growth mediums, due to failed neurulation (d ~ 8.5; Total gestation ~ 20 d), or late-gestation fetal growth restriction characterized by decreased GLUT3-mediated transplacental glucose transport (Ganguly et al., 2007). Thus suggesting that bovine utero-placental tissues preferentially utilize GLUT3 over GLUT1 as the growth curve and energy demands of the conceptus increase in order to provide sufficient glucose to prevent early embryonic mortality. In addition, the greater expression of *GLUT3* on d 50 in CAR, as well as greater still on d 34 in CAR compared with ICAR on all days of gestation provides insight into the prevalence of *GLUT3* within the developing placentome. Histological results from our laboratory localized GLUT3 in bovine utero-placental tissues, with specific localization in the caruncle on d 50 of gestation (Osei et al., 2016). Although not collected or measured, it would be of significant interest in future studies to evaluate the presence and level of expression of *GLUT3* in cotyledon and inter-cotyledonary tissue to determine if the presence and expression of *GLUT3* in fetal tissues follow patterns similar to maternal tissue.

In all three cationic amino acid transporters investigated (*SLC7A1*, *SLC7A2*, and *SLC7A3*), greater levels of mRNA expression were observed during key days of gestation; d 16 at maternal recognition of pregnancy and implantation, and d 28 to 40 the critical window of placental development beyond which little embryonic/fetal loss occurs (Thatcher et al.,1994; Bridges et al., 2013). In FM, *SLC7A1* peaked in expression on d 34 and 50, coinciding with

increased expression on d 34 in maternal tissues. Concentration of arginine was greater on d 40 compared with d 50 of gestation, following a similar pattern as seen in maternal and fetal transporter expression, being greater d 34 of gestation and decreasing to d 50. This pattern of expression coinciding with arginine abundance may indicate the increased abundance of SLC7A1 to be established in the endometrium and chorioallantois in order to transport greater quantities of arginine to the fetus.

Supplemental lysine in the diet of rats reduced proteolysis and autophagy as well as upregulating the mTOR signaling pathway (Sato et al., 2015). Additional information regarding the significance of lysine in fetal mTOR pathways was not found in ruminants; however, mean concentrations of lysine, were greater than that of arginine in our heifers. This observation indicates that lysine may potentially play a great role in stimulating protein synthesis and differentiation during the first 50 days of gestation compared with arginine. An additional hypothesis to explain the difference between arginine and lysine would be the catabolism of arginine to its catabolites ornithine and citrulline, thus decreasing the concentration of arginine in fetal fluids compared with lysine. Because lysine uses the same intracellular transporter system as arginine, competition of transport can be hypothesized (Luiking and Deutz, 2007) and subsequently there is equal probability of arginine or lysine to be transported by any one individual transporter which in theory could make total concentrations of arginine and lysine transported equal. Interestingly, when the concentration of arginine, citrulline, and ornithine are added together and compared to lysine in allantoic fluid (579.33 vs. 634.50 μmol/L, respectively), we find that concentrations of arginine and lysine are more equal than the raw means of arginine vs. lysine individually. Additionally, we see similar patterns in amniotic fluid in which arginine and ornithine together are numerically greater compared with lysine

concentrations (244.66 vs. 236.95, respectively). These data may support the hypothesis that arginine is being metabolized within the allantoic and amniotic fluid to ornithine and citrulline in order to increase the abundance of polyamines and NO. Enzymatic analysis of arginase, nitric oxide synthase, and ornithine transcarbamoylase would provide increased information to answer this question and would be of interest to future studies.

Genes were greater in mean expression across all days in either CAR (*GLUT3* and *SLC7A3*), ICAR (*SLC7A1*), or both CAR and ICAR (*GLUT1* and *SLC7A2*) compared with FM during the first 50 days of gestation. Although there was a general numerical increase in the expression of all transporters in FM; the overall expression of these transporters were never greater than the expression seen in either CAR or ICAR. These data align with findings of Gao et al. (2009 a, b) emphasizing the importance of nutrient transport into the uterine lumen during the period of placental differentiation. Investigating placental transporter expression past d 50 of gestation may deduce the time change in which placental transport exceeds that of uterine transport of nutrients. Alternatively, this time may never be reached indicating that at the time of establishment of hemotrophic nutrition, most efficient transport of nutrients is through the shared maternal/fetal blood supply. Additionally comparison of nutrient composition of histotroph to that of allantoic and amniotic fluids would provide greater insight into the transport capacity of placental transporters during the first 50 days of gestation.

Any project is not without areas of improvement. Beginning with chapter two, which was a preliminary study. A more complete study would have had tissues collected on every day of gestation from day 16 to 50 in order to better determine key dates for study for chapters three and four. However, this would have required nearly six times more animals to complete the study, and an even greater amount of time and man power. Due to constraints in time, manpower, and

funding, this was not possible; therefore, the dates chosen reflect key dates of gestation with minimal spacing between dates of surgery. The studies conducted for chapters two and three also could have been improved with a more consistent feeding approach. These heifers were switched between diets during the middle of the study due to lack of fermented feed. The diet switch for these heifers may have influenced specific nutrient flux to the conceptus, although the diets (haylage and silage) were fed to the same protein and energy requirements. Additionally, the duration of nutritional treatment in chapter 3 and 4 may not have been long enough to impact gene expression in these heifers. Finally, one of the last areas of criticism would be the idea of global nutrient restriction. Although in theory, when animals are not being fed adequately in the dry lot, pasture, or feedlot, the whole diet would be globally restricted. However, interpretation of results becomes more difficult, as we are unable to tell with certainty, which nutrient(s) were causing the changes expression and or nutrient concentrations. Limiting single nutrients at a time would more clearly begin to delineate which nutrients are in fact vital to conceptus growth and development during this stage of gestation, bringing us closer to a feeding strategy which can be implemented in a production setting in an effort to decrease spontaneous abortions during early gestation.

Future Directions

The project itself had several ways in which it could have been improved upon; however, the data elucidated has opened more doors for future research. The obvious being to conduct the study with more controlled feeding parameters, and a more selective nutrient deficiency to better elucidate the mechanisms occurring during this timeframe. Additionally, completing the amino

acid profile and including neutral and anionic amino acids would provide a more complete picture of fetal energetic demands.

On a braoder scale, which reaches beyond the scope of the current project, we have determined that expression of nutrient transporters, and nutrient concentrations in fetal fluids are affected by day of gestation. Therefore, it is understandable that a perturbation in flux of nutrients during critical time points in gestation can have detrimental effects on the growth and development of the fetus throughout gestation and postnatally as well. With this in mind, future projects could investigate timing of nutrient restriction during pregnancy on "programming" of detrimental outcomes (metabolic syndrome, growth defects, and mortality) which become imprinted into the genome. Determining whether nutritional perturbations to the fetus during specific time points would imprint susceptibilities to chronic diseases would provide greater information into how nutritional timing during gestation affects fetal growth and development. With this data we could more specifically isolate time points in gestation which will be affecting growth and development of certain organ systems to more effectively mitigate these effects and more successfully raise beef cattle to feed the growing population of the world.