INFLUENCE OF MATERNAL NUTRIENT INTAKE ON PLACENTAL VASCULAR FUNCTION IN PREGNANT BEEF COWS

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Influence of maternal nutrition on placental vascularity and uterine and mammary blood flow in pregnant beef cows

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ABSTRACT

Two experiments investigating impacts of maternal nutrition in beef cows were conducted; 1) to test the impact of duration of global nutrient restriction and subsequent realimentation throughout gestation on placentome capillary development; and 2) to test the response of protein supplementation on blood flow to the uterus and mammary gland in late gestation. In experiment one, realimentation following varying periods of nutrient restriction had minor alterations in angiogenic factor mRNA expression of as well as vascularity in the placentome. In experiment two, mammary gland blood flow and calf birth weight was not affected, while protein supplementation decreased uterine blood flow. Further research is warranted to investigate capillary function to determine how compensation is occurring when vascularity is not being drastically compromised with global maternal dietary restriction. Additionally the mechanism of how DDGS supplementation is decreasing blood flow to the uterus is a question which requires more exploration.

Keywords: beef cow, placenta, vascularity, supplementation.

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CHAPTER 1. LITERATURE REVIEW

Introduction

Profitability of cow- calf operations within the beef industry is dependent upon the successful birth and performance of calves by means of minimal stress to the dam, thereby promoting further reproductive success of the herd. Gestation is a critical time where fetal growth can be limited by the amount of nutrients available for cellular differentiation and tissue development, thus affecting functional calf performance after birth. Adequate fetal growth is supported by the placenta which itself must grow and acquire proper vascularity and function to support fetal growth. Calf performance can also be limited by nutrition available to the calf after parturition, which is associated with milk quality and quantity.

The demands of gestation and subsequent lactation to generate optimal calf growth are great expenditures for the cow, who will mobilize her own body reserves when there is need. For these reasons, dam nutrition during gestation is critical. Many producers provide supplements to support the cow's nutritional demands during gestation, especially in late gestation when demands are the greatest. Investigation as to the benefits of protein supplementation in late gestation and whether it affects calf nutrition and performance are necessary. Additionally when the pregnant cow is in a state where nutrition is inadequate, we do not know what the implications are when the cow is realimented to a normal plane of nutrition or if the duration of nutrient restriction can program vascularity of the placenta and potentially increase nutrient transfer to the calf.

The purpose of this literature review is to examine the role nutrition plays on placental growth, vascularity, and the impacts of supplementation on uterine and mammary blood flow during later pregnancy in beef cattle specifically. Examples from other species will be used to

emphasize specific points as needed. This literature review will encompass the development of the placenta and mammary gland as well as blood flow to the uterus and mammary gland. Nutritional affects such as impacts of nutrient restriction and protein supplementation will be discussed in the following main sections: development of the placenta in beef cows; development of the mammary gland in beef cows; perturbed nutritional impacts during gestation; and use of Doppler ultrasonography. Lastly, to summarize and introduce my thesis, a statement of the problem is addressed.

Development of the placenta in beef cows

As a eutherian species, bovine have placental development which occurs during pregnancy as an evolutionary mechanism to improve the survival of offspring. After the occurrence of conception, placental establishment of the conceptus ensues to facilitate vital transfer of nutrients, gases, and metabolic waste exchange between fetal and maternal tissues. Livestock offspring spend 25 to 40% of their lives, with lifespan being measured as conception to human consumption, within the uterus being supported by the placenta (Vonnahme and Lemley, 2012). In cows, as with all ruminants, the placenta is cotyledonary. The placenta is derived from the embryonic trophoblast layer of cells. Approximately 30 days after conception, the bovine placenta will develop structures responsible for exchange with maternal tissue known as cotyledons, which grow throughout gestation (Prior and Laster, 1979; Reynolds et al., 1990; Vonnahme et al., 2007). On the maternal side, the uterus has roughly 70 to 120 button-like caruncles to which cotyledonary villi attach; together, the fetal and maternal unit is known as a placentome (Schlafer et al., 2000). The majority of placental growth in cattle occurs in the first two trimesters of gestation, this ensures the organ will be adequate for support of the third trimester of gestation during which approximately two-thirds of fetal growth occurs (Winters et

al., 1942; Ellenberger, 1950; Jakobsen et al., 1956, 1957; Ferrell et al., 1976, 1982; Prior and Laster, 1979). Unlike within ewes, the placenta of cows continues to grow until term. Placental development and growth is therefore critical for normal growth and functional cellular tissue development of the fetus. A number of studies support the theory that the primary mechanism of increased transplacental exchange throughout gestation is increased blood flow rather than increased extraction (Meschia, 1983; Reynolds et al., 1986; Metcalfe et al., 1988; Ferrell, 1989; Reynolds and Redmer, 1995). In livestock species, nutrient uptake by the gravid uterus is primarily dependent on the rate of uterine blood flow (Ferrell and Ford, 1980; Morriss et al., 1980; Vorherr, 1982; Ford et al., 1984b, 1995). While trajectories for placental and fetal growth differ throughout pregnancy, the pattern uterine arterial blood supply is similar to the pattern of fetal growth. To orchestrate this necessary blood flow to the uterus in humans and other mammals, the mother herself must undergo physiological cardiovascular adaptations such as increased stroke volume of the heart, increased heart rate, and overall increasing cardiac output (Robson et al., 1989).

The uterine artery supplies nutrients and respiratory gases for the placenta of the growing fetus. Uterine and umbilical blood flows increase throughout gestation and are able to keep pace with growth of the fetus (Reynolds and Redmer, 1995; Magness, 1998; Reynolds et al., 2005). Additionally, there is greater uterine blood flow to the ipsilateral vs. contralateral horns and inversely, arterial resistance and pulsitility decrease with advancing gestation (Bollwein et al., 2002; Panarace et al., 2006; Ginther et al., 2007; Camacho et al., 2014b). These hemodynamic changes, in addition to changes in cardiac output, occur throughout gestation to facilitate optimal transfer of nutrients to the fetus. To allow this impressive increase in blood flow, in addition to

adaptations of cardiovascular capabilities of the dam, the placenta needs to develop adequate capillaries, a process which is largely mediated by the actions of angiogenic factors.

Factors involved in placental capillary development

Angiogenesis, or the formation of new blood vessels from preexisting ones, in the placenta is largely regulated by angiogenic factors produced in fetal and maternal tissues. While there are many factors that have been shown to modulate vascular development in the placenta, this literature review will focus on vascular endothelial growth factor (VEGF) and its two receptors KDR (kinase-insert domain-containing receptor) and Flt-1 (fins-like tyrosine kinase), endothelial nitric oxide synthase (eNOS) and its receptor soluble guanylate cyclase beta (sGCβ), as well as platelet endothelial cell adhesion molecule-1(PECAM-1).

Vascular endothelial growth factor mitogenically targets vascular endothelial cells and is known to potently promote their growth and migration. VEGF can induce proliferation in vascular endothelial cell tissue types including bovine aortic, human umbilical vein, and chick chorioallantoic membranes. Additionally, VEGF, its receptors, or both, have been expressed in other cell types such as trophoblast, uterine epithelium, and uterine glands implying that VEGF has a role in placental establishment (Connolly et al., 1989; Leung et al., 1989; Plouct et al., 1989; Winther et al., 1999; Winther and Dantzer, 2001). Vascular endothelial growth factor is essential in embryonic vasculogenesis, angiogenesis, and regulation of normal growth processes occurring during post-natal life (Carmeliet et al., 1996; Ferrara et al., 1996; Gerber et al., 1999). It has been indicated by increases in mRNA expression, that VEGF is regulated by oxygen tension, growth factors, and oncogenes (Grugel et al., 1995; Ferrara and Davis-Smyth, 1997; Okada et al., 1998; Neufeld et al., 1999; Dor et al., 2001; Ferrara et al., 2003).

Vascular endothelial growth factor is known to act through two tyrosine kinase receptors: KDR (kinase-insert domain-containing receptor) also known as VEGFR-2 (vascular endothelial growth factor receptor-2) and Flt-1 (fins-like tyrosine kinase) also known as VEGFR-1 (vascular endothelial growth factor receptor-1). Placental growth factor is a homologue of VEGF which shares 42% amino acid sequence identity and binds Flt-1 but not KDR, though results are mixed as to how active placental growth factor is in endothelial cell functions (reviewed by Autiero et al., 2003). In knockout mice for either of these receptors, embryonic death occurs between 8.5 and 9.5 days (Fong et al., 1995, 1999; Shalaby et al., 1995); however, there is evidence that these two receptors work in opposing ways. Mice which have for Flk-1 knockout, the gene which is also known as KDR in other mammals, have a lethal phenotype associated with lack of vasculogenesis, and mice which are knockouts for Flt-1 die at the same age from overgrowth of endothelial cells (Fong et al., 1995). It is agreed that KDR is the chief moderator of the mitogenic, angiogenic, and microvascular permeability effects of VEGF on endothelial cells (reviewed in Shibuya, 2013). Conversely, soluble Flt-1 negatively regulates VEGF (Fong et al., 1999; reviewed by Shibuya et al., 2013). This could prove to be vital to offspring survival as excessive vascular growth and maternal vascular growth into fetal tissues can be lethal in early embryonic development; therefore, VEGF must be sequestered at times (Fong et al., 1999; Borowicz et al., 2007). Shibuya et al. (2013), discusses soluble Flt-1 expression in placental trophoblast cells located in umbilical, fetal, and maternal capillaries suggesting that it is binding VEGF thereby preventing fusion of the vessels and further abnormal angiogenesis.

Blood vessel diameter is regulated by VEGF through KDR within in vitro models (Nakatsu et al., 2003). In vitro endothelial cell-derived nitric oxide (NO) causes VEGF to induce vasodilatation in a dose-dependent fashion (Ku et al., 1993). Nitric oxide, a gas molecule, plays a

role in the regulation of endothelial cell migration and proliferation, vessel tone, inflammation, and angiogenesis in wound healing, and regulates the effects of many angiogenic factors (Duda et al, 2004). Nitric oxide synthase (NOS) is the enzyme that produces NO via the oxidation of L-arginine to L-citrulline. Endothelial nitric oxide synthase (eNOS) is an endothelial cell-specific isoform of NOS, which is expressed by vascular endothelial cells. Nitric oxide produced by eNOS has been shown to function in neovascularization in vivo and also in postnatal vasculogenesis (Moncada, 1992; Aicher et al., 2003). In humans, eNOS is expressed in the endothelium of umbilical and chorionic arteries and veins, as well as in the syncytiotrophoblast layer of the placenta (Myatt et al., 1997). Additionally, eNOS is expressed in human placental tissues throughout gestation (Rossmanith et al., 1999). Nitric oxide is believed to cause vasodilation by activating soluble guanylate cyclase (sGCβ, GUCY1B3), which catalyzes the conversion of guanosine 5'-triphosphate (GTP) to the second messenger molecule, guanosine 3', 5'- monophosphate (cGMP). Accumulation of cGMP activates protein kinase G (PKG) which catalyzes the phosphorylation of a number of proteins whose function is to regulate the contractile activity of the smooth muscle cell (Denniger and Marletta, 1999; reviewed by Lincoln et al., 2001; Schlossmann et al., 2003). Relaxation of smooth muscle surrounding arteries permits more blood to flow; this is the mechanism by which the NO pathway controls local blood flow (Denniger and Marletta, 1999; Condorelli and George, 2001 and 2002).

Platelet endothelial cell adhesion molecule (PECAM-1) is a receptor expressed on surfaces of vascular endothelial cells, platelets, macrophages, neutrophils, bone marrow, and lymphocytes in mice and is highly concentrated in junctions of endothelial cells (Xie and Muller, 1993; Newman, 1997). Platelet endothelial cell adhesion molecule is involved in vascular development including initial formation between cells at lateral junctions and migration of

endothelial cells themselves as well as monocytes and neutrophils. Other functions of PECAM-1 in endothelial cells include neovascularization and the maintenance of a vascular permeability layer (reviewed in Jackson, 2003). Both placental vascularity as well as uterine and umbilical placental blood flows must be increased for the placenta to maintain transport capacity to keep up with late gestational fetal requirements (Reynolds et al., 1995, 2000).

To achieve the most optimal fetal growth possible, the placental tissue itself must grow and undergo angiogenic proliferation. This angiogenic growth must be for capillaries needed for the transfer of fetal nutrients, but also for placental cellular differentiation and growth for functionality of this organ. Additionally, the nutrients available to build this functional placental growth and fetal growth are dependent on blood supply to the uterus mainly via the uterine artery. Therefore, it is advantageous when placental vascularity and blood flow to the uterus is maximized for delivery of nutrients to support the development and growth in utero.

After parturition, however, the calf will be reliant on milk as its nutrient source; therefore, dam nutrition during gestation is essential as maternal body preserves stored will be hormonally mobilized for mammary glandular growth in late gestation. The reserves stored during gestation affect milk composition and production during lactation. Moreover, dam nutrition during gestation is essential for sustaining placental and fetal growth and for preparing to support the fetus after parturition. How these nutritional effects can augment or promote these processes, including placental development and blood flow to the uterus and mammary gland, will be conversed in the following sections.

Development of mammary gland in beef cows

Mammary gland development begins in utero. However, unlike many other organs in the body most of the development occurs during postnatal life (Bloise et al., 2010). The cyclic stages

of tissue growth and differentiation, milk secretion, and involution are largely orchestrated by the ovarian steroid hormones, progesterone, and estrogen (Connor et al., 2007). Additional regulators of mammary function and development are oxytocin, prolactin, growth hormone, placental lactogen, thyroid hormone, and glucocorticoids (Cowie et al., 1980; Forsyth, 1986; Tucker, 2000; Neville et al., 2002). Data from knockout mice models for estrogen and progesterone receptors support the current premise that estrogen is essential for ductal morphogenesis in growing animals and mammary cell proliferation. Progesterone is a critical stimulant for lobulo-alveolar development during pregnancy (Lydon et al., 2000; Walker and Korach, 2004).

After birth, little mammogenesis occurs until the onset of puberty, growth of the mammary gland is considered isometric, or proportional to the body. At pubescence, there is allometric growth where rapid extension and branching of the ductal system occurs within the mammary gland. Gestational mammary proliferation is dependent on the amount of mammary development and involution that has occurred in prior reproductive cycles. Formerly it was thought that mammary development occurred only in the first two trimesters of gestation and the growth in size that was observed in the third trimester was filling of the secretory cells within the gland (reviewed by Lamote et al., 2004). However cellular division occurs throughout gestation and continues into early lactation (Cowie et al., 1971).

Lactogenesis, or the establishment of milk secretion, consists of two stages, the first of which involves synthesis and secretion of pre-colostrum a few weeks prior to parturition. The second stage of lactogenesis involves the start of copious milk secretion and extends several days post partum (Fleet et al., 1975; Tucker, 1985). It has been established that during late pregnancy mammary ducts develop into lobulo-alveolar tissue with differentiated cells capable of producing

milk. However, Davis et al. (1979) demonstrated in the goat that although mammary blood flow, oxygen consumption, and glucose uptake had increased in the week prior to parturition, the first significant change with respect to days 7 to 9 pre-partum was apparent at 0.5 to 1 day pre-partum. Additionally, mammary glucose uptake on the day after parturition was nine times that on d 7 to 9 prepartum.

Three weeks prior to parturition, the time period when fetal growth is most rapid (Ferrell, 1991), voluntary feed intake decreases 30 to 35% (Grummer, 1995). Moreover, in rats body reserves stored during gestation are the major supply of energy for lactation, and mobilization of these fat storages are under hormonal not dietary control (Naismith et al., 1982). For these reasons, adequate nutrient supply during gestation is a necessity for optimal lactation in addition to an adequate lactational diet, as maternal intake during lactation is not the main factor contributing to energy supply for lactation.

Gestational nutrition is important for adequate nourishment of the offspring as it can impact the placenta and the development of the mammary gland. The following sections will discuss how nutrition has been reported to influence these two important ephemeral organs.

Perturbed nutritional impacts during gestation in the beef cow

It is established that nutrition received by the dam during gestation impacts both fetal and postnatal growth (Barker et al., 1995; Wu et al., 2006). In the initial two-thirds of pregnancy, maternal metabolism is in a physiologically anabolic state (Vernon et al., 1985), whereas the last third is a catabolic state (Naismith et al., 1976; Symonds and Clarke, 1996). In the first two thirds of pregnancy, the majority of fetal organogenesis occurs and nutrient deprivation can impair cellular function of those tissues for life (reviewed by Wu et al., 2006). In bovine, two-

thirds of fetal growth occurs in the last trimester of gestation requiring an increase in maternal expenditure to facilitate adequate fetal growth and proper cellular tissue development.

In the Midwest, calves are primarily born in early Spring and the last trimester of gestation is typically during harsh winter months being kept either on corn stover or in a lot, situations where forage nutrition may be poor and cows may be nutritionally restricted. Metabolizable protein content of grazed forage, particularly during winter, is low and is often insufficient for optimal reproductive performance of beef heifers or cows (Patterson et al., 2003; Ferguson, 2005). Supplementation of an energy or protein source is a viable option for producers to help meet nutritional needs of late gestating beef cattle. There is evidence that late gestational supplementation of protein and energy concentrates increases calf birth weight in heifers and cows (Clanton and Zimmerman, 1970; Bellows and Short, 1978). Nutrient availability whether in a positive (supplementation) or negative (restriction) state can impact placental growth and function. If the placenta does not have adequate growth and vascularity, it may not be able to sufficiently transfer nutrients for optimal fetal growth especially during late gestation; therefore, proper nutrition is also essential for placental growth, and is negatively affected by adverse limited nutrition.

Maternal dietary impacts on placental growth

Over or undernutrition may lead to impaired placental growth (including vascular growth) or function, possibly caused by reduced synthesis of vasodilators and metabolic regulators in the placenta, and may contribute primarily to intrauterine growth restriction (IUGR; Wu et al., 2006). In livestock, uteroplacental blood flow rate is dependent on growth of the placenta itself as well as vascular growth via angiogenesis (Vonnahme and Ford, 2004; Reynolds et al., 2005). Current studies support the notion that nutrient restriction during gestation causes

changes in blood flow and vascularity to the placenta as a compensatory mechanism in an attempt to maintain fetal growth and development. Removal of concentrate during mid-gestation in the ewe decreased mRNA expression for the angiogenic factor VEGF and later impacted placental weights at day 90 of gestation (McMullen et al., 2005). Zhu et al. (2007) demonstrated that beef cows restricted to 50% of requirements from 30 to 125 days of gestation had increased placentome efficiency (fetal weight / total placentome weight), and there was a tendency for the cotyledon to have a greater number of larger-sized blood vessels. In cows, while restriction did not influence capillary development in the placentome, upon realimentation a decrease in capillary vascularity in placentomes was observed (Vonnahme et al., 2007). Total placentome weight was lower after nutrient restriction up to day 125 of gestation and remained decreased even after realimentation until day 250 (Vonnahme et al., 2007; Zhu et al., 2007).

While there are many studies in rats that demonstrate protein restriction decreased placental size as well as total DNA quantity (reviewed in Brasel and Winick, 1972) the effects of protein restriction in beef cattle is less understood. A study by Perry et al. (1999) investigating the effects of different dietary protein concentrations in either first or second trimester for gestating beef heifers has suggested that protein restriction in the first trimester leads to a larger placenta at term and could be attributed to the greater development of placental microvilli. It is clear that while research in the area of protein restriction across species seems to be lacking, information on the impacts of protein supplementation on placental development and function is even less available.

It may be proposed that birth weight is indicative of nourishment received from the maternal source. Results as to whether dried distillers grain supplementation (DDGS), a commonly used protein supplement, during gestation can effect birth weight (which could be

indicative of placental function) in cattle are inconclusive with some reporting an increase in birth weight while others report no significant change in birth weight with DDGS supplementation (summarized in Table 1.1). Moreover it is unclear whether the changes in birth weight which have been observed are due to a change in the amount of blood flow received by the uterus. The next sections address what is currently observed from the literature regarding maternal dietary impacts on blood flow to the uterus and mammary gland.

Maternal dietary impacts on uterine blood flow

The uterine artery serves as the main supply of nutrients, oxygen, and waste removal for the placenta of the growing fetus. Uteroplacental blood flow is directly related to nutrient transport efficiency of the placenta (Reynolds and Redmer, 1995). When blood flow to the uterus is altered in any way it can greatly impact the survival and performance of offspring. Research by Camacho et al. (2014b) demonstrated that when cows experienced nutrient restriction from days 30 to 85 of gestation and were realimented to adequate nutrition until day 254 of gestation there was an increase in blood flow to the gravid horn of the uterus. Our laboratory has previously demonstrated that uterine blood flow near term in protein adequate ewes was decreased compared with protein restricted ewes (Lekatz et al., 2010b). To our knowledge no prior research has investigated the impact of protein supplementation on blood flow in gestating beef cattle. Overall, literature investigating the dietary impacts on bovine uterine blood flow is largely lacking.

Table 1.1. Calf birth weight change due to maternal dried distillers grains supplementation during late gestation

Study[1]	DDGS supplementation level and study treatments[2]	Birth weight change[3], kg
Stalker et al., 2006	2 x 2 factorial: prepartum supplement (0.45 kg/ cow/ day) and postpartum meadow grazing or drylot feeding of hay	NC[4]
Larson et al., 2009	2 x 2 factorial: wintering system (drylot or pasture) with or without supplement (0.45 kg/ cow/ day)	NC
Woods et al., 2010	• 100% Haylage (free choice haylage) • 0.7% Haylage (haylage offered at 0.5% of BW for 36 d and 0.75% for remainder with free choice access to wheat straw) • 1% Haylage (haylage offered at 1% of BW with free choice access to wheat straw) • Haylage-SBM (haylage offered at 0.5% of BW plus soybean meal with free choice access to wheat straw) • Haylage-DDGS (haylage offered at 0.5% of BW plus dried corn distillers grains plus solubles with free choice access to wheat straw)	NC
Radunz et al., 2010	• Hay (fed ab libitum; average consumption 12.4 kg/ cow/ day) • CN (5.3 kg of corn/ cow/ day plus 2.1 kg of hay, and 1.0 kg of supplement) • DDGS (4.1 kg of DDGS/ cow/ day plus 2.1 kg of hay, and 1.0 kg of supplement)	Increase 3.4 kg for calves from cows fed CN and DDGS than for those fed HY
Winterholler et al., 2012	• DGSL (DGS low; 0.77 kg/ cow/ day) • DGSI (DGS intermediate; 1.54 kg/ cow/ day) • DGSH (DGS high; 2.31 kg/day) • POS (Positive control; 1.54 kg/day of 49% midlings and 51% cottonseed meal) • NEG (Negative control; 0.23 kg/d of cottonseed hull pellet mix)	Increased linearly (1.7 kg) with respect to increasing levels of DGS
Bohnert et al., 2014	2 x 2 factorial: low or high BCS with or without supplement (0.9 kg/ cow/ day)	Increase 1.5 kg

[1] Studies reporting birth weights of calves born from dams supplemented with DDGS in late gestation.
[2] Treatments applied to cows within experiments of studies.
[3] Change in calf birth weight as effected by dam supplementation with DDGS in late gestation
[4] NC = no change in birth weight.

Maternal dietary impacts on mammary blood flow

Maternal nutrition during gestation is important for lactation. Body reserves stored will be utilized in lactation as well as for preparatory growth of alveolar tissue which occurs in late gestation (Davis et al., 1979; Naismith et al., 1982). Moreover, blood flow to the mammary gland is strongly correlated with milk yield (Kronfeld et al., 1968; Linzel et al., 1974). It has previously been reported that protein supplementation does not affect milk production in beef cattle in the period prior to grazing season or at weaning (Larson et al., 2009). However, negative effects on milk production have been observed in beef heifers receiving a high level of protein and energy supplement in the first trimester of gestation regardless of protein levels in the second trimester (Sullivan et al., 2009). Lactating rats fed protein-restricted (10% vs. 20% in control diet) or nutrient-restricted diets (the control diet at 40 and 60% of the ad libitum intake) had decreased mammary, liver, and kidney masses than rats consuming the ad libitum control diet. Milk production was decreased in both protein and food restricted rats than those of ad libitum-fed control, as evidenced by the decrease in litter live weight gains (Grigor et al., 1987). While literature has focused largely on how nutrition during lactation can affect parameters such as milk production and composition (especially within the dairy livestock species), the significance of nutrition during gestation has been less probed. Likewise, gestation nutritional effects on both mammary and uterine blood flow has been under studied. This may be due to the complexity of techniques used to measure these variables as well as the intricacy and risks associated with measuring the flow of blood though arteries over the course of gestation or lactation without inhibition of flow. Perhaps one of the reasons why uterine and mammary gland blood flows have not been investigated largely in beef cattle is due to the expense of the animals and the procedures needed to monitor blood flow. The use of Doppler ultrasonography, though not

widely used in livestock, is growing in popularity and provides a means of safely monitoring these blood flows within the same animal over time. The next section highlights some of the applications as well as benefits and disadvantages of Doppler ultrasonography.

Doppler ultrasonography

There are many applications of Doppler ultrasonography as it is largely popular in human medical applications including, but not limited to, observing sonoelasticity (i.e., using tissue elasticity to judge disease state), growth and vasculature of tumors, fluid within cystic breast tissue, assessment of kidney function and presence of renal calculi, umbilical and placental blood flow to assess fetal health, and many studies assessing cardiac function. Basically, ultrasonography works by utilizing a transducer probe placed on a body surface or cavity to emit and receive high-frequency (1 to 5 megahertz) ultrasonic waves. This is accomplished using the piezoelectric (pressure-electricity) effect, which was discovered by Pierre and Jacques Curie in 1880 (Curie and Curie, 1880, 1881) Within the probe, there are crystals, commonly lead zirconate titanate, with piezoelectric properties; the size and shape of these crystals dictate what frequency of waves are omitted. Doppler ultrasonography works via the Doppler Effect named after Christian Doppler (Doppler, 1842) who was first to analyze the effect of motion on the observer relative to the source of a wave on the perceived wave frequency (Gowda et al., 2004). The phenomenon of the Doppler Effect can be commonly demonstrated as one is passed or passes an ambulance, police car, or fire truck that omits a siren. This principal can be used to measure the flow of blood through an artery as when sound waves are omitted, there is a change in frequency of tone as the waves are reflected by red blood cells passing through the artery (Satomura, 1957).

Utilization of Doppler

Doppler ultrasonography has largely gained popularity in terms of human medicine; however, this area is still relatively novel to large animal research. Doppler ultrasonography is a non-invasive way to measure blood flow. Other methods used to measure blood flow, such as flow probes, require that animals be anesthetized and undergo surgery which causes more stress to the animal and can actually restrict the arteries. This could potentially affect results of those studies. Additionally, animals must typically be euthanized to retrieve devices placed on arteries and if devices are placed on arteries which grow (i.e., during gestation) they must be removed and multiple animals must be used to observe blood flow in different trimesters as flow probes will eventually restrict flow, whereas Doppler ultrasonography gives the ability to observe the same animal throughout gestation. Also, other methods can have large financial expenditures, as tracers that can be put into circulation of an animal such as deuterium and other radioactive ions can be expensive.

When measuring blood flow through an artery, there are factors to consider that can decrease Doppler frequency and cause error. Angle of insonation is an important factor. The angle of insonation is the angle between the ultrasound beam of the probe and the direction of the flow. A higher Doppler frequency is obtained when the beam is more aligned to flow direction, and an ideal angle of insonation is less than 60 degrees as discrepancies in velocity are observed with larger angles. Additionally, non-uniform insonation of the blood vessel can also be a source of error (i.e., probe not uniformly placed on vessel measured). Blood velocity is another factor affecting measures; if blood velocity through an artery is small, this can decrease Doppler frequency. The presence of neighboring vessels and the use of filters can lead to inaccurate reception of sound waves returning to the probe and contribute to inaccurate

information. Lastly, calculation packages from manufacturer companies used for analysis and calculations can also contribute to variations in blood flow and velocity measures (Gill, 1985). It is important for the operator to repeat measures, measure the same portion of the vessel each time using landmarks when possible, and if possible, use the same angle of insonation and apply the narrowest filters when measuring a blood vessel over time.

Statement of the problem

Optimal fetal development is dependent on growth of the placenta and its vasculature as well as blood flow to the placenta. Late gestation is a demanding time for the cow as this is when the majority of fetal growth occurs, voluntary intake decreases, and nutrition at this time and throughout gestation are vital to maintaining condition because body reserves stored will soon be mobilized in early lactation. Restriction from nutrients throughout gestation may alter vasculature of the placenta, thereby impeding fetal nutrient exchange, which could be harmful for functional tissue development and postnatal survivability of the offspring. However, the question of whether a return to adequate nutrition can offset the effects of restriction in the first or second trimesters still persists. We hypothesize that during nutrient restriction the placenta may have a decrease in vasculature but that upon realimentation of nutrients vasculature of the placenta will be increased and comparable to that of cattle that did not have a period of nutrient restriction.

In the Midwest cows are fed forages which alone may not have a nutritionally adequate crude protein concentration. Supplementation of protein during late gestation may help offset maternal expenditure and, thereby, improve fetal growth. Observations by others demonstrate that dietary protein supplemented during gestation has been shown to positively alter offspring growth and performance (Naismith et al, 1976; McNeil et al, 1997; Ocak et al, 2005; Stalker et al, 2006; Larson et al, 2009; Funston et al, 2010). Furthermore, increasing amounts of DDGS

supplementation three weeks prior to parturition linearly increased calf birth weight as well as linearly increased calf weight at d 60 and 90 of age (Winterholler et al, 2014), which may indicate that DDGS supplementation may enhance uterine blood flow, mammary blood flow, or both. We hypothesize that the offspring benefit from DDGS supplementation may be due to an increase in blood flow to the uterus, mammary gland, or both. While we know that in all livestock species blood flow to the uterus increases in gestation, there have been few studies conducted specifically in late gestating beef cattle and within those studies there are variations amongst experiments with regard to changes in blood flow. Currently there are no previous studies observing the effects of late gestational DDGS supplementation on blood flow to the uterus in beef cattle have been previously been published. Few studies have investigated blood flow to the mammary gland (Götze et al., 2010; Potapow et al., 2010; Christensen et al., 1989; Kensinger et al., 1983; Neilsen et al., 1990 and 1995; Madsen et al., 2005; Braun et al., 2008, 2012). All of those performed in cattle (Götze et al., 2009; Potapow et al., 2009; Braun et al., 2012) have been conducted in dairy breeds and during different phases of lactation. Not only does there need to be more investigation into blood flow to the mammary gland in beef cattle, there also is a need to look at blood flow during late gestation. There are no documented studies of how DDGS supplementation affects blood flow to the mammary gland in beef cattle to the knowledge of our laboratory.

Experiments were designed to answer these queries. The first objective of our experiment was to establish whether duration of nutrient restriction and subsequent realimentation would allow compensation of vascular growth in the placenta to the level of, or surpassing, cows that were not exposed to nutrient restriction. A second experiment was designed to determine the

impacts of DDGS supplementation on blood flow to the uterus and mammary gland in late gestating beef cows and how DDGS supplementation affects calf and placenta size at birth.

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CHAPTER 2. IMPACTS OF NUTRIENT RESTRICTION AND REALIMENTATION IN GESTATING BEEF COWS ON VASCULARITY IN PLACENTOMES AND mRNA EXPRESSION OF ANGIOGENIC FACTORS

Abstract

The objective of the current study was to investigate how maternal global nutrient restriction and realimentation impacts placentome vascularity and mRNA expression for angiogenic and vasoactive factors in cotyledonary (COT) and caruncular (CAR) tissues from beef cows. On d 30 of pregnancy, multiparous, nonlactating cows (620.5 ± 11.3 kg BW and 5.1 ± 0.1 BCS) were assigned to 1 of 2 dietary treatments: control (CON; 100% NRC requirements for maintenance and fetal growth; n = 18) and restricted (RES; 60% NRC; n = 30). On d 85, cows were either slaughtered (CON, n = 6; RES, n = 6), remained on control (CC; n = 12) and restricted (RR; n = 12), or were realimented to control (RC; n = 11). On d 140, cows were either slaughtered (CC, n = 6; RR, n = 6; RC, n = 5), remained on control (CCC, n = 6; RCC, n = 5), or were realimented to control (RRC, n = 6). On d 254, all remaining cows were slaughtered to collect tissues. At tissue collection, placentomes were separated and COT and CAR were snap frozen until qPCR analyses for mRNA expression of platelet endothelial cell adhesion molecule-1 (PECAM-1), soluble guanylate cyclase-β, endothelial nitric oxide synthase, vascular endothelial growth factor, fms-like tyrosine kinase, and kinase insert domain containing receptor were performed with all normalized to 18S rRNA. To determine vascularity measurements in CAR and COT, histologically processed placentomes were stained for PECAM-1, Rhodamine labeled lectin, and DAPI, and micrographs analyzed with image software. Analysis of mRNA expression revealed few treatment effects within CAR or COT tissues on any day of gestation. At d 85, COT capillary size was smaller in RES vs. CON (82.87 vs. 136.03 ± 16.19 μm^2). There

was no effect of treatments on d 140 or 254 on any vascularity measurements in CAR or COT. In CON cows, CAR tissue area decreased ($P = 0.02$) and capillary number density increased ($P < 0.01$) from d 85 to 254 (0.136 to 0.104 ± 0.008 mm^2 and 67.05 to 33.66 ± 12.23 number / μm^2). In COT, tissue area increased ($P = 0.02$) from d 85 to 140 (0.473 to 0.500 ± 0.008 mm^2) thereafter remaining similar. Capillary area and surface densities were similar ($P \geq 0.19$) on d 85 and 140 (3.25 and 2.62 ± 0.31 μm^2 / μm^2, 0.013 and 0.014 ± 0.001 μm / μm^2) and increased ($P \leq 0.04$) by d 254 (4.27, 0.026). Capillary size decreased ($P < 0.01$) from d 85 to 140 (136.03 to 70.14 ± 12.23 μm^2) however from d 140 to 254 there was no significant change in capillary size ($P = 0.14$). Capillary number density increased ($P < 0.03$) throughout gestation (256.26, 385.15, and 913.32 ± 36.88 number / μm^2). It appears that bovine COT undergo more capillary modifications as gestation advances than CAR. While our laboratory has previously reported that realimentation can augment uterine blood flow and placental arteriole vasoreactivity, the histologic and mRNA expression for angiogenic/vasoactive factors do not appear to be altered by maternal dietary intake.

Keywords: angiogenic factor, beef cow, placentome, vascularity.

Introduction

It is recognized that nutrition available to the fetus during gestation impacts both fetal and postnatal growth (Barker et al., 1995; Wu et al., 2006). The placenta is the fetal-derived organ through which all nutrients and wastes are transferred from the maternal and fetal capillary vasculatures. Optimal fetal development is dependent on growth of the placenta and its vasculature. To sustain the large amount of fetal growth that occurs in late gestation, the placenta must undergo angiogenesis to support the needs of its own functional cellular differentiation as well as to increase capillary exchange of nutrients for the growing fetus. This angiogenesis is

mediated, in part, by angiogenic factors such as vascular endothelial growth factor (VEGF) and its two tyrosine kinase receptors, KDR (kinase-insert domain-containing receptor) and Flt-1 (fins-like tyrosine kinase) and through endothelial nitric oxide synthase (eNOS) and its receptor, soluble guanylate cyclase (sGC).

Restriction from nutrients throughout gestation may alter vasculature of the placenta, thereby impeding fetal nutrient exchange (Reynolds and Redmer, 2001) which could be harmful for functional tissue development and survivability of postnatal offspring. However, the question of whether a return to adequate nutrition can offset the effects of restriction in the first or second trimesters still persists. We hypothesized that during nutrient restriction the placenta would have a decrease in vasculature, but upon realimentation of nutrients, vasculature of the placenta will increase and be comparable to those with no nutrient restriction. The objectives of this experiment were to measure vascularity and expression of mRNA for angiogenic factors and a vascularity marker in both cotyledon (COT) and caruncular (CAR) portions of the placentome. More specifically, we set out to determine how mRNA expression and vascularity were changed 1) within specific days of cows experiencing different nutritional planes; 2) across gestation in adequately fed cows; 3) in cows experiencing 2 durations of restriction; and 4) in cows experiencing two different times of realimentation.

Methods

Animal procedure and tissue collection

All animal procedures were approved by the North Dakota State University Animal Care and Use Committee (#A10001). Detailed methods for animal procedures are previously published from our laboratory by Camacho et al. (2014a). Maternal body weight, placental size, fetal size, and umbilical blood flow for the animal model used here have been previously

published (Camacho et al., 2014a, Camacho et al., 2014b). Briefly, all cows were fed chopped grass hay [8.02% CP and 57.93% TDN (DM basis)] and a mineral and vitamin supplement was top - dressed three times per week. From the time of artificial insemination to day 30 of gestation, all cows received 100% of NRC requirements for maintenance and fetal growth. On d 30 of pregnancy, cows were randomly assigned to dietary treatments (n = 4 to 5/pen with greater than 1 dietary treatment per pen): control (CON; 100% NRC; n = 18) and nutrient restriction (RES; 60% NRC; n = 30). On d 85 cows were slaughtered (CON, n = 6; RES, n = 6), remained on control (CC; n = 12) and restricted (RR; n = 12) treatments, or were realimented to control (RC; n = 11). On d 140 cows were slaughtered (CC, n = 6; RR, n = 6; RC, n = 5), remained on control (CCC; n = 6; RCC; n = 5), or were realimented to control (RRC; n = 6). On d 254 all remaining cows were slaughtered (CCC, n = 6; RCC, n = 5; RRC, n = 6).

At slaughter, cows were euthanized via captive bolt followed by exsanguination. The uterus was removed at the cervix and the fetus was dissected from the gravid uterine horn. Fetal membranes were removed and two representative placentomes were dissected from the uterine wall, weighed, and either had CAR and COT tissues 1) separated, weighed, flash frozen, and stored at -80°C for mRNA analysis or 2) immersion fixed in 10% formalin and embedded in paraffin blocks.

Placentome gene expression determination

Frozen CAR and COT tissues were suspended in RLT lysis buffer (Qiagen, Valencia, CA, USA) and directly homogenized using a Polytron homogenizer (Kinematica Type PT10/35, Brinkman Instrument Inc., Westbury, NY, USA) fitted with a 7 mm generator. Total mRNA was extracted using a QIAshredder spin column and an RNeasy Mini Kit (Qiagen) following the

manufacturer's suggested protocol. The RNA was quantified by measuring absorbance on a Nanodrop 2000c spectrophotometer.

For each CAR and COT portion of the placentome, mRNA was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Genomic DNA was removed as part of the reverse transcription protocol, and quantitative RT-PCR on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was performed. The probe and primer sequences used for mRNA gene expression analysis are listed in Table 2.1. Primers and probes for VEGF, KDR, Flt-1, and eNOS were purchased from Applied Biosystems (Foster City, CA, USA). Primers for PECAM-1 and sGCβ were newly designed and synthesized from Life Technologies (Grand Island, NY, USA).

Table 2.1. List of primer and probe sets for genes used in q-PCR mRNA quantification in caruncular and cotyledonary placentome tissue.

Gene	Sequence	
VEGF	FP	5'-GCATGTCTACCAGCGCAGC-3'
	RP	5'-TCTGGGTACTCCTGGAAGATGTC-3'
	Probe[1]	5'(6FAM)-TCTGCCGTCCCATTGAGACCCTG -(TAMRA)-3'
KDR	FP	5'-GCAGTCATGGCGTCTTCTGTAA-3'
	RP	5'-GCTCCAGTATCATTTCCAATCACTT-3'
	Probe	5'(6FAM)-ATGCTCACAATTTCA-(MGBNFQ)3'
Flt-1	FP	5'-CGCCTGAAATCTACCAGATCATG-3'
	RP	5'-TCCACGAATCTTGGCCTTTCT-3'
	Probe	5'(6FAM)-TGGACTGCTGGCACAAAGACCCAA-(TAMRA)-3'
eNOS	FP	5'-CAGCGCCTGGTACATGAGC-3'
	RP	5'-TTGTAGCGGTGAGGGTCACA-3'
	Probe	5'(6FAM)-CGGAGATTGGCACGCGGAACC-(TAMRA)-3'
sGCβ	FP	5'-GCCCGAAGCAGACAGCATA-3'
	RP	5'-CAGGTCGTCCAGGTTCATGA-3'
	Probe	5'(6FAM)-TTCTCCTGTGCTCACCAAG-(MGBNFQ)-3'
PECAM-1	FP	5'-GTCACTGTGCGGGAATCCTT-3'
	RP	5'-ACCTTTCCCTCAGGGATGATG-3'
	Probe	5'(6FAM)-CAAATCCCAGTTCC-(MGBNFQ)-3'

[1] All probes utilized the same reporter FAM while there were two types of quenchers TAMRA and MGBNFQ.

All reactions were carried out in a 12.5 µL reaction mixtures containing, 2 µl of cDNA and 10.5 µL of PCR Master Mix (TaqMan universal PCR master mix (2X), forward primer; 1 µM reverse primer; 1 µM probe; 200 nM and ddH_2O). The endogenous control, 18s rRNA was used for normalization of the raw data. The comparative Ct method of data analysis was used to generate relative fold changes in gene expression.

Placental tissue area and vascularity

From each placentome, 5-µm tissue sections were cut and mounted onto positively charged glass slides. Tissue sections were deparaffinized and rehydrated by incubation in Histoclear and a graded series of water / ethanol solutions. Following antigen retrieval (10 mM sodium citrate, 0.05% Tween 20, pH 6.0; 120 ^{o}C, 15 min), the slides were cooled to room temperature for 20 minutes. Maternal and fetal blood vessels were identified by immunofluorescent labeling with an antibody (c / n: ab28364, ABCAM) against PECAM-1 antigen, and goat anti-rabbit IgG labeled with CF 633 (c / n: 20122, Biotium). Labeling for PECAM-1 resulted in bright fluorescent localization of all blood vessels. The trophoblast layer was stained with Rhodamine labeled BS1 lectin (c/n:FL-1102; Vector; 20 µg/ml). Positive lectin staining of fetal trophoblast allowed for visual enhancement of differentiation between fetal and maternal compartments (Redmer et al., 2013). Fetal and maternal placentome nuclei were counterstained with Vectashield Hardset Mounting Media with DAPI (Vector Labs). Five to seven images per slide (placentome) were randomly taken (excluding the area near the hilus portion) with a upright Zeiss Imager.M2 epifluorescence microscope using AxioCam HRc camera (Zeiss Microimaging, Thornwood, NY, USA), at 10X magnification. Three of these photomicrographs were randomly selected for image analysis. Each image was comprised of over-laid pixel intensity values collected at three

wavelengths. DAPI stained nuclei were visualized at 325 nm, fetal tissue was seen at 555 nm, and PECAM-stained blood vessels were detected at 625 nm.

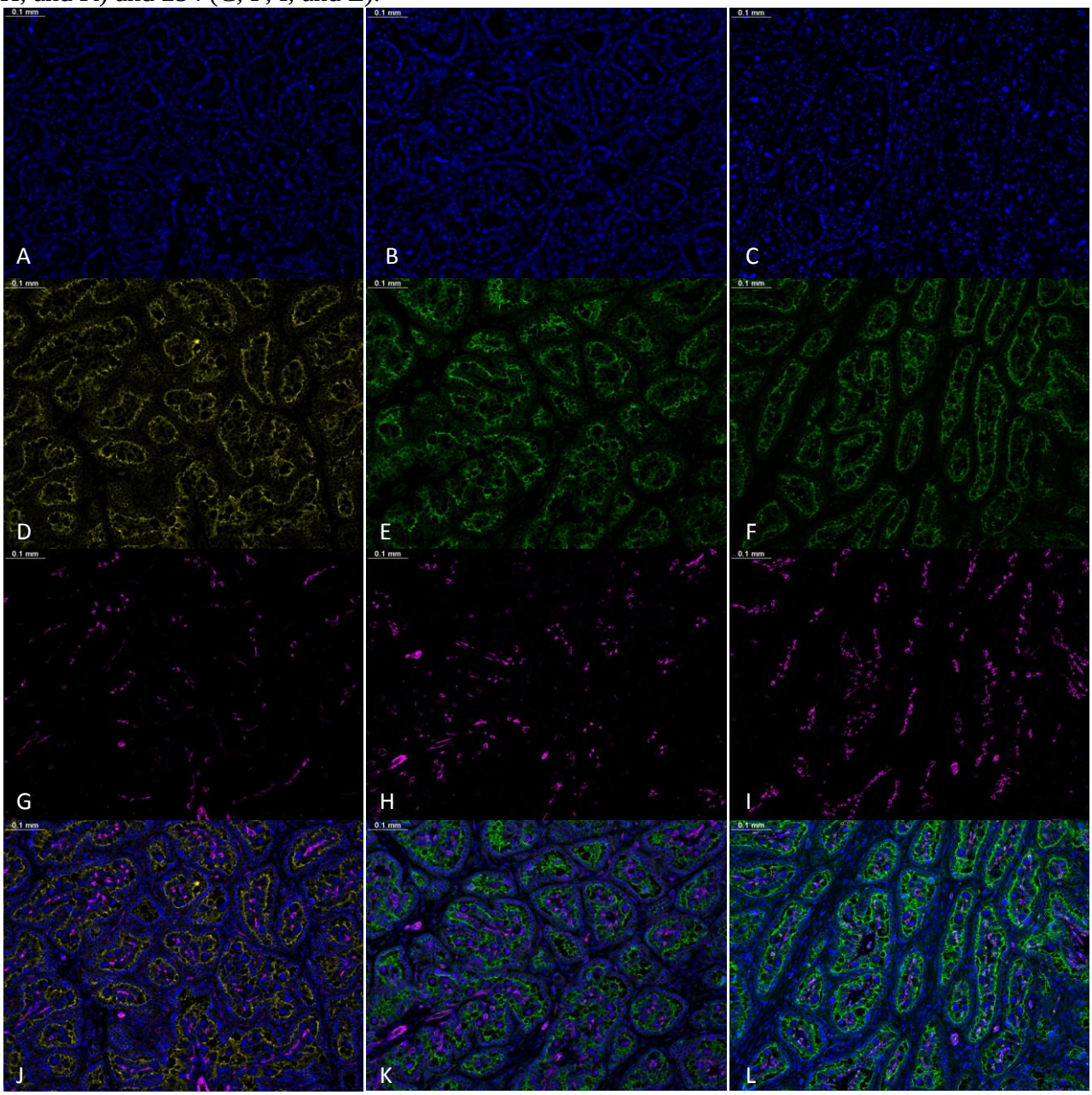
Tissue area and vascularity measurements were analyzed with image analysis software (Image Pro Premier 9.0, Media Cybernetics, Silver Spring, MD, USA). For each placentome, an image of the overlay of 325 nm, 555 nm, and 625 nm wavelengths was used, and fetal villi were outlined outside of the trophoblast including all cells deemed to be fetal by one technician. Tissue area was measured in the fetal villi, and this area was subtracted from the total area of the image to collect maternal tissue area. For vascularity measures, outlines of fetal villi versus maternal tissue were applied to PECAM-1 staining for each placentome, and the resulting values were used to calculate the capillary area density (CAD, total capillary area as a proportion of tissue area), capillary number density (CND, total number of capillaries per unit of tissue area), and capillary surface density (CSD, total capillary circumference per unit of tissue area) for both maternal (CAR) and fetal (COT) compartments (reviewed in Reynolds et al. 2005a; Vonnahme et al., 2007). To provide a measure of average capillary size, the average cross-sectional area per capillary (APC) for caruncular and cotyledonary tissue was calculated by dividing the CAD by the CND. Equations for microscopy measures are listed in Table 2.2.

Table 2.2. Equations for microscopy measures used in immunohistochemical analysis.

Microscopy variable[1]	Equation / definition*
Area	Area of tissue, mm^2
Capillary Area Density, CAD	Total capillary area, μm^2 / μm^2 of tissue area, %
Capillary Surface Density, CSD	Capillary surface area or perimeter, μm / μm^2 of tissue area
Capillary Number Density, CND	Number of capillaries / μm^2 of tissue area
Area per Capillary, APC	Area density / μm^2 of number density

[1] Variables formulated from measures obtained from microscopy images by Image Pro software.
* Equation used in calculation of vascularity variables used in statistical analysis.

Figure 2.1. Immunohistochemical staining of bovine placentomes for image analysis. Fluorescent stainging of 4', 6-diamidino-2-phenylindole (DAPI, A- C), Rhodamine labeled BS1 lectin (D- F), platelet endothelial cell adhesion molecule- 1 (PECAM-1, G- I) and an overlay of all stains (J- L) used in bovine placentome image analysis on day 85(A, D, G, and J), 140 (B, E, H, and K) and 254 (C, F, I, and L).



Statistics

All mRNA expression and microscopy measures were analyzed using the GLM procedure of SAS 9.3 (Cary, NC). For each collection day (i.e., d 85, 140 and 254), all variables were analyzed for the main effect of tissue (i.e. CAR vs. COT), maternal dietary treatment, and their interaction. There were no interactions in this analysis on any day; therefore, only the main effects of maternal dietary treatment were analyzed within day and within tissue. To determine how variables would change across time, only control cows were used (i.e., CON, CC, and CCC cows) in the analysis. Initial analysis was performed to include the interaction of day of gestation and tissue; however, there were no significant interactions, so only main effects of day of gestation and tissue are presented.

To examine how restriction duration (i.e., a 55 day reflected by the RES group, or a 110 day reflected by the RR group) impacted variables of vascularity and mRNA expression, analysis of variance was conducted using data from CON, RES, CC and RR cows, and main effects of maternal dietary treatment and day of gestation, as well as their interaction were determined. Analysis of variance using RR, RC, RRC and RCC cows were used to test whether duration of realimentation impacted variables of vascularity or mRNA expression, with main effects of maternal dietary treatment and day of gestation, as well as their interaction tested. All data are presented as LS Means ± SEM and were considered significant with when $P \leq 0.05$. Correlations of mRNA expression with microscopy measures were analyzed within day and treatment, as well as across day and across treatments, using the correlations procedure of SAS.

Results

Maternal dietary treatment impacts on vascularity measures and mRNA expression within day and tissue

There were no interactions of maternal dietary treatment and tissue (i.e., COT and CAR) within each day analyzed. Main effects of maternal dietary treatment (Tables 2.3 to 2.8) and main effects of tissue (Tables 2.9 to 2.11) are reported. While COT capillary size was smaller ($P = 0.04$) in RES vs. CON cows (Table 2.3), there was no impact of maternal dietary treatment in CAR tissue on d 85 (Table 2.4). On day 85 Flt-1 was not detectable in any COT or CAR samples. There was no impact of maternal dietary treatments on either COT or CAR tissues on d 140 of gestation (Table 2.5 and 2.6). While there were no impacts of maternal dietary treatment in COT tissue measures on d 254 (Table 2.7), CAR mRNA expression of PECAM-1 was reduced in RRC vs. CCC and RCC cows, which did not differ (Table 2.8).

Table 2.3. Vascularity and angiogenic factor mRNA expression in day 85 cotyledonary tissue.

	Dietary treatments[1]			
Variable	CON	RES	SEM	P-value
n	6	6		
Histologic measures				
Tissue area, mm^2	0.473	0.470	0.008	0.85
CAD[a], %	3.25	2.31	0.38	0.11
CSD[b], $\mu m / \mu m^2$	0.0135	0.0112	0.0008	0.08
CND[c], $\# / \mu m^2$	256.26	286.93	19.03	0.28
APC[d], μm^2	136.03	82.87	16.19	0.04
mRNA expression[e]				
VEGF	1.59	1.06	0.45	0.42
KDR	1.07	1.15	0.29	0.84
Flt-1	-[f]	-	-	-
eNOS	1.20	0.80	0.18	0.15
sGC	1.09	0.99	0.22	0.77
PECAM-1	1.16	1.04	0.26	0.74

[1] Cows received either control (100% NRC) diet (CON) or restricted (60% of CON) diet (RES) from d 30 to 85.

[a] Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area, %)

[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)

[c] Capillary number density (Number of capillaries $/ \mu m^2$ of tissue area)

[d] Area per capillary (Area density $/ \mu m^2$ of number density)

[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

[f] Not detectable

Table 2.4. Vascularity and angiogenic factor mRNA expression in day 85 caruncular tissue.

	Dietary treatments[1]			
Variable	CON	RES	SEM	P-value
n	6	6		
Histologic measures				
Tissue area, mm^2	0.136	0.138	0.008	0.85
CAD[a], %	3.45	3.17	0.70	0.78
CSD[b], $\mu m / \mu m^2$	0.021	0.021	0.004	0.98
CND[c], $\# / \mu m^2$	638.02	702.65	115.66	0.70
APC[d], μm^2	67.06	43.96	13.10	0.24
mRNA expression[e]				
VEGF	1.52	1.89	0.49	0.61
KDR	1.14	0.61	0.27	0.19
Flt-1	-[f]	-	-	-
eNOS	1.08	0.65	0.17	0.09
sGC	1.18	0.71	0.27	0.25
PECAM-1	1.08	0.63	0.22	0.17

[1] Cows received either control (100% NRC) diet (CON) or restricted (60% of CON) diet (RES) from d 30 to 85.
[a] Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area; %)
[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)
[c] Capillary number density (Number of capillaries $/ \mu m^2$ of tissue area)
[d] Area per capillary (Area density $/ \mu m^2$ of number density)
[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.
[f] Not detectable

Table 2.5. Vascularity and angiogenic factor mRNA expression in day 140 cotyledonary tissue.

	Dietary treatments[1]				
Variable	CC	RC	RR	SEM	P-value
n	6	5	6		
Histologic measures					
Tissue area, mm^2	0.500	0.498	0.477	0.011	0.25
CAD[a], %	2.62	2.93	2.59	0.25	0.57
CSD[b], $\mu m / \mu m^2$	0.014	0.015	0.013	0.001	0.42
CND[c], # / μm^2	385.15	400.44	359.04	33.49	0.66
APC[d], μm^2	70.17	73.85	72.08	4.78	0.85
mRNA expression[e],					
VEGF	1.64	0.84	1.30	0.50	0.51
KDR	2.63	0.79	1.07	0.90	0.34
Flt-1	3.92	1.44	0.98	1.85	0.46
eNOS	2.05	0.98	1.27	0.42	0.16
sGC	2.47	0.79	1.06	1.00	0.42
PECAM-1	2.67	0.49	1.01	1.10	0.33

[1] Cows received the control diet (100% NRC) from d 30 until 140 (CC), restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 140 (RC), and restricted from d 30 to 140 (60% NRC; RR).
[a] Capillary area density (Total capillary area, μm^2 / μm^2 of tissue area; %)
[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)
[c] Capillary number density (Number of capillaries / μm^2 of tissue area)
[d] Area per capillary (Area density / μm^2 of number density)
[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

Table 2.6. Vascularity and angiogenic factor mRNA expression in day 140 caruncular tissue.

	Dietary treatments[1]				
Variable	CC	RC	RR	SEM	P-value
n	6	5	6		
Histologic measures					
Tissue area, mm^2	0.11	0.11	0.13	0.01	0.21
CAD[a], %	4.14	5.36	3.56	0.82	0.30
CSD[b], $\mu m / \mu m^2$	0.026	0.032	0.021	0.005	0.31
CND[c], $\# / \mu m^2$	874.14	1085.14	667.51	173.33	0.24
APC[d], μm^2	59.37	49.50	69.61	10.46	0.45
mRNA expression[e]					
VEGF	0.74	0.59	1.79	0.57	0.25
KDR	0.66	0.69	1.72	0.42	0.18
Flt-1	2.20	2.75	2.62	0.89	0.89
eNOS	1.41	0.54	3.37	1.66	0.45
sGC	0.23	0.19	0.54	0.19	0.34
PECAM-1	0.23	0.19	0.61	0.16	0.13

[1] Cows received the control diet (100% NRC) from d 30 until 140 (CC), restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 140 (RC), and restricted from d 30 to 140 (60% NRC; RR).

[a] Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area; %)

[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)

[c] Capillary number density (Number of capillaries $/ \mu m^2$ of tissue area)

[d] Area per capillary (Area density / μm^2 of number density)

[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

Table 2.7. Vascularity and angiogenic factor mRNA expression in day 254 cotyledonary tissue.

	Dietary treatments[1]				
Variable	CCC	RCC	RRC	SEM	P-value
n	6	4	6		
Histologic measures					
Tissue area, mm^2	0.505	0.506	0.492	0.011	0.52
CAD[a], %	4.27	5.88	4.73	0.50	0.08
CSD[b], $\mu m / \mu m^2$	0.026	0.032	0.028	0.002	0.20
CND[c], $\# / \mu m^2$	913.32	1113.57	976.36	118.86	0.45
APC[d], μm^2	47.57	54.62	49.17	5.38	0.60
mRNA expression[e]					
VEGF	1.40	1.96	1.32	0.50	0.66
KDR	0.88	2.22	1.27	0.48	0.16
Flt-1	0.77	1.69	0.98	0.40	0.27
eNOS	1.64	1.52	0.90	0.62	0.63
sGC	1.48	1.79	1.28	0.57	0.81
PECAM-1	1.32	1.74	1.08	0.48	0.60

[1] Cows received the control diet (100% NRC) from d 30 until 254 (CCC), restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 254 (RCC), and restricted from d 30 to 140 (60% NRC) and then realimented to 100% of the NRC requirements until d 254 (RRC).

[a] Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area; %)

[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)

[c] Capillary number density (Number of capillaries $/ \mu m^2$ of tissue area)

[d] Area per capillary (Area density / μm^2 of number density)

[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

Table 2.8. Vascularity and angiogenic factor mRNA expression in day 254 caruncular tissue.

	Dietary treatments[1]				
Variable	CCC	RCC	RRC	SEM	P-value
n	6	4	6		
Histologic measures					
Tissue area, mm^2	0.104	0.104	0.117	0.011	0.52
CAD[a], %	5.39	5.63	6.24	0.98	0.75
CSD[b], $\mu m / \mu m^2$	0.036	0.034	0.041	0.005	0.55
CND[c], $\# / \mu m^2$	1690.95	1425.56	1822.86	196.37	0.32
APC[d], μm^2	33.66	37.51	34.42	4.26	0.77
mRNA expression[e]					
VEGF	1.25	1.18	0.53	0.30	0.20
KDR	1.58	1.11	0.69	0.37	0.21
Flt-1	1.32	1.40	0.86	0.19	0.09
eNOS	1.40	1.01	0.72	0.20	0.06
sGC	0.92	1.20	0.64	0.16	0.07
PECAM-1	1.06^{A}	1.20^{A}	0.61^{B}	0.14	0.02

[1] Cows received the control diet (100% NRC) from d 30 until 254 (CCC), restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 254 (RCC), and restricted from d 30 to 140 (60% NRC) and then realimented to 100% of the NRC requirements until d 254 (RRC).
[a]Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area; %)
[b]Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)
[c] Capillary number density (Number of capillaries / μm^2 of tissue area)
[d] Area per capillary (Area density / μm^2 of number density)
[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA
[AB]LSMeans ± SEM with different superscripts within a row differ; $P < 0.05$

On d 85, CAR tissue area and capillary size were smaller than COT (Table 2.9). However, CSD and CND were larger in CAR vs. COT whereas CAD was not different between tissue (Table 2.9). There were no effect of tissues on mRNA expression; however, it should be noted that mRNA expression of Flt-1 was below our detectable limits. On d 140, CAR tissue area and capillary size were smaller than COT, and CAD, CND, and CSD were all greater in CAR vs. COT (Table 2.10). Moreover, sGC mRNA expression was increased in CAR vs. COT tissue on d 140. By d 254, CAR tissue area remained smaller than COT tissue area. Area per capillary was also reduced in CAR vs. COT (Table 2.11). Capillary surface density and number

density as well as CAD were greater in CAR vs. COT. There were no differences between CAR and COT mRNA expression of the angiogenic factors that were measured (Table 2.11).

Table 2.9. Vascularity and angiogenic factor mRNA expression for cotyledon and caruncle tissue comparison at day 85.

	Tissue[1]			
Variable	COT	CAR	SEM	P-value
n	6	6		
Histologic measures				
Tissue area, mm^2	0.472	0.137	0.005	<0.01
CAD[a], %	2.78	3.31	0.40	0.51
CSD[b], µm / $µm^2$	0.012	0.021	0.002	0.06
CND[c], # / $µm^2$	271.60	670.34	58.61	<0.01
APC[d], $µm^2$	109.45	55.51	10.41	<0.01
mRNA expression[e]				
VEGF	1.33	1.71	0.33	0.42
KDR	1.11	0.87	0.20	0.41
Flt-1	-[f]	-	-	-
eNOS	1.00	0.89	0.12	0.51
sGC	1.04	0.94	0.18	0.71
PECAM-1	1.10	0.86	0.17	0.32

[1] COT (cotyledon) and CAR (caruncle) tissues from cows which received either control (100% NRC) diet (CON) or restricted (60% of CON) diet (RES) from d 30 to 85.
[a] Capillary area density (Total capillary area, $µm^2$ / $µm^2$ of tissue area; %)
[b] Capillary surface density (Capillary surface area or perimeter, µm / $µm^2$ of tissue area)
[c] Capillary number density (Number of capillaries / $µm^2$ of tissue area)
[d] Area per capillary (Area density / $µm^2$ of number density)
[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.
[f] Not detectable.

Table 2.10. Vascularity and angiogenic factor mRNA expression for cotyledon and caruncle tissue comparison at day 140.

	Tissue[1]			
Variable	COT	CAR	SEM	P-value
n	17	17		
Histologic measures				
Tissue area, mm^2	0.492	0.117	0.006	< 0.01
CAD[a], μm^2 / μm^2	2.71	4.37	0.34	0.02
CSD[b], μm / μm^2	0.014	0.026	0.002	< 0.01
CND[c], # / μm^2	381.54	882.40	68.00	< 0.01
APC[d], μm^2	72.02	58.40	4.80	0.26
mRNA expression[e]				
VEGF	1.04	1.26	0.29	0.60
KDR	1.02	1.50	0.03	0.43
Flt-1	2.52	2.07	0.65	0.63
eNOS	1.66	1.43	0.64	0.80
sGC	0.32	1.44	0.39	0.05
PECAM-1	0.34	1.39	0.43	0.10

[1] COT (cotyledon) and CAR (caruncle) tissues from cows which received the control diet (100% NRC) from d 30 until 140 (CC), restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 140 (RC), and restricted from d 30 to 140 (60% NRC; RR).

[a] Capillary area density (Total capillary area, μm^2 / μm^2 of tissue area; %)

[b] Capillary surface density (Capillary surface area or perimeter, μm / μm^2 of tissue area)

[c] Capillary number density (Number of capillaries / μm^2 of tissue area)

[d] Area per capillary (Area density / μm^2 of number density)

[e] mRNA expression as $\Delta\Delta$ Ct value normalized to 18S rRNA.

Table 2.11. Vascularity and angiogenic factor mRNA expression for cotyledon and caruncle tissue comparison at day 254.

	Tissue[1]			
Variable	COT	CAR	SEM	P-value
n	16	16		
Histologic measures				
Tissue area, mm^2	0.501	0.108	0.005	<0.01
CAD[a], %	4.96	5.76	0.40	0.30
CSD[b], $\mu m / \mu m^2$	0.028	0.037	0.002	0.04
CND[c], $\# / \mu m^2$	1001.08	1646.46	82.64	<0.01
APC[d], μm^2	50.45	35.20	2.47	<0.01
mRNA expression[e]				
VEGF	0.99	1.56	0.25	0.11
KDR	1.13	1.75	0.32	0.18
Flt-1	1.31	1.15	0.20	0.55
eNOS	1.04	1.08	0.61	0.88
sGC	1.09	1.30	0.22	0.50
PECAM-1	1.09	1.38	0.21	0.35

[1] COT (cotyledon) and CAR (caruncle) tissues from cows which received the control diet (100% NRC) from d 30 until 254 (CCC), restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 254 (RCC), and restricted from d 30 to 140 (60% NRC) and then realimented to 100% of the NRC requirements until d 254 (RRC).
[a] Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area; %)
[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)
[c] Capillary number density (Number of capillaries $/ \mu m^2$ of tissue area)
[d] Area per capillary (Area density $/ \mu m^2$ of number density)
[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

Impacts of gestational day on vascularity and mRNA expression

To assess how advancing gestation impacted vascularity and mRNA expression of angiogenic factors, data from control-fed animals (i.e., CON, CC, and CCC) were analyzed over time. While there was no impact of gestational day on mRNA expression of any angiogenic factor in either COT or CAR (Tables 2.12 and 2.13), there were alterations in capillary vascularity. There was an interaction of gestational day and tissue for tissue area and area per capillary. For tissue area (Figure 2.1), COT tissue area was greater than CAR tissue area at each gestational day measured. Moreover, as COT tissue area increased from d 85 to 140, and remained similar at d 254, CAR tissue area decreased from d 85 to 140 and remained similar at

254 (Figure 2.1). As gestation advanced, COT capillary size decreased from day 85 to 140 and remained similar on day 254. There was a decrease in CAR capillary size between day 85 and 254 with day 140 being intermediate. Moreover, COT capillary size on d 85 was greater than any other day or tissue bed (Figure 2.2). There were main effects of gestational day of control cows within the COT and CAR tissues. In the COT, CAD and CSD were similar between d 85 and 140, but both increased by d 254 of gestation. As gestation advanced, CND in the COT increased at each day measured (Table 2.12). In the CAR, there was no effect of gestational day on CAD and CSD. Similar to COT, within CAR tissue, CND increased from day 140 to 254 (Table 2.13). Expression of mRNAs was not altered within control CAR or COT tissue throughout gestation.

Figure 2.2. Tissue area comparison of cotyledonary and caruncular tissue in cows fed control diets of 100% NRC requirements from day 30 to 85, 140, or 254.

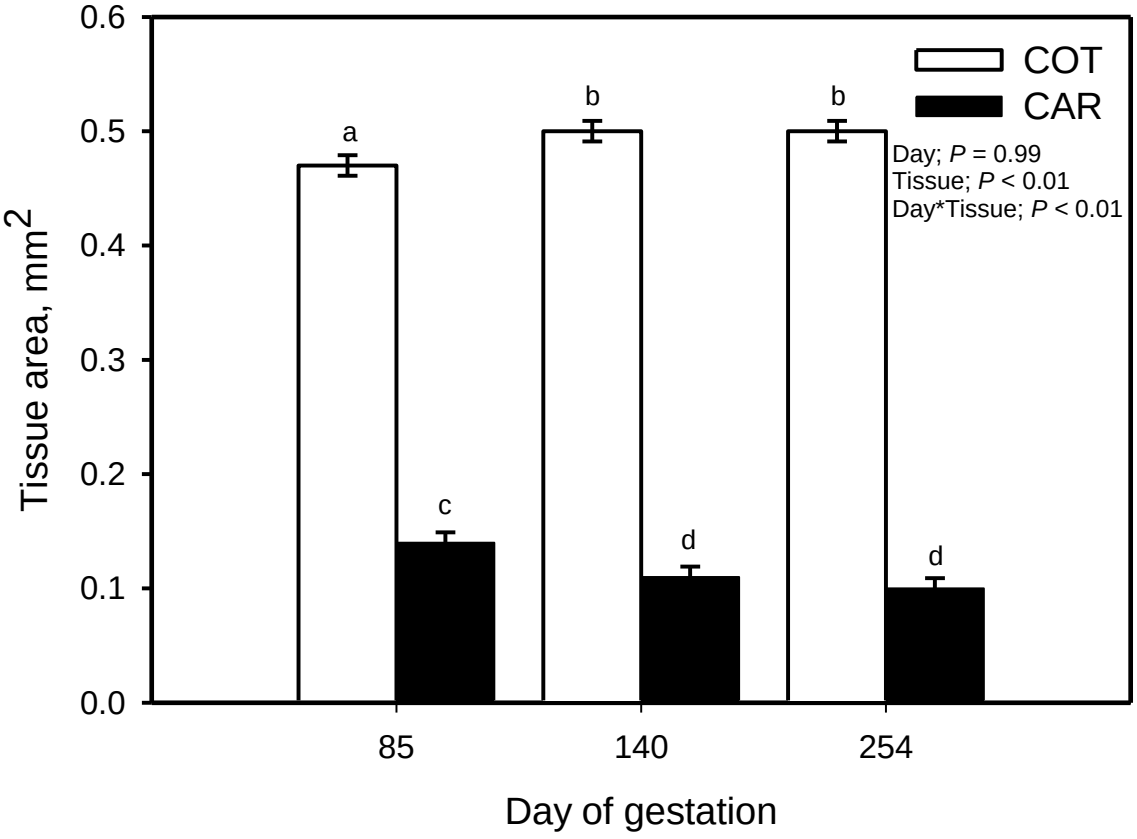


Figure 2.3. Capillary size comparison of cotyledonary and caruncular tissue in cows fed control diets of 100% NRC requirements from day 30 to 85, 140 or 254.

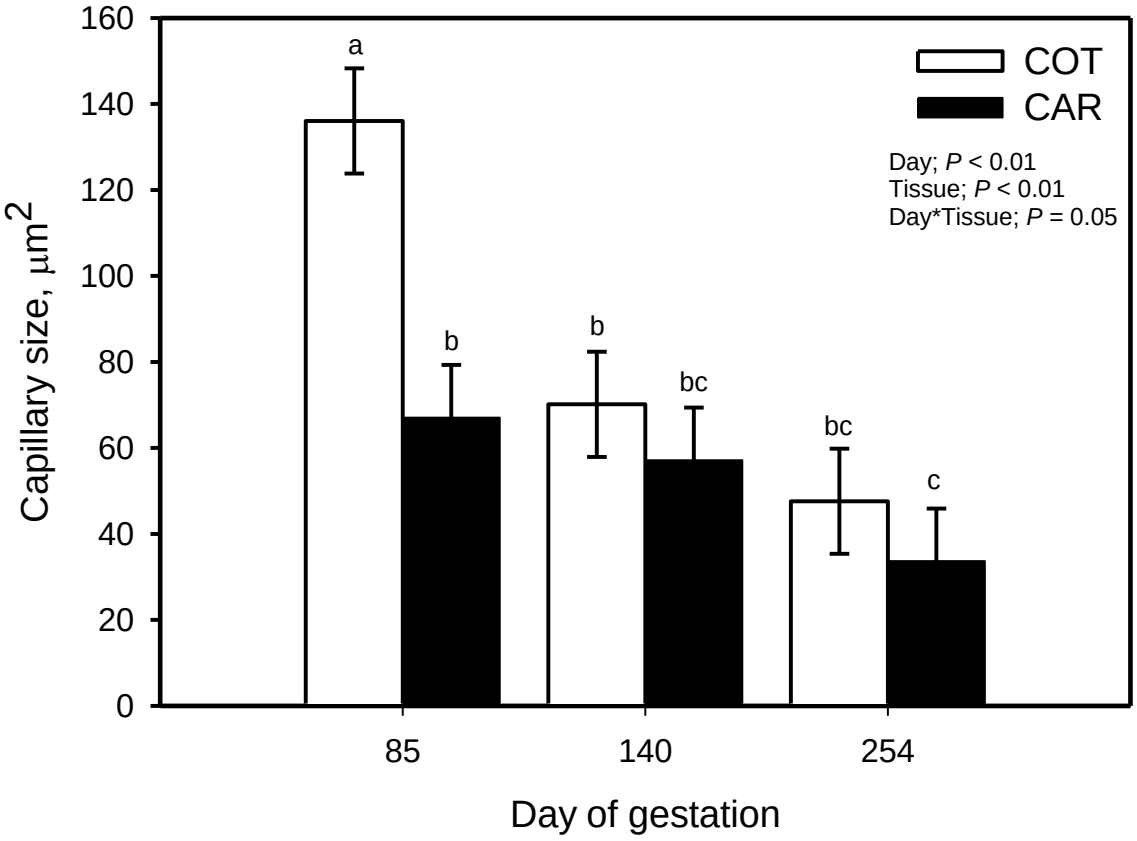


Table 2.12. Vascularity and angiogenic factor mRNA expression for cotyledonary tissue in cows fed control diets at day 85, 140 and 254.

	Dietary treatments[1]				
Variable	CON	CC	CCC	SEM	P-value
n	6	6	6		
Histologic measures					
Tissue area‡, mm^2					
CAD[a], %	3.25^{A}	2.62^{A}	4.27^{B}	0.31	0. 01
CSD[b], μm / μm^2	0.013^{A}	0.014^{A}	0.026^{B}	0.001	< 0.01
CND[c], # / μm^2	256.26^{A}	385.15^{B}	913.32^{C}	36.88	< 0.01
APC[d‡], μm^2					
mRNA expression[e]					
VEGF	1.59	1.30	1.40	0.45	0.90
KDR	1.07	1.07	1.77	0.53	0.57
Flt-1	-[f]	1.30	0.77	0.25	0.16
eNOS	1.20	1.27	1.64	0.51	0.81
sGC	1.09	1.06	1.48	0.40	0.72
PECAM-1	1.16	1.01	1.32	0.36	0.83

[1] Cows received control (100% NRC) diet from d 30 to 85 (CON; n = 6), d 30 to 140 (CC; n = 6), or d 30 to 254 (CCC; n = 6).

‡ Interactive means and P-values presented in Figures 2.1 and 2.2.

[a] Capillary area density (Total capillary area, μm^2 / μm^2 of tissue area; %)

[b] Capillary surface density (Capillary surface area or perimeter, μm / μm^2 of tissue area)

[c] Capillary number density (Number of capillaries / μm^2 of tissue area)

[d] Area per capillary (Area density / μm^2 of number density)

[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

[f] Not detectable.

[ABC]LSMeans ± SEM with different superscripts within a row differ; P < 0.05

Table 2.13. Vascularity and angiogenic factor mRNA expression for caruncular tissue in cows fed control diets at day 85, 140 and 254.

	Dietary treatments[1]				
Variable	CON	CC	CCC	SEM	P-value
n	6	6	6		
Histologic measures					
Tissue area‡, mm^2					
CAD[a], %	3.45	4.29	5.39	0.68	0.15
CSD[b], $\mu m / \mu m^2$	0.021	0.026	0.036	0.004	0.07
CND[c], # / μm^2	638.02^{A}	897.41^{A}	1690.95^{B}	147.78	<0.01
APC[d‡], μm^2					
mRNA expression[e]					
VEGF	1.52	1.79	1.25	0.57	0.80
KDR	1.14	1.72	1.59	0.52	0.71
Flt-1	-[f]	2.62	1.68	0.64	0.32
eNOS	1.13	0.94	1.40	1.42	0.44
sGC	1.18	0.54	1.43	0.39	0.28
PECAM-1	1.08	0.61	1.45	0.31	0.20

[1] Cows received control (100% NRC) diet from d 30 to 85 (CON; n = 6), d 30 to 140 (CC; n = 6), or d 30 to 254 (CCC; n = 6)
‡ Interactive means and P-values presented in Figures 2.1 and 2.2.
[a] Capillary area density (Total capillary area, μm^2 / μm^2 of tissue area; %)
[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)
[c] Capillary number density (Number of capillaries / μm^2 of tissue area)
[d] Area per capillary (Area density / μm^2 of number density)
[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.
[f] Not detectable.
[AB]LSMeans ± SEM with different superscripts within a row differ; $P < 0.05$

Impacts of restriction duration on vascularity and mRNA expression

There was an interaction of maternal dietary treatment and gestational day for COT capillary size, where capillaries from CON cows on d 85 had larger capillaries than any other group (Figure 2.3). There was a main effect of gestational day where COT CND was greater on d 140 compared with d 85 (Table. 2.14). Moreover, while length of dietary restriction did not impact angiogenic factor mRNA expression, there was a main effect of gestational day with eNOS mRNA expression being higher on d 140 compared with d 85 (Table 2.14).

There were no interactions in CAR tissue. Moreover, there were no impacts of gestational day or maternal dietary treatment on any vascularity measurement. There was, however, an

effect of maternal dietary treatment on PECAM-1 and KDR mRNA expression with CON cows having increased expression of both genes compared with RES cows. Gestational day impacted PECAM-1 mRNA expression decreasing from day 85 to 140 (Table 2.15).

Figure 2.4. Capillary size comparison of caruncular tissue in cows fed control diets (from day 30 to 85 or 140) or nutrient restricted diets (from day 30 to 85 or 140).

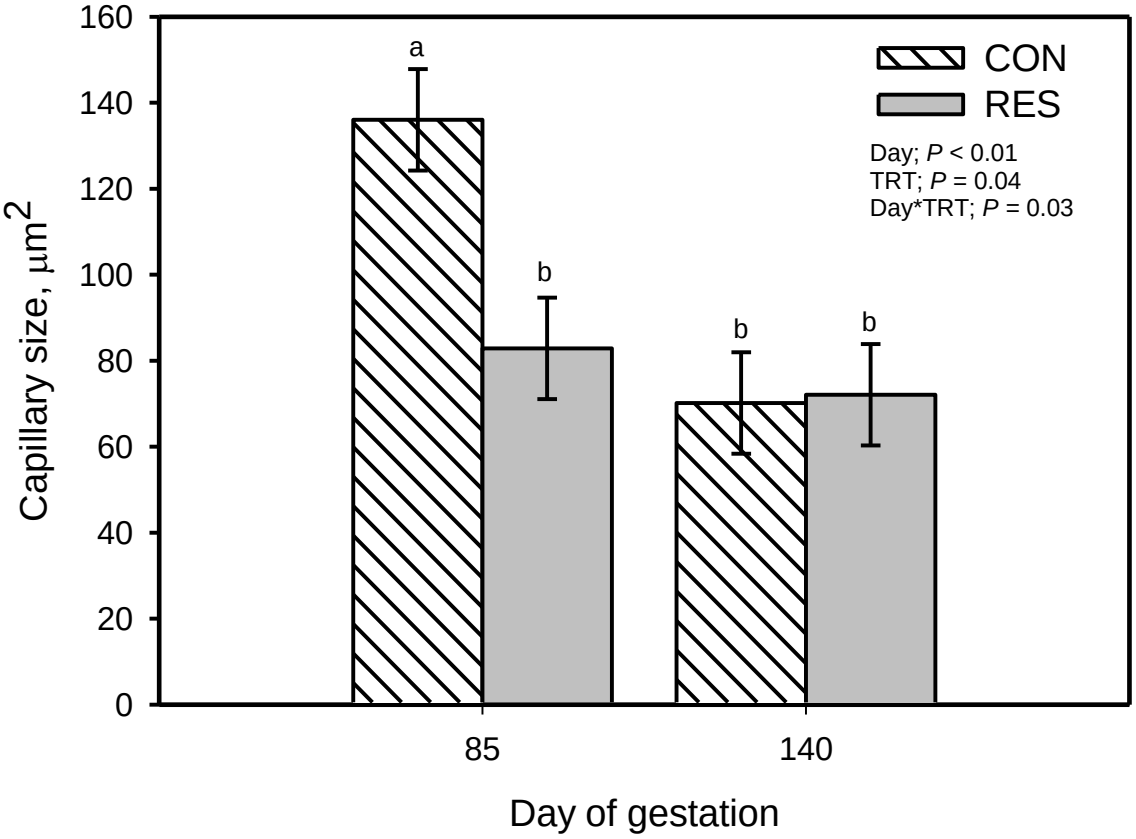


Table 2.14. Vascularity and angiogenic factor mRNA expression for comparison of cotyledonary tissue in cows fed control diets (from day 30 to 85 or 140) or nutrient restricted diets (from day 30 to 85 or 140).

Variable	Treatment[1]			Day			P-values		
	CON	RES	SEM	85	140	SEM	Trt	Day	Trt x Day
n	12	12		12	12				
Histologic measures									
Tissue area, mm^2	0.487	0.474	0.007	0.472	0.489	0.007	0.21	0.11	0.30
CAD[a], %	2.93	2.45	0.22	2.78	2.60	0.22	0.13	0.59	0.16
CSD[b], µm / $µm^2$	0.0137	0.0123	0.0006	0.0124	0.0136	0.0006	0.12	0.16	0.33
CND[c], # / $µm^2$	320.71	322.99	18.75	271.60	372.10	18.75	0.93	< 0.01	0.30
APC[d§], $µm^2$									
mRNA expression[e]									
VEGF	1.44	1.35	0.34	1.33	1.47	0.34	0.85	0.77	0.37
KDR	1.07	1.89	0.55	1.11	1.85	0.55	0.30	0.35	0.35
Flt-1[€]									
eNOS	1.24	1.43	0.23	1.00	1.66	0.23	0.56	0.05	0.08
sGC	1.08	1.73	0.55	1.04	1.77	0.55	0.41	0.36	0.34
PECAM-1	0.89	0.71	0.14	0.86	0.75	0.14	0.37	0.60	0.20

[1] Cows received control (100% NRC) diets from d 30 to 85 (CON; n = 6), and d 30 to 140 (CC; n = 6), or nutrient restriction (60% of control) diets from d 30 to 85 (RES; n = 6), and d 30 to 140 (RR; n = 6)

[a] Capillary area density (Total capillary area, $µm^2$ / $µm^2$ of tissue area; %)

[b] Capillary surface density (Capillary surface area or perimeter, µm / $µm^2$ of tissue area)

[c] Capillary number density (Number of capillaries / $µm^2$ of tissue area)

[d] Area per capillary (Area density / $µm^2$ of number density)

[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

[§] Data and interactions for APC located in Figure 2.3.

[€] No analysis due to no detection of Flt-1 on d 85.

Table 2.15. Vascularity and angiogenic factor mRNA expression for comparison of caruncular tissue in cows fed control diets (from day 30 to 85 or 140) or nutrient restricted diets (from day 30 to 85 or 140).

Variable	Treatment[1]			Day			P-values		
	CON	RES	SEM	85	140	SEM	Trt	Day	Trt x Day
n	12	12		12	12				
Histologic measures									
Tissue area, mm^2	0.122	0.135	0.007	0.137	0.120	0.007	0.20	0.11	0.28
CAD[a], %	3.87	3.36	0.49	3.31	3.92	0.49	0.48	0.38	0.75
CSD[b], $\mu m / \mu m^2$	0.024	0.021	0.003	0.021	0.023	0.003	0.53	0.57	0.51
CND[c], $\# / \mu m^2$	767.72	685.08	93.20	670.34	782.46	93.20	0.54	0.41	0.28
APC[d], μm^2	62.09	56.79	8.96	55.51	63.36	8.96	0.68	0.54	0.18
mRNA expression[e]									
VEGF	1.66	1.31	0.39	1.71	1.26	0.39	0.54	0.43	0.21
KDR	1.43	0.63	0.27	0.87	1.19	0.27	0.05	0.43	0.50
Flt-1[€]									
eNOS	2.25	0.86	0.88	0.89	2.22	0.88	0.28	0.30	0.47
sGC	1.92	0.88	0.55	1.35	1.46	0.55	0.19	0.89	0.37
PECAM-1	0.85	0.43	0.14	0.86	0.42	0.14	0.05	0.04	0.87

[1] Cows received control (100% NRC) diets from d 30 to 85 (CON; n = 6), and d 30 to 140 (CC; n = 6), or nutrient restriction (60% of control) diets from d 30 to 85 (RES; n = 6), and d 30 to 140 (RR; n = 6)
[a] Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area; %)
[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)
[c] Capillary number density (Number of capillaries $/ \mu m^2$ of tissue area)
[d] Area per capillary (Area density / μm^2 of number density)
[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.
[€] No analysis due to no detection of Flt-1 on d 85.

Effects of duration of realimentation on measures of vascularity and mRNA expression

In COT tissue, there were no interactions of treatment by day on any measures; therefore, only main effects will be discussed (Table 2.16). Duration of realimentation did not impact cotyledonary tissue area; however, there was an effect of day ($P < 0.01$) for capillary area, number, and surface densities which all increased from d 140 to 254. Capillary size decreased from d 140 to 254. No measures of mRNA expression were impacted by duration of realimentation.

Table 2.16. Vascularity and mRNA expression measures for realimentation duration (days 85 to 140 or 140 to 254) comparison within cotyledonary tissue.

	Trt			Day			P-values		
Variable	RC	RR	SEM	140	254	SEM	Trt	Day	Trt x Day
Histologic measures									
Tissue area, mm^2	0.502	0.484	0.008	0.487	0.499	0.008	0.14	0.32	0.74
CAD[a], %	4.41	3.66	0.32	2.76	5.30	0.30	0.09	<0.01	0.34
CSD[b], $\mu m / \mu m^2$	0.023	0.021	0.002	0.014	0.030	0.001	0.16	<0.01	0.57
CND[c], $\# / \mu m^2$	757.0	667.7	67.9	379.9	1045.0	65.3	0.33	<0.01	0.60
APC[d], μm^2	64.23	60.62	3.41	79.96	51.89	3.08	0.43	<0.01	0.69
mRNA expression[e]									
VEGF	1.40	1.48	0.40	1.24	1.64	0.39	0.89	0.47	0.20
KDR	1.51	1.95	0.67	1.71	1.74	0.64	0.64	0.97	0.14
Flt-1	1.56	2.23	0.80	2.46	1.33	0.80	0.55	0.32	0.23
eNOS	1.25	1.47	0.31	1.51	1.21	0.29	0.60	0.47	0.06
sGC	1.29	1.87	0.67	1.63	1.53	0.64	0.52	0.83	0.24
PECAM-1	0.74	1.58	0.71	0.67	1.65	0.68	0.40	0.32	0.56

[1] RC includes cows restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 140 or 254. RR includes cows restricted from d 30 to 140 (60% NRC) and cows which were restricted from d 30 to 140 thereafter receiving realimentation (100% NRC) until day 254.

[3] Day 140 includes all treatment cows which were restricted from d 30 to 85 thereafter receiving either 60 or 100% NRC. Day 254 includes all cows realimented to 100% NRC from day 140 until day 254 having previously been fed either restricted (60% NRC) or control (100% NRC) diets.

[a] Capillary area density (Total capillary area, $\mu m^2 / \mu m^2$ of tissue area; %)

[b] Capillary surface density (Capillary surface area or perimeter, $\mu m / \mu m^2$ of tissue area)

[c] Capillary number density (Number of capillaries $/ \mu m^2$ of tissue area)

[d] Area per capillary (Area density / μm^2 of number density)

[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

In the CAR, there was an interaction between gestation day and maternal dietary treatment on CND (Figure 2.4). Within a day, there was no effect of maternal dietary treatment on CND. While there was no change in CND in cows experiencing a longer realimentation, there was an increase in CND from d 140 to 254 in cows receiving a shorter realimentation. Moreover, there was an interaction of gestational day and maternal dietary treatment on sGC mRNA expression (Figure 2.5). The expression of sGC increased from d 140 to 254 in both maternal dietary treatments, but there was more sGC mRNA expression in those cows experiencing a

longer realimentation period compared with the shorter realimentation period. As gestation advanced, capillary size decreased while a greater than 5-fold increase in Flt-1 mRNA expression was observed (Table 2.17).

Figure 2.5. Capillary number density comparison of caruncular tissue in cows with different durations of realimentation following nutrient restriction from days 85 to 140 or 140 to 254.

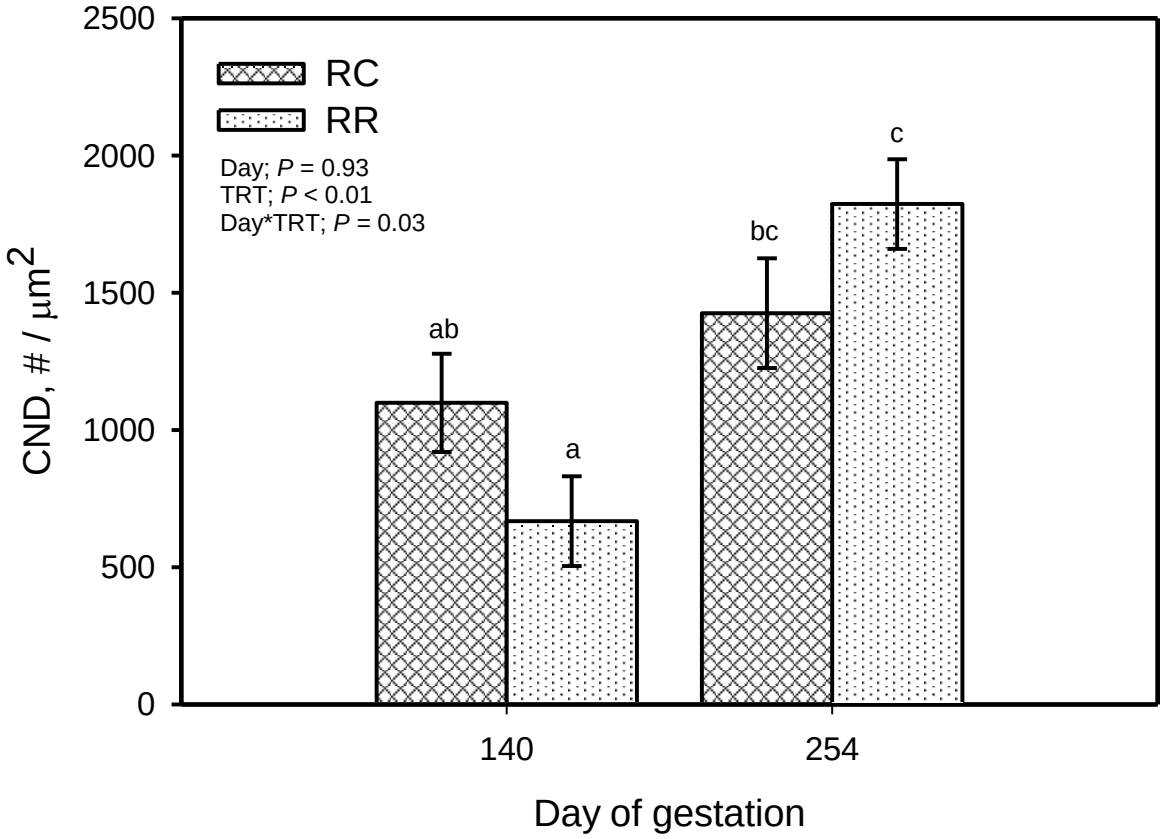


Figure 2.6. Comparison of sGC mRNA expression within caruncular tissue of cows with different durations of realimentation following nutrient restriction from days 85 to 140 or 140 to 254.

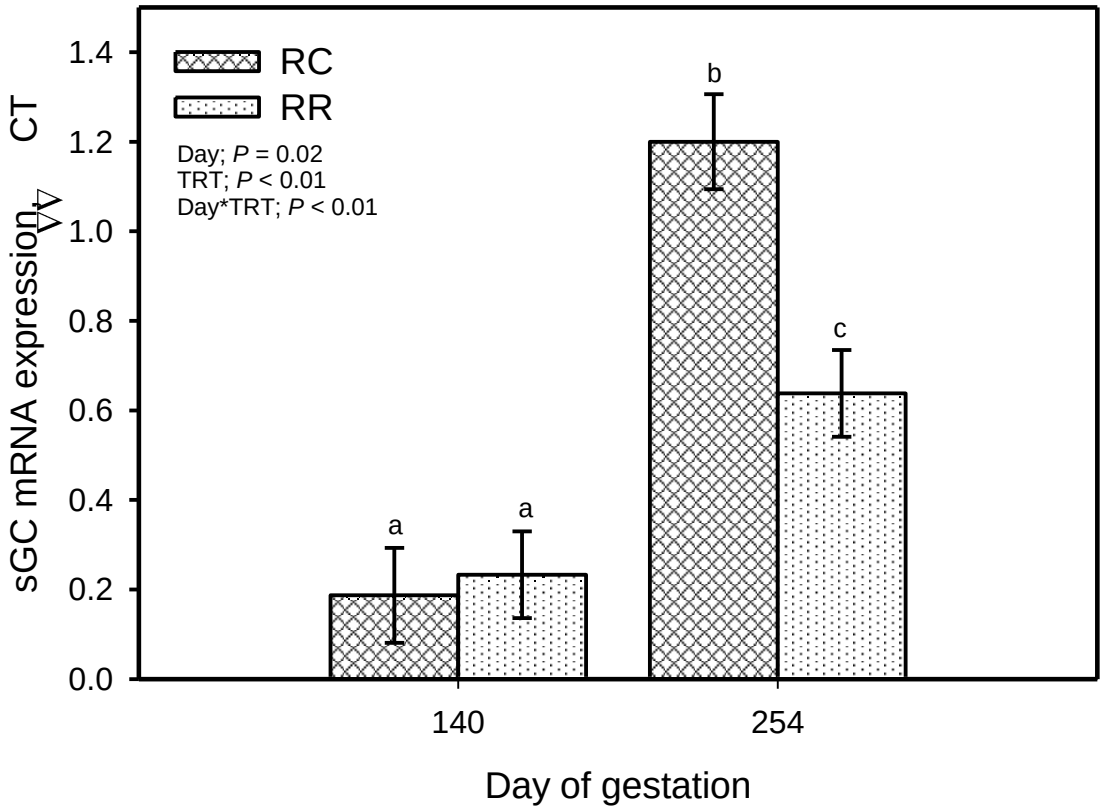


Table 2.17. Vascularity and mRNA expression measures for realimentation duration (days 85 to 140 or 140 to 254) comparison within caruncular tissue.

	Treatment[1]			Day[3]			P-values		
Variable	RC	RR	SEM	140	254	SEM	Trt	Day	Trt x Day
Histologic measures									
Tissue area, mm^2	0.104	0.125	0.009	0.118	0.110	0.008	0.08	0.49	0.52
CAD[a], μm^2 / μm^2	5.51	4.90	0.72	4.46	5.94	0.70	0.53	0.14	0.22
CSD[b], μm / μm^2	0.033	0.031	0.004	0.027	0.037	0.004	0.65	0.06	0.10
CND[c], # / μm^2	[§]								
APC[d§], μm^2	43.34	52.01	6.64	59.39	35.96	6.39	0.34	0.02	0.20
mRNA expression[e]									
VEGF	0.89	0.98	0.33	0.66	1.21	0.31	0.83	0.23	0.92
KDR	0.90	0.67	0.15	0.67	0.90	0.14	0.28	0.29	0.36
Flt-1	2.07	1.53	0.42	0.40	2.47	1.13	0.42	0.04	0.99
eNOS	0.78	0.90	0.19	0.81	0.87	0.18	0.63	0.83	0.13
sGC[§]									
PECAM-1	1.63	1.09	0.28	1.31	1.41	0.27	0.18	0.80	0.75

[1] RC includes cows restricted from d 30 to 85 (60% NRC) and then realimented to 100% of the NRC requirements until d 140 or 254. RR includes cows restricted from d 30 to 140 (60% NRC) and cows which were restricted from d 30 to 140 thereafter receiving realimentation (100% NRC) until day 254.

[3] Day 140 includes all treatment cows which were restricted from d 30 to 85 thereafter receiving either 60 or 100% NRC. Day 254 includes all cows realimented to 100% NRC from day 140 until day 254 having previously been fed either restricted (60% NRC) or control (100% NRC) diets.

[a] Capillary area density (Total capillary area, μm^2 / μm^2 of tissue area; %)

[b] Capillary surface density (Capillary surface area or perimeter, μm / μm^2 of tissue area)

[c] Capillary number density (Number of capillaries / μm^2 of tissue area)

[d] Area per capillary (Area density / μm^2 of number density)

[e] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.

[§] Interactive means and P-values located within Figures 2.4 and 2.5.

Correlations of mRNA expression with microscopy measures

Of the correlations that were analyzed amongst all mRNA expression genes with all microscopy measures, the only ones correlated were CAR PECAM-1 expression with the CAR microscopy measures CAD, CSD, and CND (see Table 2.18). Within CAR tissue of CON cows, there was a negative correlation ($P = 0.01$) for PECAM-1 with CAD, CSD, and CND. In RC and RCC cows, there were strong positive correlations ($P \leq 0.04$) for PECAM-1 expression and CAD

and CSD. There was no correlation ($P \geq 0.07$) of PECAM-1 and CND. At day 85, all cows had a moderately strong correlation ($P \leq 0.05$) of PECAM-1 and CAD, CSD, and CND.

Table 2.18. Correlation of CAR PECAM-1 mRNA expression with CAR vascularity measures in CON, RC, RCC, and combined CON and RES treatment cows.

PECAM-1[1]	CAD[a], μm^2 / μm^2	CSD[b], μm / μm^2	CND[c], # / μm^2
CON[3]	-0.90[d]	-0.91	-0.90
	0.01[e]	**0.01**	**0.01**
RC	0.90	0.89	0.78
	0.03	**0.04**	0.12
RCC	0.96	0.99	0.93
	0.04	**0.01**	0.07
Day 85[4]	-0.57	-0.61	-0.60
	0.05	**0.04**	**0.04**

[1] mRNA expression as ΔΔ Ct value normalized to 18S rRNA.
[3] Cows received the diets of 100% NRC from d 30 to 85 (CON; n = 6), 60% NRC from d 30 to 85 followed by realimentation to 100% NRC until d 140 (RC; n = 5), and 60% NRC from d 30 to 85 followed by realimentation to 100% NRC until d 254 (RCC; n = 4).
[4] All cows on treatments CON (100% NRC from d 30 to 85) and RES (60% of CON diet from d 30 to 85).
[a] Capillary area density (Total capillary area, μm^2 / μm^2 of tissue area; %)
[b] Capillary surface density (Capillary surface area or perimeter, μm / μm^2 of tissue area)
[c] Capillary number density (Number of capillaries / μm^2 of tissue area)
[d] Correlation values for variables compared.
[e] P-values for above correlations; values in bold font are $P < 0.05$.

Discussion

We reject the hypothesis that vascularity is significantly impacted by restriction or realimentation. While we have established that realimentation can increase ipsilateral uterine blood flow (Camacho et al., 2014b), in the current study capillary densities and mRNA expression for angiogenic / vasoactive factors do not appear to be altered by maternal dietary intake to the degree which we hypothesized. Therefore maternal gestational diet may instead be affecting function of placental capillaries; we have already demonstrated that realimentation can affect placental arteriole vasoreactivity (Reyaz et al., unpublished data) by increasing sensitivity to vasodilators such as bradykinin within the same placentomes utilized in the current study. Moreover, in a 2 x 2 factorial study investigating CAR and COT vascularity in ewes when

receiving gestational nutrient restriction and melatonin supplementation there was an interaction of melatonin by nutritional level on sodium nitroprusside-induced relaxation of COT arteries where ewes nutrient restricted without melatonin were more sensitive to sodium nitroprusside, (thereby sensitivity to nitric oxide) compared to those adequately fed the inverse was true of those receiving melatonin (Shukla, 2014).

A similar bovine nutrient restriction / realimentation study conducted by Vonnahme et al. (2007) has also demonstrated little effects of nutrient restriction on placental vascularity by d 125 of gestation when exposure to nutrient restriction occurred from d 30 to 125 of gestation. However, in this study, upon realimentation, alterations in vascularity became apparent by d 250 of gestation, suggesting a placental programming effect but methods for realimentation of cows between this study and are different as well as the durations of restriction and realimentation. In the study by Vonnahme et al. (2007), cows were fed to achieve a BCS equal to their control group contemporaries by d 220 of gestation. For the cows used in the current study, body condition score was similar (Camacho et al., 2014a).

Maternal dietary treatment and gestational day impacts on vascularity measures and mRNA expression within day and tissue in control and nutrient restricted cows

Data previously collected in our laboratory (Camacho et al., 2014b), demonstrated that nutrient restricted cows have greater number of placentomes at day 85 and 140 than controls though placentomes were similarly sized across treatments. Perhaps because there were more, there was no need for capillary vascularity to be enhanced within the placentome to maintain adequate nutrients. This is supported by larger restricted fetuses at day 85 and similarly sized fetuses on day 140 and 254 (Camacho et al., 2014b). Another study in gestating beef cows which had different durations of restriction (days 30 to 125) and realimentation (days 125 to 250) show

no effects of diet or gestational time on placentome number or fetal birth weight (Vonnahme et al., 2007; Zhu et al., 2007).

In this study, capillary changes throughout gestation were more prevalent in COT vs. CAR placentome tissue. Within all days the same trend applied where COT had greater tissue area and APC and CAR had greater CAD, CSD, and CND. A similar trend has been evidenced previously where CAR has increased CAD, CSD, and CND on day 125 and 250 of gestation, although this study reported increased APC in CAR tissue on day 125 (Vonnahme et al., 2007). On day 85, area per capillary, a measure of capillary size, was greater in CON vs. RES cows. Prior research (Borowicz et al., 2007) suggests that when capillary size is decreasing there is more capillary branching (angiogenesis) occurring which is an idea supported by a numerical increase in capillary number density in addition to an APC decrease. Within all treatments and tissues, APC decreased as gestation advanced, and values for CND increased. However, though CND is numerically greater in RES than that of CON, the difference is not significant and tissue area is the same between treatments. In cotyledonary arteries of cows nutrient restricted from day 30 to 125 of gestation, Zhu et al. (2007) reported an increase in phosphorylated Akt and ERK½ which play a signaling role in angiogenic pathways.

By day 140 tissue area increased in COT tissue and decreased in CAR tissue which demonstrates that, over time, the fetal tissue of the placentome increased and the area of maternal tissue within the analyzed placentome decreased. Capillary surface density, historically a measure of potential nutrient transfer, also increased over time in both tissues. Within this experiment it may appear that there was more angiogenesis occurring in COT tissues, as size of these capillaries declined more so than that of CAR tissue. This would support work that previously demonstrated more branching occurs in the COT than within the CAR in sheep

(Borowicz et al., 2007). Moreover, Vonnahme et al. (2007) report an increase in cotyledonary cellular proliferation on day 250 compared with 125 of gestation in the cow.

On day 85, Flt-1 was not detectable in either cotyledonary or caruncular tissues. This is an early time point of gestation and it may simply be that, at this stage, expression is not yet high enough for detection, but does not necessarily mean that Flt-1 is not present or having a physiologic impact on VEGF-mediated angiogenesis within bovine placentomes. In CAR at day 254, there was a decrease in expression of PECAM-1 within cows given a shorter realimentation period in comparison to controls and those receiving longer duration of realimentation; however, there were no differences in microscopy measures of vascularity. This indicates that what we see in mRNA expression may not always be the best measure to show what is going on at the tissue level, because these are transcripts for protein expression, and may not necessarily reflect what functionally is happening within the tissue.

Maternal dietary treatment vascularity measures and mRNA expression within control fed cows

On day 85 COT APC was greater than in other gestational days measured in either COT or CAR tissue. After the decrease in COT APC from day 85, APC was similar from day 140 to 254. Within control fed cows, CAR tissue capillary size was similar on day 85 and 140 of gestation thereafter it was smaller thereby again potentially evidencing more angiogenic growth occurring sooner from the COT portion of the placentome. In the current study COT tissue of control cows over time had increased CAD, CSD, and CND whereas in CAR only CND was significantly increased at each gestational time point. These measurements within the bovine placentome appear to be in agreement with that of ovine, in addition to the decrease in capillary size of COT tissue. However, this study may be evidencing a species or placental type difference: CAR capillary size in the bovine was relatively similar throughout gestation only

differing from day 85 to 254 of gestation, but in ovine CAR tissue, capillary size increases 2.2 fold from mid to late gestation (Borowicz et al., 2007). A review by Reynolds et al. (2004) characterizes growth within the maternal placental capillary bed in the ewe as mainly through increases in capillary size with only small changes in capillary number or surface densities. Moreover in ovine COT tissue, APC inversely decreases 2.2 fold (Borowicz et al., 2007), where as finding of this study show no significant change from mid to late gestation however APC decreases 2.86 fold from day 85 to 254 of gestation. In a review from Vonnahme et al. (2013) CAR and COT CAD are compared between the sheep and cow from mid to late gestation where in the ewe, CAR and COT increases 200 and 400% respectively and in the cow CAR CAD decreased 30% and COT CAD increased 190%. In this study CAR CAD increased 26% and COT CAD increased 63%. The variation amongst cow CAD measures could be due to variations in treatments, as well as the time interval measured: days 125 to 250 of gestation in Vonnahme et al., study (2007) vs. days 140 to 254 in the current study. From early gestation to mid gestation this study had an increase (25%) in CAR CAD and a decrease in COT CAD (19%).

Duration of restriction on vascularity and mRNA expression

Day 85 capillaries in cotyledons of CON cows were larger than any d 140 or d 85 restricted capillaries. Perhaps early nutrient restriction from d 30 to 85 had already programmed capillaries to undergo angiogenesis. If this were true, conceivably the d 85 RES capillaries would begin branching sooner and, therefore, be a smaller size that they would eventually reach at d 140 earlier than CON capillaries. This could serve as evidence for nutrient restriction programming the placenta. Other data from our laboratory collected from the same cows utilized in this study support this theory as RES cows had heavier fetuses as well as more placentomes than CON cows when dams were nutrient restricted from day 30 to 85 of gestation (Camacho et

al., 2014b). However, other studies show no observed differences in placentomes vascularity for cows nutrient restricted from d 30 to 125, and changes did not become apparent until realimentation (Vonnahme et al., 2007). Amongst COT of restricted and control cows, CND increased from d 85 to 140 which indicates that capillary numbers increased regardless of dietary treatment. Expression of eNOS changed similarly in COT tissue also increasing by day 140, perhaps meaning that the placentomes were becoming more vasoreactive. Expression of KDR was higher in CON CAR tissue than in RES, indicating that perhaps more angiogenesis may be occurring, but was not detectable by histology or that the tissue was gearing up for vascular growth later in gestation. Additionally, while PECAM-1 mRNA was greater in CON vs. RES and increased within both treatments from day 85 to 140 however there were no differences in vascularity. In light of these findings, mRNA expression may not necessarily confer what is happening functionally at the cellular level within tissues.

Duration of realimentation on measures of vascularity and mRNA expression

In COT tissue, all capillary densities increased and capillary size decreased from d 140 to 254 in cows with varying exposure to nutrient realimentation which is a trend seen in all analyses. It is curious that duration of realimentation does not appear to affect these measures of vascularity and angiogenic factor expression in cotyledonary tissue. In agreement with data in the current study, Vonnahme et al. (2007) observed decreases in cotyledonary CAD, CND, and CSD of cows receiving realimentation to achieve similar BCS to controls after nutrient restriction from d 30 to 125 of gestation.

In CAR tissue, cows which had a shorter realimentation period (longer duration of restriction) had an increase in CND from d 140 to 254. Perhaps cows which were restricted longer needed more maternal capillaries to supply nutrients for the vast fetal growth that occurs

in late gestation. Though many factors are involved, this may partly help explain why there were no differences across treatments in fetal weight of calves collected from the same cows utilized in this study (Camacho et al., 2014b). Research from Vonnahme et al. (2007) shows that cows realimented (after being nutrient restricted from d 30 to 125) have an increase in CAR CSD, while in the current study this difference was not observed however this study only had one period of realimentation during a different gestational time interval. Interestingly, in CAR tissue, cows receiving a longer duration of realimentation had elevated sGC mRNA expression. Our laboratory (Reyaz et al., unpublished data) has previously demonstrated that placental arteries from RC cows tend to be less sensitive to bradykinin-induced relaxation than CC cows, whereas RR cows are most sensitive in both CAR and COT arteries. Perhaps as an alternative, vasoactivity within RC placentomes is more directed via sGC vs. bradykinin-induced eNOS relaxation. Additionally, Flt-1 expression was increased 5-fold by d 254 in CAR tissue of realimented cows. In a study by Vonnahme et al. (2007), there is a tendency for Flt-1 to be increased in realimented (d 125 to 250) after restriction (d 30 to 125) compared with controls. Current literature supports activity of Flt-1 to be inhibitory of VEGF-mediated angiogenesis which is believed to keep vascular growth 'in check'. Elevated mRNA expression of Flt-1 in CAR tissues may be functioning to allow more angiogenic growth to occur in fetal tissue instead by regulating CAR capillary growth within placentomes.

Correlations of mRNA expression with microscopy measures

Of the correlations that were analyzed amongst all mRNA expression genes with all microscopy measures, the only significant findings were the correlations of CAR PECAM-1 expression with the CAR microscopy measures CAD, CSD, and CND, all of which were vascularity measures that increased with gestational time. Expression PECAM-1 mRNA was

greater in CON vs. RES cows overall; however, there were no differences in cows with different durations of realimentation. The treatments where significant correlations were observed were on day 85 and cows which were realimented. In RC and RCC cows, strong positive correlations for PECAM-1 expression and CAD and CSD could be indicative of an increase in vasculature. However, these results further imply that mRNA expression and vascularity correlations may not be useful to apply when seeking a measure of vascularity.

Conclusion

Results of the current study were contrary to those hypothesized where it was predicted that realimentation and duration of realimentation would provide equivalent or excess vascularity to control cow placentomes compared with those nutrient restricted. This was the first study in bovine investigating placental vascularity in early gestation and differing lengths of restriction and realimentation. These results demonstrate there could be species differences between placentome vascular growth between bovine and ovine, and assumptions of similarity should be cautiously made across the two ruminant species. Moreover, the results warrant further investigation as to whether mRNA expression is a candid way to determine what is going on functionally within a tissue.

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CHAPTER 3. SUPPLEMENTATION OF DDGS DURING LATE GESTATION IN BEEF COWS CONSUMING A LOW QUAILITY FORAGE DECREASES UTERINE BLOOD FLOW WITHOUT IMPACTING MAMMARY GLAND BLOOD FLOW

Abstract

Positive effects have been observed in offspring from beef cattle supplemented with corn dried distillers grain with solubles (DDGS) in late gestation. The hypothesis was that late gestational DDGS supplementation to beef cows would increase blood flow to the gravid uterus, mammary gland, or both during the time of supplementation. Multiparous late pregnant beef cows were fed either a control (CON) diet of a low quality hay (n = 4) or a supplement diet (SUP) of low quality hay with DDGS (1.7 g/kg of BW; n = 5). Blood flow to the uterus and mammary gland was monitored at 21 d intervals during late gestation. Hay intake of cows was not affected by supplementation or by interaction of supplementation and stage of gestational ($P > 0.09$) but was affected by progression of pregnancy ($P < 0.01$). Intake of DDGS in SUP cows was not different over time ($P = 0.44$). Heart rate and blood flow to the contralateral horn of the uterus or mammary gland blood flow were not altered by treatment, day of gestation, or the interaction between treatment and gestational time ($P > 0.06$). The uterine parameters for total blood flow, blood flow to the ipsilateral uterine horn, ipsilateral pulsitility index, contralateral pulsitility index, and contralateral resistance index were all impacted by day of gestation ($P < 0.01$). In addition, total uterine blood flow and flow to the ipsilateral horn increased over time, and there was an effect of treatment and the interaction of treatment and gestation day ($P \leq 0.04$), but these effects were not measured in the rest of the uterine parameters ($P > 0.28$).

Key words: beef cows, late gestation, mammary blood flow, supplementation, uterine blood flow.

Introduction

It is well established that nutrition during gestation impacts both fetal and postnatal growth (Barker et al., 1995; Wu et al., 2006). In the initial two-thirds of pregnancy, maternal metabolism is in a physiologically anabolic state (Vernon et al., 1985), whereas the last third is a catabolic state (Naismith et al., 1976; Symonds and Clarke, 1996,). In bovine, two-thirds of fetal growth occurs in the last trimester of gestation, which requires an increase in maternal nutrients to facilitate adequate fetal growth and proper cellular tissue development. Maternal nutrition during gestation is important for lactation because preparatory growth of alveolar tissue occurs in late gestation, and stored body reserves (i.e. fat) will be utilized in lactation (Davis et al., 1979, Naismith et al., 1982).

Dietary protein supplemented during gestation has been shown to positively alter offspring growth and performance (Naismith et al, 1976, McNeil et al, 1997, Ocak et al, 2005, Funston et al, 2010, Larson et al, 2009, Stalker et al, 2006), while impacts on maternal lactational performance is highly variable and has been shown to increase (Winterholler et al., 2014), decrease (Sullivan et al., 2009), or not change (Larson et al., 2009). Increasing levels of DDGS supplementation three weeks prior to parturition linearly increased calf birth weight and calf BW at d 60 and 90 of age (Winterholler et al, 2014). These results may be attributed to an increased uterine blood flow, mammary blood flow, or both due to extra energy and protein that the DDGS would provide.

Blood flow to the mammary gland is highly correlated with milk yield (Kronfeld et al., 1968; Linzel et al., 1974). Our laboratory has previously published that gestational nutritional intake can impact milk yield in ewes (Meyers et al., 2011). The hypothesis of the current study is

that supplementation with DDGS during late gestation will augment uterine blood flow, as well as enhance blood flow to the mammary gland in preparation for lactation.

Materials and methods

Procedures for the animal experimentation were approved by the North Dakota State University Animal Care and Use Committee (#A12046).

Animal Procedures

Multiparous beef cattle (average age = 6.0 ± 2.5 (SD) yr, average parity = 4 ± 3 (SD)) were transported from the NDSU Beef Unit to the NDSU Beef Cattle Research Complex (distance 1.3 km) before day 150 of gestation (120 days average). Cows were acclimated to Insentec Roughage Intake Control feeders for a 3-week period. Insentec RIC feeders permitted entrance to feed troughs based on RFID ear tags and allowed individual intake data for each animal. Cows were assigned to treatments: control (CON) which were fed 2 % BW of low quality forage (n = 4) or supplemented (SUP) which were fed corn dried distiller's grain with solubles (DDGS) at 1.7 g/kg of BW in addition to the CON diet (n = 5). Nutrient composition of hay and DDGS can be found in Table 1. Cows were weighed and diets were adjusted for cow BW every 21 days coinciding with ultrasonography scans.

Table 3.1. Nutrient composition of feedstuffs fed to cows receiving or not receiving DDGS supplementation during late gestation.

	Feed		
Component, % of DM	Hay[1]	Hay[2]	DDGS
CP	6.35	9.54	27.44
ADF	76.33	72.06	8.15
NDF	48.11	44.21	29.36
ASH	8.42	11.63	10.06

[1]Hay fed from October 15, 2012 until December 12, 2012.
[2]Hay fed from December 12, 2012 until February 8, 2013.

Determination of MP and NEm in the diet

Hay samples were collected for analysis 5 times over the 4-month experiment; 3 samples for the first lot of hay used and 2 samples for the second lot of hay used. The change-over to a new lot of hay occurred on December 12th, 2012. All samples were analyzed for DM, ash, CP, lignin, and EE (AOAC, 2010; methods 934.01, 942.05, 2001.11, 973.18, and 920.39, respectively); NDF and ADF (ANKOM fiber analyzer, somewhere, NY); and CP associated with NDF and ADF. A DDGS sample was taken from the single lot of DDGS and analyzed at a single time-point for DM, ash, CP, NDF, and ADF. Individual daily intakes for all cows were collected via RIC feeders (Insentec BV, Marknesse, The Netherlands). Individual cow requirements were calculated (NRC, 1996) for three 28 - day periods prior to day of parturition. Data calculated were calorie maintenance requirement, calorie pregnancy requirement, metabolizable protein maintenance requirement, and metabolizable protein pregnancy requirement. Individual cow age, calf birth BW, and average cow BW for the 28-d period, average temperature for each 28-d period, and previous month's average temperature were entered into the program. Average cow weight for each 28 day period was predicted with regression equations derived from each cow made from two day BW taken at the same time as ultrasound time points.

To estimate the nutrition cows were getting from feed, energy prediction equations from Weiss et al. (1992) were used to calculate NEm for the hay. The calculation of MP was based on NRC equations (1996). Values from NRC (1996) were used for DDGS, TDN, and DDGS ruminally degraded protein and hay ruminally degraded protein for each hay. Balance was calculated based on supply minus requirement.

Uterine and mammary artery ultrasonography measurements

To minimize variation, all ultrasonography scans were conducted by one trained individual and one individual was used to run the Doppler ultrasound software (model SSD-3500; Aloka UST-672 equipped with a 7.5 MHz linear finger-probe transducer, Aloka, Wallingford, CT. Scans were conducted starting at 1300 and ending at 1700 where 3 to 4 cows were scanned within each period. All scans were performed at a constant flow gain setting of 10. Uterine artery ultrasonography scans were performed as previously published (Camacho et al., 2014b). Briefly, transrectal Doppler ultrasounds were performed by positioning a linear finger probe dorsally in hand on the abdominal aorta, detecting the origin of the external iliac, and tracing this artery caudally to locate the internal iliac artery to establish the branch point of the uterine artery. All measurements were taken immediately after this branch point to insure the same portion of artery was measured each time. Based on uterine blood flow measurements, we were determined the ipsilateral horn (Bollwein et al., 2002; Panarace et al., 2006; Camacho et al., 2014b).

For mammary gland blood flow, the pudendoepigastric arterial trunk was used to determine mammary gland blood flow as described by Götze et al. (2010) and Potapow et al. (2010). The linear finger-probe was directed dorsally in hand while moving cranially within the rectum. The abdominal aorta was located, and branches of the common iliac arteries were followed; the probe was directed ventrally so that the external iliac artery was visualized on the monitor. Thereafter, branching was followed as it became the femoral artery, passing the caudal epigastric branch to the pudendoepigastric arterial trunk where measurements were obtained.

We used the same anatomy as uterine blood flow when denoting the ipsilateral and contralateral sides of the mammary gland blood supply. For all arteries examined, hemodynamic

measurements obtained included: pulsatility index (PI); resistance index (RI); heart rate; uterine blood flow (ipsilateral, contralateral, total); and mammary blood flow (ipsilateral, contralateral, total). Last ultrasounds were performed on average, 40 days prior to calving. Equations for measurements used can be found in Table 3.2.

Table 3.2. Equations of Doppler ultrasonography hemodynamic measurements

Measure	Equation
Pulsatility index (PI)	[PSV[a] (cm/s) – EDV[b] (cm/s)] / MnV[c] (cm/s)
Resistance index (RI)	[PSV[a] (cm/s) – EDV[b] (cm/s)] / PSV[a] (cm/s)
Blood flow (BF)	MnV[c] (cm/s) x CSA[d] (cm^2) x 60 s

[a]PSV = Peak systolic velocity
[b]EDV = End diastolic velocity
[c]MnV = Mean velocity
[d]CSA = cross sectional area

Hemodynamic measurements were assessed from three separate scans with three wave-forms each (nine total wave-forms were evaluated per measure) as previously described (Lemley et al., 2012; Camacho et al., 2014b). Following baseline data collection, blood hemodynamics were reassessed in 21 d intervals for 3 time points prior to parturition. Blood samples were collected for all cows on the day prior to ultrasonography.

Parturition procedure

When cows were observed in labor (visible contractions and / or expelled water-bag) they were brought to stalls indoors for direct observation of calving. Data that were recorded during parturition included calving ease score (scale defined as: 1 = unobserved / unassisted, 2 = easily pulled, 3 = difficultly pulled, and 4 = excessive force or surgery needed). At parturition, after maternal bonding occurred (approximately 15 minutes,) calves were removed from dams prior to nursing for data collection. Crown-rump-length, heart-girth (i.e., circumference around the chest with measurement being taken immediately caudal to the front legs) and birth BW were measured for each calf. Placentas were collected immediately after delivery (all placentas were

delivered within 4 h) checked to be complete and weighed. Thereafter, cotyledons were dissected, counted, and weighed. The smallest and largest cotyledons from each placenta were individually weighed and recorded. Cows were weighed approximately 15 to 30 min after calving and again at 24 h. Thereafter, the cow-calf pairs were managed with the entire NDSU beef herd and weaning weights were obtained.

Blood serum analysis

Blood serum samples were analyzed for non-esterfied fatty acids (NEFA) and urea by methods previously utilized in our lab (Lekatz et al., 2010b). Samples were run in a single assay, and the CV within those assays were 3.2% for urea and 5.6% for NEFA. Progesterone was analyzed via Immulite (Siemens, Worcester, MA), as described previously (Galbreath et al., 2008; Martin et al., 2008); CV within the assay was 3.2%.

Statistical analysis

All data were analyzed using generalized least squares procedure (mixed procedure, SAS Institute, Cary, NC). Ultrasound parameters, NEm balance, MP balance, NEFA, urea, and body weight were analyzed with treatment, day of gestation, and the interaction of treatment by day of gestation in the model. Solutions for linear and quadratic regression coefficients were generated for blood hemodynamics, metabolites, and cow body weight. Day of gestation was treated as a continuous variable. All calving and placental data were analyzed with treatment as the only effect in the model.

Results

There was a tendency for the effect of treatment by period on intake (hay + DDGS) where intake from SUP cows decreased over time whereas CON cows maintained similar intakes throughout the study Table 3.3. The consumption of DDGS in SUP cows was not altered over time (P = 0.44). As predicted, SUP cows received more MP than CON cows; there was an effect of treatment (P < 0.01) and day of gestation (P < 0.01), and a tendency for the interaction of treatment by day of gestation (P = 0.09) on the balance of MP (Table 3.4). The NEm balance (Table 3.5) was less (P = 0.06) for CON cows than SUP cows. For NEm, there was no interaction between treatment and gestation day (P = 0.23), but there was an effect for gestation day (P = 0.02). Body weight of all cows was affected by day of gestation (P = 0.06) where weight was gained during gestation climaxing around 60 days prepartum thereafter gradually declining until parturition. There were no effects of treatment (P = 0.27 or an interaction of gestational time and maternal diet (P = 0.21) on body weight. Regression equations for cow body weight have CON and SUP cows weighing 704.52 ± 21.92 and 659.58 ± 19.66 kg 110 days prepartum, 724.53 ± 21.92 and 690.19 ± 19.66 kg 60 days prepartum, and 674.23 ± 21.92 and 623.32 ± 19.66 kg at parturition.

Table 3.3. Feed intake of cows receiving or not receiving DDGS supplementation during late gestation.

	CON[1]			SUP[1]						
	Period[2]			Period[2]				P-values		
Feed	1	2	3	1	2	3	SE	Trt	Period	Trt x Period
Hay, kgs	12.60	12.10	12.04	13.08	11.92	11.36	0.61	0.34	0.003	0.09
DDGS, kgs	-	-	-	1.53	1.53	1.61		-	0.44	-

[1]Treatments were Control (CON) low quality forage fed at 2% BW; protein supplemented (SUP) fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g/kg of BW.
[2]Periods are 28 day intervals prior to parturition where period 1 is closest to parturition.

Table 3.4. Metabolizable protein intakes of cows receiving or not receiving DDGS supplementation during late gestation.

	CON[1]			SUP[1]			P-values		
Period[2]	1	2	3	1	2	3	Trt	Period	Period x Trt
Intake, g	791	801	841	1177.5	1172	1168	0.002	0.002	0.09
Required, g	664.5	601	559	701.2	628.2	578			
Balance[3], g	126.9	200	281	476.3	543.7	590			

[1]Treatments were Control (CON) low quality forage fed at 2% BW; protein supplemented (SUP) fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g/kg of BW.
[2]Periods are 28 day intervals prior to parturition.
[3]Balance = Intake-required.

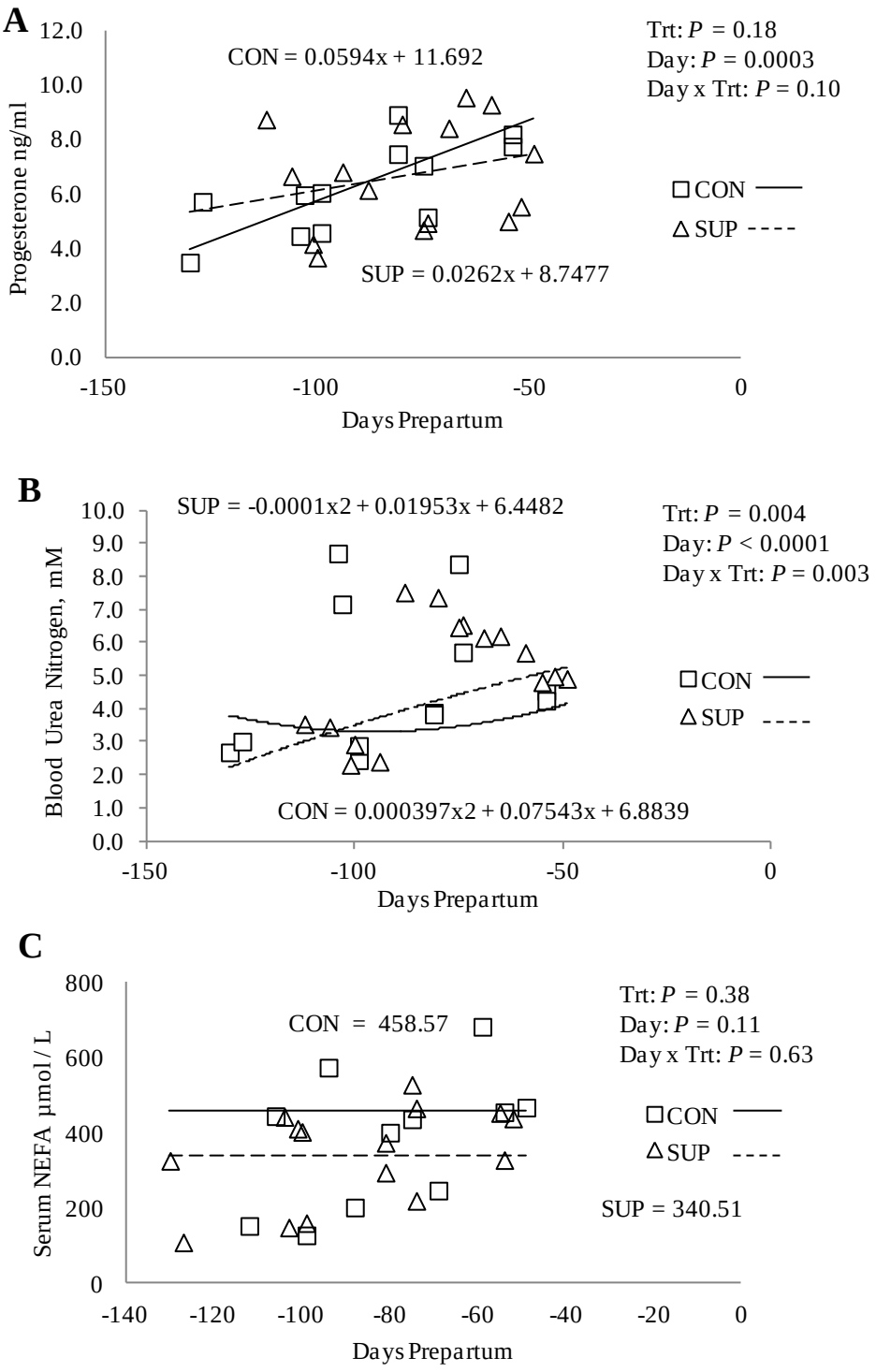
Table 3.5. Net energy maintenance measures of cows receiving or not receiving DDGS supplementation during late gestation.

	CON[1]			SUP			P-values		
Period[2]	1	2	3	1	2	3	Trt	Period	Period x Trt
Intake, kg	14.85	13.84	13.06	18.75	16.68	15.79	0.06	0.02	0.29
Required, kg	16.28	14.20	12.63	17.30	14.94	13.08			
Balance[3], kg	-1.42	-0.36	0.44	1.45	1.74	2.71			

[1]Treatments were Control (CON) low quality forage fed at 2% BW; protein supplemented (SUP) fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g/kg of BW.
[2]Periods are 28 day intervals prior to parturition.
3Balance = Intake-required.

Progesterone concentration tended (P = 0.10) to be affected by the interaction between day and treatment (Figure 3.1), by the end of gestation CON cows had more progesterone. There was an interaction of day and treatment (P = 0.003) on serum urea where SUP cows had an increase throughout gestation, whereas CON cows did not appear to change or increase to the degree of SUP cows during this point of gestation. Serum NEFA were not altered (P = 0.38) by treatments nor where they affected by day or the interaction between day (P = 0.11) and treatment (P = 0.63).

Figure 3.1. Blood metabolites for cows fed control low quality forage at 2% BW (CON; n = 4) and protein supplemented fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g / kg of BW (SUP; n = 5).



Heart rate was not affected in uterine or mammary ultrasounds by treatment, day of gestation, or the interaction between treatment and day of gestation ($P \geq 0.16$; Tables 3.6 and 3.7). There was an interaction of treatment and day of gestation on ipsilateral blood flow and total blood flow to the uterus where blood flow to the uterus increased throughout gestation and the SUP dams had a decrease in blood flow compared to CON (Table 3.6 and Figure 3.2). Supplementation of DDGS negatively affected blood flow to the ipsilateral horn ($P = 0.03$) as well as total blood flow ($P = 0.04$) to the uterus; however, it did not impact blood flow to the contralateral horn ($P = 0.63$) compared with the CON cows. Contralateral and ipsilateral PI as well as contralateral RI for all cows decreased with gestational time ($P \leq 0.01$) but were not affected by treatment or the interaction between gestation day and treatment ($P > 0.60$). While ipsilateral RI decreased throughout gestation for all cows, there was no effect of treatment, day of gestation, or the interaction of treatment by day of gestation ($P > 0.25$).

Table 3.6. Uterine arterial ultrasonography measurements obtained from cows during late gestation receiving or not receiving DDGS supplementation

	Treatments[1]			P-values		
Ultrasonography measure	CON	SUP	SEM	Trt	Day	Trt x day
Heart rate, beats / min	65	69	4.63	0.48	0.16	0.74
*Total BF[2], L / min	22.07	16.74	27.79	0.03	< 0.01	0.03
*Ipsilateral BF[2], L / min	17.62	12.74	27.65	0.04	< 0.01	0.04
*Contralateral BF[2], L / min	45.55	40.43	10.69	0.63	0.06	0.71
§Ipsilateral PI[3]	0.79	0.79	0.03	0.74	< 0.01	0.62
§Contralateral PI[3]	1.00	1.01	0.09	0.86	< 0.01	0.86
§Ipsilateral RI[4]	0.55	0.54	0.01	0.30	0.68	0.28
§Contralateral RI[4]	0.60	0.64	0.02	0.97	< 0.01	0.69

[1]Treatments were Control (CON) low quality forage fed at 2% BW; protein supplemented (SUP) fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g/kg of BW.
[2]BF = blood flow
[3]PI = pulsitility index
[4]RI = resistance index
*Refer to Figure 3.1 for day and day by treatment interaction results.
§ Refer to Figure 3.2 for day and day by treatment interaction results.

Figure 3.2. Uterine blood flow for cows fed control low quality forage at 2% BW (CON; n = 4) and protein supplemented fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g / kg of BW (SUP; n = 5).

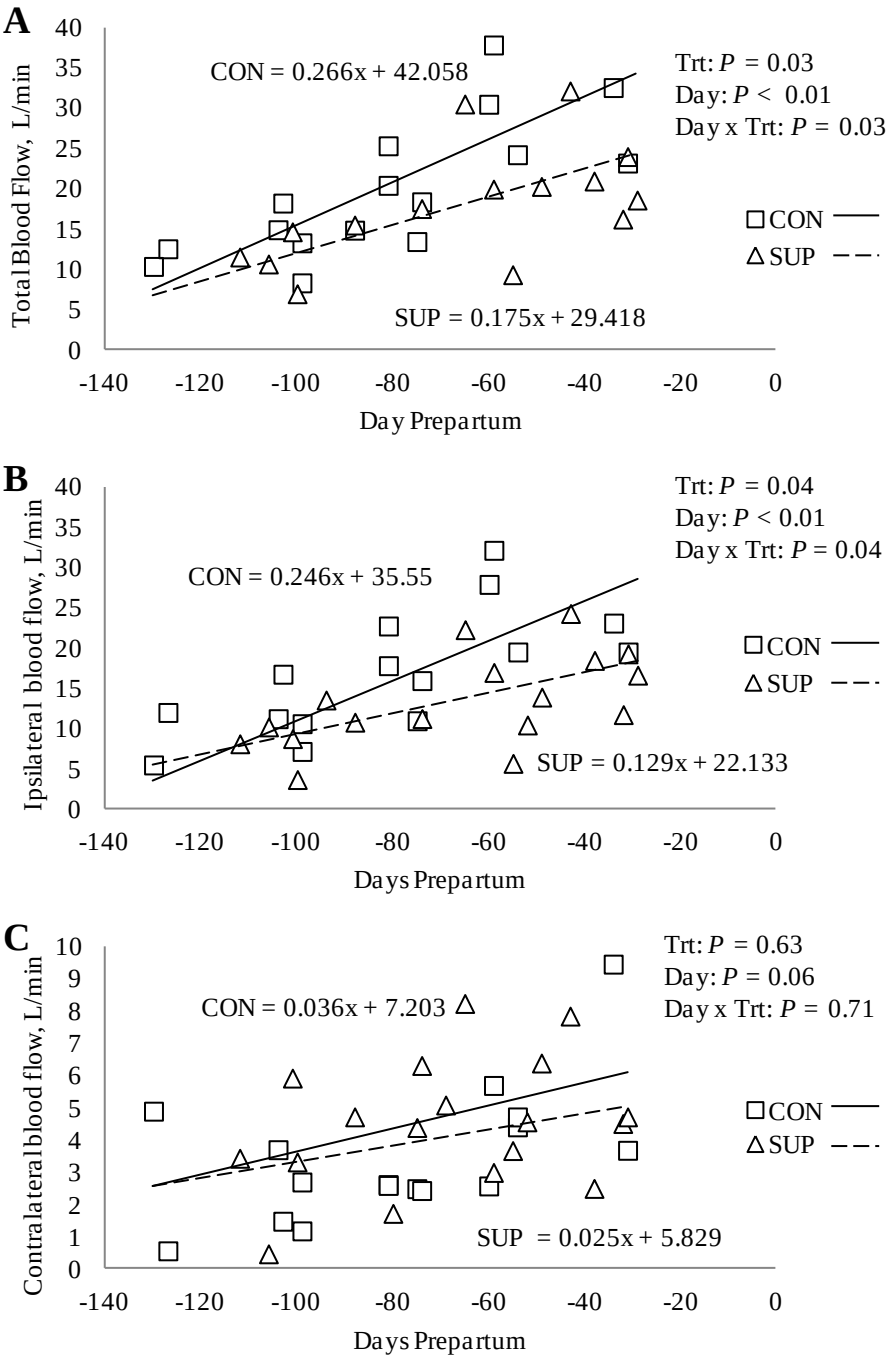
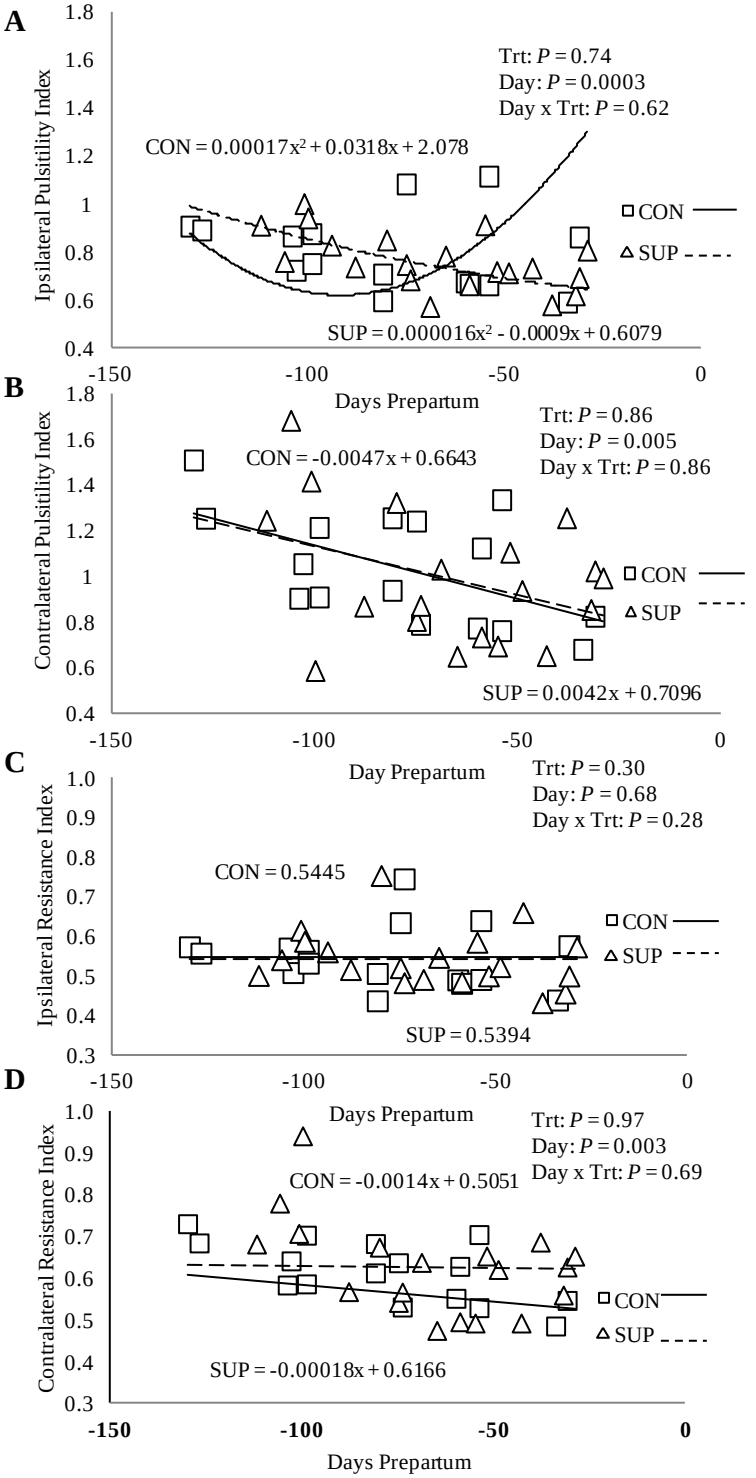


Figure 3.3. Uterine arterial pulsitility and resistance indices for cows fed control low quality forage at 2% BW (CON; n = 4) and protein supplemented fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g / kg of BW (SUP; n = 5).



There were no effects of treatment, day of gestation, or the interaction of treatment by day of gestation on any mammary blood flow parameters (P > 0.17; Table 3.7) except for an effect of the interaction of treatment by day of gestation on contralateral RI (P = 0.004).

Table 3.7. Mammary arterial ultrasonography measurements obtained from cows during late gestation receiving or not receiving DDGS supplementation.

				P-value		
Ultrasonography measure	CON[1]	SUP[1]	SEM	Trt	Day	Trt x day
Heart rate, beats /min	67.21	71.85	4.43	0.55	0.26	0.79
*Total BF[2], L / min	5.15	5.94	0.81	0.26	0.29	0.33
*Ipsilateral BF[2], L / min	2.72	3.17	0.48	0.49	0.68	0.64
*Contralateral BF[2], L / min	2.50	2.85	0.36	0.28	0.17	0.34
§Ipsilateral PI[3]	1.49	1.58	0.13	0.68	0.57	0.83
§Contralateral PI[3]	1.54	1.53	0.15	0.39	0.26	0.23
§Ipsilateral RI[4]	0.73	0.75	0.02	0.24	0.77	0.41
§Contralateral RI[4]	0.74	0.73	0.02	0.26	0.05	0.004

[1]Treatments were Control (CON) low quality forage fed at 2% BW; protein supplemented (SUP) fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g/kg of BW.
[2]BF = blood flow
[3]PI = pulsitility index
[4]RI = resistance index
*Refer to figure 3 for day and day by treatment interaction results.
§ Refer to figure 4 for day and day by treatment interaction results.

Figure 3.4. Mammary blood flow for cows fed control low quality forage at 2% BW (CON; n = 4) and protein supplemented fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g / kg of BW (SUP; n = 5).

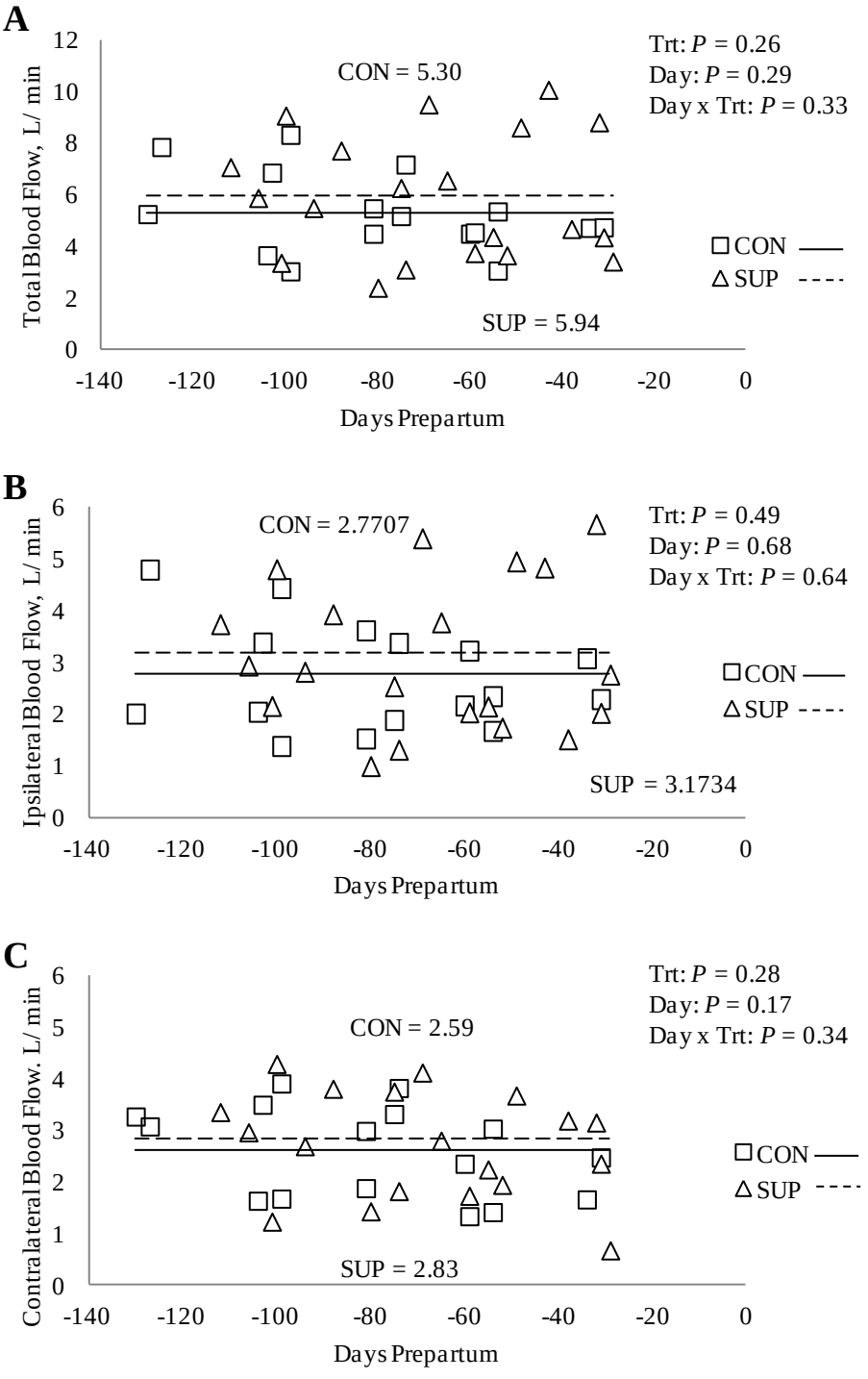
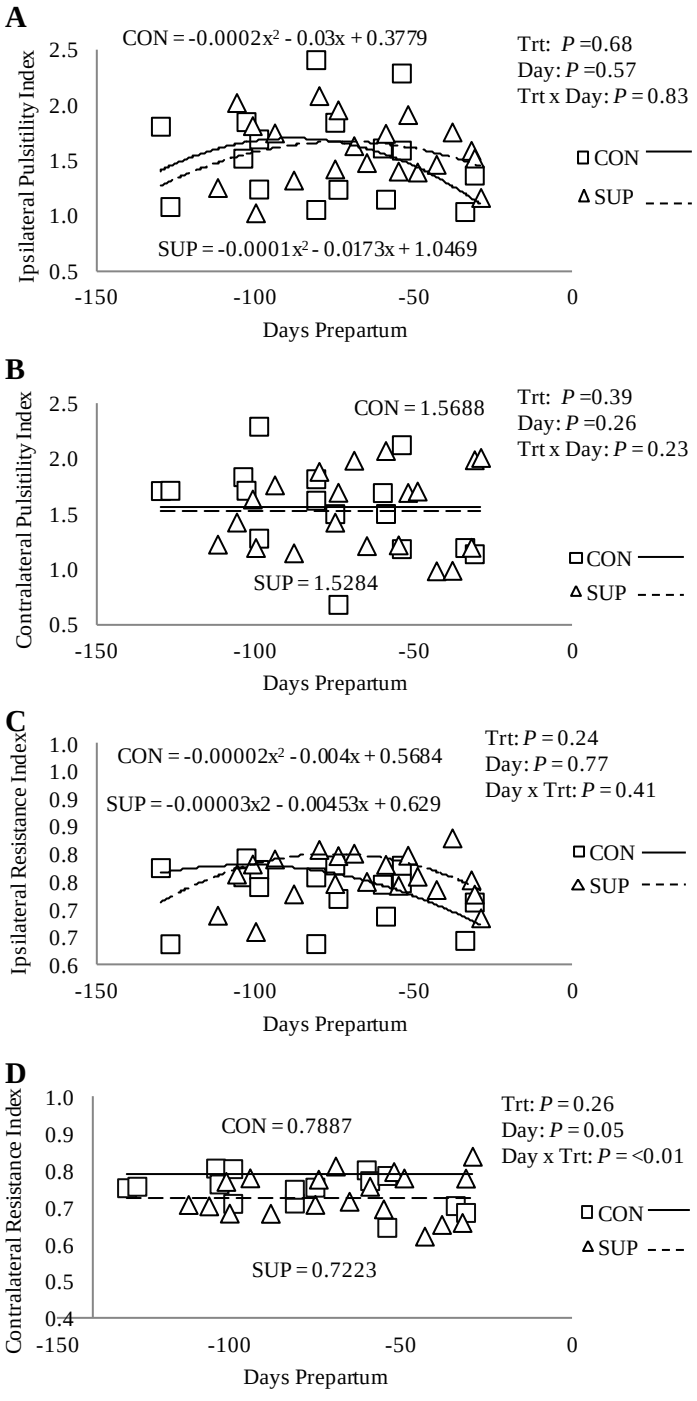


Figure 3.5. Mammary arterial pulsitility and resistance for cows fed control low quality forage at 2% BW (CON; n = 4) and protein supplemented fed CON diet plus corn dried distiller's grain with solubles (DDGS) to 1.7 g / kg of BW (SUP; n = 5).



All calf data is summarized in Table 3.8. Protein supplementation did not affect calf birth weight, crown-rump-length, heart-girth, or weaning weight ($P \geq 0.64$). In addition, placental measures (total placental weight, total cotyledon number, total cotyledon weight, largest cotyledon weight, and smallest cotyledon weight) also were not influenced by late gestational DDGS supplementation ($P \geq 0.18$).

Table 3.8. Calf measurements obtained where dams received or did not receive DDGS supplementation during late gestation.

Measure	CON[1]	SUP[1]	Pooled SE	P-value
Calf birth weight, kg	39.43	37.91	3.15	0.73
Crown-rump-length, cm	81.28	82.80	3.28	0.74
Heart girth, cm	78.89	77.72	1.78	0.64
Total placental weight, kg	3.56	3.55	0.0047	0.99
Total cotyledon weight, g	1547.28	1635.80	345.08	0.85
Total cotyledon number	79.50	101.20	21.48	0.48
Largest cotyledon weight, g	70.30	52.74	17.33	0.47
Smallest cotyledon weight , g	1.20	0.24	0.48	0.18
Weaning weight ,kg	284.57	285.22	11.97	0.97

[1]Treatments were dams fed control ,CON) diet of 2 % BW of low quality forage or supplemented ,SUP) fed corn dried distiller's grain with solubles , DDGS) to 1.7 g/kg of BW in addition to the CON diet

Discussion

Contrary to our hypothesis, it appears that supplementation with DDGS in this study during late gestation decreases uterine blood flow. In spite of this, we observed no differences in calf BW or placental weight at birth. We can only speculate that perhaps nutrient transporters or umbilical blood flow were similar across treatments in the cows in this study. While not measured, if maternal amino acid concentrations were elevated in the SUP cows, the placenta would have needed to compensate for their uptake with increased transport capacity. It has been previously reported in ewes that within a range of uterine blood flow, there is not a direct correlation of umbilical blood flow or fetal amino acid uptake during late gestation (Wilkening et al., 1985).

While there may be several underlying factors contributing to the mechanism of decreased blood flow in SUP cows it is possible that supplementation can cause negative effects for the fetus if it is not needed or in great excess. Reports from human studies show that high amounts of dietary protein result in babies being small for their gestational age which tend to be preterm deliveries, and have increased perinatal mortality rates (Rush et al., 1980; Say et al., 2003; Brown et al., 2011). While we did not observe any alterations in birth weights or early life mortality rates, it appears that protein intake does impact uterine blood flow and if we fed our cows a protein supplement for a longer time period, we may have experienced similar outcomes as the human studies cited above. The mechanism as to why protein intake would alter blood flow to the uterus is not known.

The reduced uterine blood flow of the SUP cows may be due to increased steroid metabolism, particularly estrogen. This notion is supported by the tendency in SUP cows to have decreased progesterone concentration in the current study. Conversely, Radunz et al. (2010) demonstrated an increase in progesterone with DDGS and corn supplementation. Estrogens are known to have vasodilator affects and have been demonstrated to increase uterine blood flow in oophorectomized ewes (Killam et al. 1973; Rosenfeld et al., 1973; Resnik et al., 1974). Moreover, in sheep, dietary protein caused an increase in hepatic P450 enzyme activity, which oxidizes steroids (Dziuk et al., 1992). There is further evidence in the literature that protein supplementation alters estrogen concentrations where high protein feeding during early gestation decreases estrone sulfate in beef heifers (Sullivan et al., 2009.)

The use of Doppler ultrasonography as a tool to determine whether supplementing late pregnant beef cows with DDGS can alter mammary blood flow does not exist in current literature. Most of the research utilizing Doppler ultrasound has been directed towards responses

of blood flow to the mammary gland with regards to: phase of lactation, variability amongst cows, stage in estrous cycle, mammary gland infection, hormones, and blood flow leaving the milk vein, and supplementation of lysine and methionine (Götze et al., 2010; Potapow et al., 2010; Christensen et al., 1989; Kensinger et al., 1983; Neilsen et al., 1990, 1995; Madsen et al., 2008; Braun et al., 2008, 2012). The pudendoepigastric arterial trunk was used as a measure for blood flow to the mammary gland, it has been noted previously that while the pudendoepigastric trunk branches into the caudal epigastric artery in addition to the external pudendal artery (sole blood supply to mammary gland), the muscles supplied by the caudal epigastric artery are small and the amount of blood flow is insignificant in comparison to the amount of blood flow of the external pudendal artery; therefore, the pudendoepigastric trunk is a reliable measure for blood flow to the mammary gland in cattle (Götze et al. 2010; Potapow et al., 2009; Braun et al., 2012).

In this study, there was no effect of supplementation on blood flow to the mammary gland throughout late gestation or between the two sides of the cow. Findings by Potapow et al. (2010) and Götze et al. (2010) have also demonstrated no differences between blood flow to the sides of the cow's pudendoepigastric trunks. Larson et al., (2009) reported that DDGS supplementation does not affect milk production in beef cows in the period prior to grazing season or at weaning. In contrast, Sullivan et al., (2009) have reported that beef heifers receiving a high amount of protein and energy supplement in the first trimester of gestation have a decrease in milk production. While milk yield and mammary blood flow are correlated, milk yield was not measured in the current study and, therefore, we cannot confidently predict if milk production would be altered in the cows of the current study.

The findings of this study do not necessarily rule out the possibility that positive effects in offspring evidenced by other studies are not due to mammary benefits. In other words, while it

appears cows may experience a decrease in uterine blood flow during late gestation, perhaps colostrum and milk production are enhanced (i.e., we observed similar weaning weights across treatments), aiding to negate any harmful effects that were experienced during late gestation in utero. It is possible that the window of timing in the present study has not captured the period of mammary gland preparation for lactation. In the present study, our last ultrasound was, on average, 40 d before calving. It is likely that the increase in arterial supply to the mammary gland necessary for lactation preparation occurred after our mammary gland data was collected. It has been established that during late pregnancy mammary ducts develop into lobulo-alveolar tissue with differentiated cells capable of producing milk, but Davis et al. (1979) has demonstrated in the goat that although mammary blood flow, oxygen consumption, and glucose uptake increased in the week prior to parturition, the first significant change with respect to days 7 - 9 pre-partum was apparent at 0.5 - 1 day pre-partum. Additionally, in the first 12 wk of lactation, total blood flow to the mammary gland was highest in German Holstein cattle the day after parturition (Götze et al. 2010).

Distiller's grain with solubles has been utilized by producers as both a protein and energy source. The NRC reports DDGS to be 30.4 ± 3.55 % CP and 10.7 ± 3.12 % EE. In the current experiment, the DDGS fed was of 27.44 % CP and 11.02% EE. Larson et al. (2009) have attributed the crude protein of DDGS as the component accountable for the impacts seen in calves. Calculated MP balance and serum urea values of SUP cows were higher than those of CON cows in this study while both treatments had similar forage intakes. Additionally, there appears to be a pattern within this data where duration of DDGS supplementation increases circulating urea, whereas the control cows appeared similar throughout the trial. Sullivan et al. (2009) found in the first two trimesters in gestating beef heifers, urea is higher when cows are

supplemented with a protein and energy source; moreover, this study also reported no differences in plasma NEFA amongst treatments. In the current study, while CON cows did have negative NEm balance during the two 28 d periods prior to parturition and SUP cows had positive NEm balance values throughout the trial, there were no differences in serum NEFA between the treatments possibly indicating that despite being in a negative balance, CON cows were not in a metabolic state different than SUP cows. A similar study has observed differences in plasma NEFA concentrations between cows supplemented and unsupplemented, with those unsupplemented having higher serum NEFA levels than protein supplemented (Sletmoen-Olson et al., 2000). Diets in this study were not isocaloric, and increased energy for SUP cows may have contributed to differences seen in blood metabolites between treatments.

The present study demonstrated that DDGS supplementation given to dams in late gestation did not affect the birth weight or weaning weight of calves. Other studies are inconclusive as to whether maternal DDGS supplementation affects calf birth weight; some studies reported an increase in birth weight (Clanton and Zimmerman, 1970; Larson et al., 2009; Winterholler et al., 2012, Radunz et al., 2010) and others reporting no differences in birth BW (Bohnert et al., 2002; Currier et al., 2004; Stalker et al., 2006). The lack of statistical differences among calf birth weight and placental measures because of treatments in this study could be due to the limited number of cows in the experiment. Cotyledon number was increased in heifers receiving high protein diets in the first two trimesters cotyledons compared with those receiving a high or low protein diet in either of the first trimesters (Sullivan et al., 2009). No significant differences in cotyledon number were observed with DDGS supplementation in the third trimester within this experiment. In cattle, approximately two-thirds of fetal growth occurs in the third trimester; however, the majority of placental growth occurs during the first two trimesters

of gestation (Winters et al., 1942; Ellenberger, 1950; Jakobsen et al., 1956, 1957; Ferrell et al., 1976, 1982; Prior and Laster, 1979), this may be why observed placental measures were not different amongst treatments as the majority of placental growth would have occurred prior to supplementation. That being said, one cannot assess placental function by weight alone.

Supplementation with DDGS decreased blood flow to the uterus and did not impact blood flow to the mammary gland at the current point in gestation. Further research is required to understand the mechanism of enhanced postnatal performance of calves whose dams were supplemented with protein sources.

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CHAPTER 4. GENERAL DISCUSSION AND FUTURE DIRECTIONS

General conclusions

This thesis presents two experiments investigating maternal dietary conditions in gestating beef cows including 1) effects of maternal nutrient restriction and realimentation on vascularity of placentomes and 2) impacts of protein supplementation on uterine and mammary blood flow. Chapter 1 examined how placental and mammary development occurs in the beef cow and how gestational nutrition affects these organs. Current literature about the roles of angiogenic factors affecting placental vascularity was also reviewed within this chapter.

The literature review discussed how uterine and mammary blood flow may be impacted by maternal nutrition which demonstrated that more research is warranted in these areas. Principles and applications, in addition to the advantages and limitations, of Doppler ultrasonography are also assessed in the literature review. It was evident from this review that the effect for protein supplementation on blood flow to the uterus and mammary gland as measured by Doppler ultrasound does not exist in literature; furthermore, to our knowledge, no studies investigating this have been published in gestating beef cows. By the same token, there are few studies which have explored how length and exposure of nutrient restriction and realimentation during gestation affects vascularity of placentomes. Other studies have investigated a restriction period covering days 30 to 125 of gestation (Zhu et al., 2006, Vonnahme et al., 2007). However, no research has shown the potential changes in vascularity at an earlier or later point of gestation.

Chapter 2 highlights mRNA expression of angiogenic factors as well as mRNA expression and immunohistochemical presence (as measured by microscopy) of platelet endothelial cell adhesion molecule-1(PECAM-1) within beef cow placentomes when exposed to varying lengths of nutrient restriction and realimentation. Expression of mRNA for angiogenic

factors was not altered by duration of restriction or realimentation in fetal or maternal portions of the placentome. Moreover, expression of PECAM-1 mRNA was not altered throughout gestation. This was not reflected in the histology which shows changes in vascular measures with gestational time. However, mRNA expression is the transcript for cellular protein expression and may not truly tell us what is going on at the functional tissue level.

Whilst there were only effects of treatment on vascularity measures at d 85, when differences between fetal and maternal tissues are examined, we see that tissue area, capillary area, and capillary surface area and number of capillaries were different at every day of gestation. Tissue area was greater in COT than CAR each d. Capillary number and surface densities were greater at each d in CAR tissues. Whereas capillary area, though decreasing in both tissues throughout gestation, was larger in COT tissues at each time point demonstrating that capillary changes throughout gestation were more prevalent in COT than CAR placentome tissue.

Chapter 3 presents data investigating blood flow to the uterus and mammary gland, feed intake, body weight, blood metabolites, birth weight and size of calves, and placental weight when cows receive late gestational DDGS supplementation. While blood flow to the uterus increased in both treatments (CON and SUP) throughout gestation, blood flow of SUP cows was decreased compared with CON. Mammary blood flow in both treatments remained unaffected throughout the experiment. Perhaps in this study, mammary blood flow was not measured close enough to parturition to observe possible changes in mammary blood flow. Measures collected at parturition, including calf size, birth weight, and placental weight, were unaffected by maternal dietary treatment.

Future directions

Future studies are needed to determine why blood flow was decreased in supplemented cows. In light of the hypothesis presented by Dzuik (1992), we hypothesize decreased blood flow with supplementation could be due to an increase in hepatic enzymes such as cytochrome P 450 which could be decreasing the amount of circulating estrogens, which have been demonstrated to increase blood flow (Killam et al. 1973, Rosenfeld et al., 1973, Resnik et al., 1974.) Exploration of gestational supplementation impact on mammary blood flow closer to parturition and in early lactation are warranted.

The research presented in this thesis evaluated maternal nutritional conditions of supplementation in late gestation varying exposure lengths of nutrient restriction and realimentation throughout gestation. If these studies were to be repeated with a larger number of animals within all of the treatments, more accurate results can be obtained. However, both of these experiments were novel and served as pilot studies for future research.

The nutrient restriction and subsequent realimentation study provided some interesting results as we still do not understand exactly what is happening in terms of vascularity that appears to be preventing negative consequences of nutrient restriction. We know that adverse maternal nutrition can consequently alter offspring post-natal performance (Barker et al., 1995; Wu et al., 2006). In an ideal future study, in addition to more animals, vascularity should be measured within multiple placentomes of each animal and measured throughout the entire placentome. This would give us a better picture of what is going on within the vasculature of the whole placentome not just within a screen shot. Moreover, vascular responsiveness should be measured as well; just because there are the same numbers of capillaries does not necessarily mean that they are all functioning similarly within the placenta across treatments.

In the supplementation study, the effects of DDGS supplementation on blood flow to the uterus or mammary gland in beef cattle had never been conducted. Results of this study were contrary to the hypothesis that the potential increase in blood flow with gestational supplementation is responsible for the positive characteristics observed in offspring. Therefore, if this study was to be repeated, the hypothesis by Dzuik (1986, 1992) should be tested. The next study should investigate whether protein supplementation augments liver enzymatic activity or production, thereby decreasing circulating hormones (like estrogen) which increase blood flow. Additionally, this study should measure blood flow to the mammary gland later in gestation as well as during lactation to see if milk production or quality could be the reason why there is evidence for offspring developmental programming. Investigation as to whether there is postnatal programming should be pursued to determine whether carcass quality and reproductive traits are affected by dam supplementation. Furthermore, whether DDGS is serving as a supplement source of protein, energy, or both within the maternal diet in late gestation should be examined.

In conclusion, data presented within Chapters 2 and 3 provide a foundation for further evaluation of the impacts of maternal protein supplementation as well as nutrient restriction and realimentation. While results of these studies were not anticipated, they have interesting implications which after further research can be of benefit to livestock industry producers specifically those who may be considering nutritional strategies within their own cow / calf operations.

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