EFFECTS OF NUTRIENT RESTRICTION, REALIMENTATION, AND TWINNING ON PLASMA VOLUME, UMBILICAL HEMODYNAMICS AND PLACENTAL CHARACTERISTICS IN THE PREGNANT ADOLESCENT EWE

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Animal Sciences

November 2019

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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ABSTRACT

Reproductive physiology in production animals is a key economic component of longevity and profitability of animal farming. There are several components that can benefit or compromise adequate pregnancy periods. Sheep production is not only a very important economic activity for farmers around the United States, but sheep are also an important medical and surgical model to study human diseases. Our findings suggest that estradiol-17β could be involved in acute increased plasma volume early in gestation which can benefit overall gestation. We report that umbilical blood flow decreases upon nutrient restriction in adolescent ewes and does not recover upon realimentation. Finally, we suggest that a similar umbilical blood flow, placental development and plasma volume expansion in twins and singleton pregnancies could be enough to obtain similar birthweights in singletons and twins.

ACKNOWLEDGEMENTS

As for my previous degree I would like to start by thanking my advisor, Kim for giving me this opportunity and for believing so much in me all these years. Your encouragement and enthusiasm to teach all your students is what has made your program what it is. Thank you not only for being a great academic mentor but also for teaching us how to maintain our humanity in this competitive environment.

Another special thank you for Kendall Swanson, who has not only been part of my committee for my previous degree and this one but has also been a great support in academic and personal matters. Thank you for your willingness to always help me in anything I have asked.

I would also like to thank my committee members Dr. Anna and Dr. Greives, also Dr. Bauer, all of you have been of invaluable help during my academic training. Our discussions have encouraged me to always look a step further.

Thank you to all the technicians in the repro lab that helped me during the completion of this degree Jim, Sheri, Veselina.

Thank you to all the graduate students from the Vonnahme lab, specially to Keloides and Amelia.

I special thank you to all the graduate students in Animal Sciences some of whom became not only colleges but great friends.

I would like to thank all the ANPC crew and the undergraduate students that helped me during my projects.

Finally, I would like to thank my family back in Ecuador for whom I started this path. To the people that made me see the days in color when they seemed gray. To all my friends here and in Ecuador.

Thank you!

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CHAPTER 1. LITERATURE REVIEW

Adaptations of the maternal system to support pregnancy

Pregnancy is a major physiological challenge for the mother. There are many changes that occur in maternal physiology to compensate for the stress of conceptus formation and growth. We will briefly focus on three major adaptations: cardiovascular adaptations of the maternal system to support pregnancy, nutritional needs and nutrient delivery to the fetus, and additional adaptations for multiple pregnancies.

Cardiovascular adaptations

When pregnant, the mother needs to compensate for the new metabolic demands of the enlarged uterus and counteract the blood loss of parturition (Pritchard, 1965; Torgersen and Curran, 2006). These modifications require changes in maternal cardiac output, blood volume and heart rate.

Cardiac output

In ewes, total cardiac output (liters of blood pumped by the heart per minute) and cardiac output per unit of body weight increase during pregnancy (Metcalfe and Parer, 1966; Rosenfeld, 1977; Jacobson et al., 1998). In sheep, both indices reach a maximum value the last 30 days of pregnancy (Metcalfe and Parer, 1966; Rosenfeld, 1977; Jacobson et al., 1998). Similarly, cardiac output and perfusion rate of non-reproductive tissues increase as gestation advances (Rosenfeld, 1977). Uterine vascular resistance increases during mid-gestation, decreasing dramatically during late gestation (Rosenfeld, 1977). Systolic and diastolic blood pressures seem to remain similar in pregnant and non-pregnant ewes. However, peripheral blood vessel resistance decreases in pregnant animals, especially during the last third of gestation (Metcalfe and Parer, 1966; Rosenfeld, 1977). Similarly, during late gestation placental and mammary vascular beds have decreased vascular resistance (Metcalfe and Parer, 1966).

In women, sheep, and goats, uterine and mammary blood flows increase in late pregnancy (Metcalfe and Parer, 1965; Rosenfeld, 1977; Thornburg et al., 2000). Moreover, the distribution of cardiac output to the uterus and mammary gland increases during late pregnancy (Rosenfeld, 1977; Thornburg et al., 2000). Nevertheless, the proportion of cardiac output to the liver decreases during late gestation (Rosenfeld, 1977). Comparably, the proportion of cardiac output received by the kidneys decreases significantly from early to late pregnancy (Rosenfeld, 1977).

In women, body oxygen consumption increases by term of gestation by 33% (Thornburg et al., 2000). Correspondingly, alveolar ventilation increases as progesterone levels increase during gestation (Thornburg et al., 2000). Alveolar PCO_2 is decreased during pregnancy in women (Thornburg et al., 2000). In sheep, it is estimated that by the end of gestation, 20% of the maternal cardiac output is directed towards the uterus to provide nutrients and oxygen to support the growing conceptus, primarily the fetus (Gootwine et al., 2007). In women, cardiac index (cardiac output per square meter of tissue) is increased in twins when compared to singletons (Rovinsky and Jaffin, 1966).

Blood volume

In every physiological state an adequate blood volume is necessary for normal nutrient and oxygen delivery to the tissues. Similarly, blood is necessary for the collection and elimination of metabolic waste products, including CO_2 from the body. In sheep and practically all other placental mammals, blood volume increases during pregnancy (Barcroft et al., 1939; Caton et al., 1975; Daniel et al., 1989).

An adequate blood volume increase is also necessary to protect the mother and fetus from the deleterious effects of reduced venous blood return and cardiac output (Pritchard, 1965; Torgersen and Curran, 2006). Pregnant women can handle more blood loss than non-pregnant women. They can lose up to 35% of their blood volume before showing signs of hypovolemia (Pritchard, 1965; Torgersen and Curran, 2006).

In women, blood volume increases during pregnancy at a moderate rate in the first trimester, and it increases rapidly during the second trimester with the last third experiencing a slight increase in blood volume (Pritchard, 1965). Plasma volume increases at high rates during the first two trimesters and stabilizes in the third, being the principal reason of blood volume increase during the first two thirds of pregnancy in women (Longo, 1983). The increase in hematocrit is usually lengthier with erythrocyte formation reaching plasma volume formation rate just prior to parturition (Pritchard, 1965). Plasma volume increase is fundamental for healthy pregnancies. In mammals an inadequate blood volume (i.e. inadequate plasma volume increase) during pregnancy can have detrimental physiological consequences to the mother and fetus. Plasma volume increase during pregnancy will be further described in the following sections.

Heart rate

Heart rate increases during late gestation but not during mid gestation in pregnant ewes (Metcalfe and Parer, 1966; Rosenfeld, 1977). Moreover, in humans, pregnancy produces an increase in left ventricular wall thickness (Hunter and Robson, 1992). This increase is necessary to compensate for the augmented cardiac output and adequate distribution of blood to the added vasculature (Hunter and Robson, 1992).

Nutritional needs and nutrient delivery during pregnancy

Various environmental factors affect fetal and placental development. Diet during pregnancy is an important modifiable element that can have a substantial influence on the viability and body composition of the newborn (Symonds et al., 2010). Changes in the amount or composition of feed consumed by the mother prior to ovulation through to lactation have the potential to significantly reset the growth trajectory of the majority of fetal organs and tissues (Symonds et al., 2010). Similarly, in sheep, global over and under nutrition decrease blastocyst formation after in vitro fertilization (Borowczyk et al., 2006; Grazul-Bilska et al., 2012). Maternal nutritional status can program nutrient partitioning and ultimately growth, development, and function of the major fetal organ systems (Vonnahme et al., 2013).

Nutrient delivery from the mother to the fetus is carried out by the placenta. The placenta consumes approximately two thirds of oxygen and one half of the glucose transferred from the uterine circulation to the conceptus (Gootwine et al., 2007). In general, the size of the placenta is correlated with its glucose and amino acid transfer capacity (Gootwine et al., 2007). This is determined by the abundance of transporters (Gootwine et al., 2007). Interruption of placental growth through environmental challenges is thought to increase the amount of transporters to increase placental efficiency (Gootwine et al., 2007).

Many nutritional models have been developed and analyzed to study the detrimental effects of inadequate nutrition during pregnancy in dams and offspring. We will briefly review some of the most common models in animals, however, this literature review focuses on the effects of global nutrient restriction in sheep.

Overfed adolescent pregnant ewes

Overfeeding pregnant adult sheep generally results in increased lamb birth weight (Muñoz et al., 2009; Symonds et al., 2010). On the contrary, when pregnant adolescent sheep are overfed, paradoxical

effects on the fetus can be seen (Wallace et al., 1998; Wallace, 2019). Overfeeding adolescent sheep during the full length of pregnancy results in decreased birth weights, placental weights, cotyledon weights and cotyledon numbers relative to moderately fed adolescent ewes (Wallace et al., 1998; Wallace et al., 2002; Wallace, 2019). Similarly, all major organ weights, with the exception of the adrenal glands, are decreased in the fetus (Wallace et al., 1998; Wallace et al., 2002; Wallace, 2019). Uterine arteries in the dam and umbilical vein and artery blood flow in the fetus are decreased when adolescent ewes are overfed (Wallace et al., 2002; Wallace, 2019). Oxygen and glucose concentrations are also decreased in both ewe and fetal blood (Wallace et al., 2002; Wallace, 2019). Hematocrit, however, is increased in over-nourished animals (Wallace et al., 2002).

Abruptly decreasing the amount of feed to previously overfed young sheep, after the first third of pregnancy, neutralizes the detrimental effects in fetal and placental weights (Wallace et al., 1998). On the other hand, if adolescent ewes are overfed after the first third of pregnancy, lamb birth and placental weights are affected (Wallace et al., 1998).

Nutrient restriction models in animals

In rats and guinea pigs protein and global nutrient restriction were shown to limit fetal growth and induce raised blood pressure in the offspring during their postnatal life (McMullen and Mostyn, 2009). Similar effects in postnatal blood pressure are caused by high fat and iron restriction diets (McMullen and Mostyn, 2009). Low protein diets in rats, mouse, guinea pigs, rabbits, and sheep cause reductions in nephron numbers (McMullen and Mostyn, 2009). Moreover, diets low in methyl donors produce offspring that exhibit insulin resistance and elevated blood pressure (McMullen and Mostyn, 2009), showing that nutrition might cause epigenetic changes that influence later development. As mentioned before, global nutrient restriction in pregnant sheep will be further discussed in the following sections of this literature review.

Additional physiological challenges of multiple fetus gestations

In eutherian mammals, adequate physiological maternal adaptations when carrying multiple fetuses are key for prenatal development and an adequate birth weight. Important adaptations include sufficient placental development for each fetus, adequate nutrient exchange between mother and fetuses, and an appropriate increase in plasma volume during pregnancy. Placental modifications and nutrient

exchange will be briefly discussed. Plasma volume in twin pregnancies will be discussed in the next section of this literature review.

Commonly, fetal body weights are decreased in multiple pregnancy gestations when compared to singleton pregnancies (McCoard et al. 2000a; McCoard et al., 2000b; Dwyer et al., 2005; Gootwine 2005; Echternkamp et al., 2006; Gootwine, 2007). Additionally, gestation lengths are decreased with increasing number of fetuses (Gootwine et al., 2007). In women cardiac output in twin pregnancies is 15% greater than singleton pregnancies (Hunter and Robson, 1992). This increase is secondary to a greater heart rate and a greater increase in left atrial diameter which represents an increased volume overload (Hunter and Robson, 1992).

Placental adaptations to multiple pregnancies

In sheep, placentomes increase in size until day 80 of gestation (Redmer et al., 2004; gestation length: 143 to 147 days). After day 80, placentomes discontinue growing, however fetal growth is maximum during the last 50 days of gestation (Redmer et al., 2004). Placentomes increase their efficiency of nutrient and oxygen transport by increasing their vascularity after day 80 (Redmer et al., 2004). This is especially important in twin pregnancies since placentome sizes and weights are reduced per fetus compared to singleton pregnancies (McCoard et al., 2000a; McCoard et al., 2000b; Gootwine et al., 2007).

It is hypothesized that if each twin had a similar placental size, or similar nutrient exchange across their placentas as singleton lambs, birth weights would be similar given adequate uterine space (Gootwine et al., 2007).

These characteristics of twin pregnancies make them interesting for research involving placental and blood flow adaptations to multiple pregnancies in the dam. Understanding the differences that might make twins less competitive than singletons after birth is very important to counteract those effects. These topics will be furtherly discussed in the following sections.

Plasma volume during pregnancy

Blood volume variations during the reproductive cycle in animals

In rats, total blood volume and red cell volume are increased in proestrus (i.e. increased estradiol-17β (E2) and decreased progesterone concentrations) when compared to metestrous females (i.e. decreased E2 and increased progesterone concentrations; Simmer and Blair, 1996). However, plasma

volume while greater in proestrus, was not statistically different in rats (Simmer and Blair, 1996). Additionally, basal hematocrit was decreased in metestrus when compared to proestrus (Simmer and Blair, 1996). Posthemorrhagic restitution of plasma volume is faster in proestrus than metestrus in rats (Simmer and Blair, 1996). It is suggested that estradiol is responsible for this rapid plasma volume restitution (Simmer and Blair, 1996). Meanwhile progesterone seems to influence plasma protein restitution (Simmer and Blair, 1996).

Importance of an adequate plasma volume increase during pregnancy

An adequate blood volume increase is necessary to protect mother and fetus from the deleterious effects of a reduced venous blood return and cardiac output (Pritchard, 1965; Torgersen and Curran, 2006). Moreover, failure to increase blood volume during pregnancy could be the cause or the consequence of many fetomaternal illnesses. In women, failure to increase blood volume during pregnancy has been related to pregnancy-induced toxemia (preeclampsia), fetal growth retardation, and premature labor (Goodlin et al., 1981).

Mechanisms of plasma volume increase during pregnancy

While it is well established why maternal blood volume increases would need to occur, there is still debate on how blood volume increases. There are two theories that attempt to explain blood volume expansion: the decreased vascular resistance theory and the endocrine theory.

Decreased vascular resistance theory

This theory describes a mechanism by which blood volume could increase during pregnancy (Schrier and Briner, 1991; Duvekot et al., 1993). When the female becomes pregnant a new vascular system is added to the main vascular system (Schrier and Briner, 1991; Duvekot et al., 1993). This new addition decreases the total vascular resistance of the cardiovascular system of the mother (Schrier and Briner, 1991; Duvekot et al., 1993). This in turn increases the heart rate in the mother, which activates the plasma volume regulating mechanisms in the liver, kidneys, and adrenal glands (Schrier and Briner, 1991; Duvekot et al., 1993). As plasma volume increases, blood volume increases as well (Schrier and Briner, 1991; Duvekot et al., 1993).

The endocrine control theory

This theory suggests a fetal influence on blood volume in the pregnant female (Longo, 1983). As gestation advances, the fetus, and its adrenal glands increase in size (Longo, 1983). As adrenal gland size increases there is an increasing production of dehydroepiandrosterone, a hormone that stimulates E2 production in the mother (Longo, 1983). Estradiol-17β then stimulates the renin-angiotensin system, which ultimately increases plasma volume (Longo, 1983). This theory also suggests a mechanism through which erythrocytes increase during pregnancy. During gestation, placental size increases and as placental tissue grows there is an increasing production of somatomammotropin (i.e. placental lactogen) and progesterone (Longo, 1983). These two hormones stimulate the production of erythropoietin in the mother, which finally stimulates the production of erythrocytes (Longo, 1983).

Estradiol effects in plasma volume

Previous work has theorized that E2 could potentially have an endocrine effect for increasing plasma volume in pregnant females (Longo, 1983). This effect is hypothesized to happen during mid and late gestation, when the fetus is developed enough to stimulate the maternal renin-angiotensin-aldosterone system through its own adrenal gland production of dehydroepiandrosterone (Longo, 1983). Indeed, a previous study conducted in ovariectomized ewes demonstrated that after 3 weeks of exogenous estradiol-17β, plasma volume expanded (Ueda et al., 1986).

During early gestation, as the size of the corpus luteum increases, progesterone production increases in sheep (Gonzalez de Bulnes et al., 2000). In sheep, this increased progesterone production has been correlated with a greater fertilization rate (Parr et al., 1987). However, the size of the corpus luteum is determined by the size of the ovulatory follicle and the size of the follicle is usually positively correlated with the amount of estradiol-17β secreted into the maternal blood stream (Webb and England, 1992; Souza et al., 1997). Additionally, estradiol-17β causes a left ventricle chamber enlargement in pregnant ewes without a change in mass (Jacobson et al., 1998) and it seems to increase cardiac stroke volume (Jacobson et al., 1998). This raises the question if an early effect of estradiol-17β could be beneficial for a favorable fertilization and early pregnancy. The importance of an adequate plasma volume increase to achieve successful healthy pregnancies has been described previously by many researchers (Pritchard et al., 1965; Goodlin et al., 1981; Longo, 1983; Daniel et al., 1989). Moreover, estradiol-17β increases

during proestrus and estrus. This suggests that perhaps plasma volume expansion may occur early on due to high estradiol-17β levels from the follicle.

Estradiol-17β and the renin-angiotensin-aldosterone system

Plasma renin activity is a measure of the angiotensin-forming capacity of plasma (Lumbers and Pringle, 2013). Additionally, angiotensinogen production parallels that of estrogens (Lumbers and Pringle, 2013) and both are greatly produced during pregnancy (Lumbers and Pringle, 2013). In women, estradiol-17β acts through angiotensinogen to stimulate angiotensin II (Lumbers and Pringle, 2013). This is very important during early pregnancy for the increase of plasma and blood volume (Lumbers and Pringle, 2013). Through this pathway, the plasma volume increase is independent of dietary variations in sodium intake (Lumbers and Pringle, 2013). After mid gestation, renin is the major hormone responsible for the maintenance of increased plasma volume (Lumbers and Pringle, 2013). Moreover, in humans, male and female dogs and ovariectomized rats administered different doses of estradiol-17β stimulated the renin-angiotensin-aldosterone system, and water and sodium retention (Thorn and Harrop, 1937; Woolley and Timiras, 1964; Skowsky et al., 1979).

During pregnancy, plasma volume must be increased with the concomitant increase in blood flow to certain tissues (uterus, mammary) while maintaining adequate blood pressure and perfusion to the rest of the body. Angiotensin II acting through angiotensin receptor 1 causes vasoconstriction and aldosterone formation (Lumbers and Pringle, 2013). Whereas, angiotensin II through angiotensin receptor 2 causes vasodilation (Lumbers and Pringle, 2013). During gestation, angiotensin II receptor 2 expression is increased in uterine and mammary tissues while angiotensin II receptor 1 is decreased in the same tissues but present in the rest of the body (Lumbers and Pringle, 2013). This way, through angiotensin II receptor 2 activation blood flow to the uterus and fetus increase (Lumbers and Pringle, 2013). However, the rest of the circulatory system maintains an adequate blood pressure through angiotensin II receptor 1 (Lumbers and Pringle, 2013). Moreover, angiotensin II and aldosterone stimulate VEGF production in vitro (Lumbers and Pringle, 2013), suggesting a further influence on plasma volume and the circulatory system. In conclusion, estrogens stimulate angiotensin II for plasma volume expansion and blood flow through angiotensin receptor 2 in specific tissues (Lumbers and Pringle, 2013).

Plasma volume differences between twin and singleton pregnancies

In women, there is a significant relationship between plasma volume and birth weight in singleton and twin pregnancies (Rovinsky and Jaffin, 1965; Campbell and MacGillivray, 1984), with greater plasma volumes in twin pregnancies (Campbell and MacGillivray, 1984). Furthermore, it is suggested that greater plasma volume is an indicator of greater birth weights in twins (Campbell and MacGillivray, 1984). Red blood cell numbers in the blood also increase as number of fetuses increase (Rovinsky and Jaffin, 1965). Similarly, in sheep, a decrease in systemic vascular resistance, an increase in cardiac output and an expansion in blood volume are greater for ewes with multiple fetuses than singleton fetuses (Gootwine et al., 2007).

Dietary influences on plasma volume

A diet with greater concentrations of carbohydrates and potassium and decreased concentrations of protein, fat and sodium decreased blood volume, plasma volume and extracellular fluid (Murphy, 1950). These changes were hypothesized to be related with decreased sodium intake (Murphy, 1950). Indeed, sodium retention is very important in pregnancy for maintaining increased blood volume (Lumbers and Pringle, 2013). Additionally, it was reported that pure glucose diets and complete fasting diets also decrease extracellular fluid volume in humans (Murphy, 1950).

Nutritional impacts on placental characteristics and fetal size

The placenta as the organ responsible for nutrient exchange

In sheep, the fetal placenta attaches to discrete sites in the uterine wall called caruncles (Vonnahme, 2012). These caruncles are aglandular and appear as knobs along the uterine luminal surface of non-pregnant animals. The placental membranes attach to these sites via chorionic villi in areas termed cotyledons. The caruncular-cotyledonary unit is called a placentome and it is the primary functional area of physiological exchange between mother and fetus. At different stages of gestation, the relative growth rates of the placenta and fetus vary greatly (Robinson et al., 1995). Normal placental growth is a requirement for normal fetal growth (Robinson et al., 1995). Offspring born at an above average weight have an increased chance of survival compared to those born at a below average weight in all domestic livestock species (Vonnahme, 2012).

Global nutrient restriction in pregnant sheep, impacts on placental and fetal development

Maternal nutritional status can program nutrient partitioning and ultimately growth, development, and function of the major fetal organ systems (Vonnahme et al., 2013). However, the differences in the impacts of nutrient restriction may depend on the duration and intensity of the restriction (Reynolds et al., 2005; Vonnahme, 2012). Before implantation the local maternal environment can determine the size of the future placenta, hence the fetus (Robinson et al., 1995). Maternal nutrition, partly through the regulation of ovarian activity, modifies the maternal endocrine environment required for maintenance of early pregnancy (Robinson et al., 1995). Maternal nutrition influences the variation of nutrient exchange with the conceptus in early pregnancy, and subsequently, is thought to alter the growth rate of the placenta (Robinson et al., 1995). The majority of placental growth happens in the first two-thirds of pregnancy, with the placenta reaching its maximum weight by day 80 to 90 in sheep (Redmer et al., 2004).

In humans, small placental volumes at mid gestation are associated with small-for-gestational-age babies at term (Robinson et al., 1995). Placental growth retardation precedes fetal growth retardation (Robinson et al., 1995). In the ewe, the growth of the cotyledonary mass is exponential during the first 70 or 80 d of pregnancy, slowing during the last third of pregnancy (Vonnahme, 2012). Similarly, at mid-gestation, the fetal metabolic rate and visceral organ growth are at their greatest (McMullen et al., 2005). From mid to late gestation, the capillaries in the caruncle increase in area and number, but this is modest compared to the cotyledon (Vonnahme, 2012). The increase in cotyledonary vasculature is critical for the rapid fetal growth during the second half of gestation (Ma et al., 2011). The large increase in transplacental exchange, which supports the exponential increase in fetal growth during the last half of gestation, depends on the dramatic growth of the utero-placental vascular beds during the first half of pregnancy (Vonnahme, 2012). Also, at mid-gestation, the fetal metabolic rate and visceral organ growth are at their highest (McMullen et al., 2005).

Exponential fetal growth occurs during the last third of pregnancy (Redmer et al., 2004). Therefore, decreased birth weight is a common result of late-gestation nutrient restriction with several studies reporting intrauterine growth restriction (Ferrazzi et al., 2002; Redmer et al., 2004; Lemley et al., 2012). There is evidence in sheep that the reduction in birth weight in late pregnancy is greatest when maternal protein intakes are low (Luther et al., 2005). Nevertheless, sometimes there is a lack of difference in birth weights,

yet there are reported differences in postnatal performance (Vonnahme et al., 2013). Early postnatal health may be better predicted by birth weight, however many of the phenotypes that are economically important to producers (reproductive function, milking ability or carcass quality) may not be predicted by birth weight alone (Vonnahme et al., 2013). It is hypothesized that growth trajectory, including prenatal growth that is dependent on placental function, is a better predictor of postnatal performance in livestock (Vonnahme et al., 2013). In sheep reduction of fetal growth during the last period of pregnancy (late gestation) sets three days after maternal under nutrition begins (Mellor and Matheson, 1979).

In adolescent ewes, periconceptional maternal body condition seems to have an effect on the degree of the maternal undernutrition impact on the fetus (Wallace, 2019). Similarly, the growth rate of the mother when the placenta is growing affects final placental size, if the mother is growing at a greater pace, the placenta is more affected (Wallace, 2019). Similarly, in undernourished adolescent dams plasma IGF-1 and leptin concentrations is decreased when compared to controls (Wallace, 2019). During late gestation glucose, amino acids, urea, and NEFAs are reduced in undernourished adolescent ewes (Wallace, 2019).

Blood flow to the fetus during nutrient restriction

In sheep, placental weight decreases slightly after day 80, however DNA does not decrease suggesting that the reduction in weight is secondary to hydration levels or restructuring, not a decrease in cellularity (Gootwine et al., 2007). After placentome growth seizes, an increase in capillary number occurs in cotyledons and an increase in capillary diameter happens in the caruncles (Borowicz et al., 2007; Gootwine et al., 2007).

Utero-placental blood flow increases dramatically to support the nutritional demands of the rapidly growing fetus (Vonnahme at al., 2013). In the undernourished model, nutrient availability in maternal plasma is reduced, therefore uptake by the gravid uterus is limited (Vonnahme, 2012). In ewes, an increased placental blood flow, combined with an increase in the extraction of glucose and oxygen helps to maintain the fetal supply of these essential substrates (Robinson et al., 1995). In addition, placental consumption of glucose and oxygen is reduced (Robinson et al., 1995). In normal pregnancies, resistance of the utero-placental arteries decreases as gestation advances (Vonnahme, 2012). In women and sheep, increased umbilical artery resistance (PI and RI) is correlated with reduced birth weights (Robinson et al., 1995; Vonnahme, 2012). Along with these findings, greater umbilical and uterine artery blood flows have are

associated with heavier fetuses and lambs (Galan et al., 1998; Rigano et al., 2001; Ferrazi et al., 2002; Reynolds et al., 2005; Lemley at al., 2012). During mid and late-gestation nutrient restriction umbilical blood flow decreases (Lemley et al., 2012). Similarly, the percentage of increase in PI is greater in restricted animals compared to control (Lekatz et al., 2013). The fractional distribution of cardiac output to the uterus increases from 0.5% in the non-pregnant ewe to over 16% in the late pregnant ewe (reviewed in Vonnahme et al., 2013). Of the overall increase in blood flow to the gravid uterus by late gestation, more than 85% is directed towards the caruncular vascular beds (reviewed in Vonnahme et al., 2013).

Nutrient restriction effects in steroidal hormones during pregnancy

The placenta modulates fetal growth and maternal metabolism through the synthesis and secretion of steroid and peptide hormones, growth factors and cytokines (Robinson et al., 1995). It also has been suggested that estradiol and progesterone can modulate the expression of growth factors such as IGF-1 (Robinson et al., 1995). Estrogens can contribute to vasodilation via NO (White et al., 2002). It has been shown that arteries from reproductive tissues exhibit greater sensitivity to estradiol than vessels from non-reproductive tissues (Royal et al., 2011). Progesterone maintains pregnancy through the promotion of uterine growth and the suppression of myometrial contractility (Graham and Clarke, 1997). Both hormones are key for the development of a successful pregnancy and can vary in animals undergoing nutrient restriction.

Realimentation in pregnant sheep

Only a handful of studies have analyzed separately the effects of nutrient restriction and if realimentation was able to counteract the effects of such restriction. Most of the studies restricted the dam during various periods of time and measured the results towards the end of pregnancy or at lambing. A majority of the studies on nutrient restriction in sheep have been done during mid-gestation and refed them during a later period of gestation, they have shown no difference in lamb or placental weights at birth (Gilbert et al., 2005; Sebert et al., 2008; Kotsampasi et al., 2009; Sharkey et al., 2009; Ma et al., 2011).

One of the first studies that investigated the direct effects of realimentation in the sheep focused on the last 60 days of pregnancy (Mellor and Matheson, 1979). They found that fetal growth stops during the last third of pregnancy under severe undernourished conditions in ewes, and it continues after realimentation (Mellor and Matheson, 1979). It is possible that placental efficiency is enhanced in mothers

that were previously nutrient restricted and realimented (Symonds et al., 2010). When mothers are nutrient restricted during mid-gestation, where glucose concentrations drop (Ma et al., 2011). This drop in glucose can alter placental nutrient transport. For example, it can increase the capacity of the placenta to transport triglycerides (Ford et al., 2007; Ma et al., 2011). Upon realimentation, glucose concentrations normalize; however, some of the effects of nutrient restriction on the placental phenotype remain, changing the fetal body composition with an increased body fat deposition (Ma et al., 2011). Similarly, variations in nutrient availability during mid-gestation have consequences on glucose and fat metabolism later in life as well as in the cardiovascular and urinary systems of the offspring (Gilbert et al., 2005; Sharkey et al., 2009; Ma et al., 2011; Hou et al., 2013).

Realimentation and blood flow

In the undernourished adolescent ewe, there is a 20% decrease in capillary area density within the maternal caruncle during mid and late gestation (Wallace, 2019). Similarly, there is a 22% decrease in uterine blood flow (Wallace, 2019). However, refeeding to control levels rescues fetal weights and nutrient availability without increasing capillary area density (Wallace, 2019). Intra uterine growth restriction in primiparous ewes at a young age is associated with reduced expression of factors such as VEGF, angiopoietin, angiopoietin receptor 2 and endothelial NO synthase in the placentome (Gootwine et al., 2007).

Studies on the effects of nutrient restriction and realimentation on umbilical and uterine artery blood flows in farm animals are limited. In cows, a 40% nutrient restriction during mid-gestation did not decrease uterine blood flow (Camacho et al., 2014). However, realimentation increased total and ipsilateral uterine artery blood flow (Camacho et al., 2014). Our lab previously reported that nutrient restriction (to 60% of nutrient requirements) does not decrease umbilical blood blow in adult pregnant ewes during mid gestation (Vasquez Hidalgo, 2016). To our knowledge, there are no studies that investigate the effects of nutrient restriction and realimentation on umbilical blood flow in adolescent sheep and it remains unknown if an increase in fetal body weight after realimentation is secondary to an increase in umbilical blood flow in sheep.

Twin vs singleton pregnancies

Physiological adaptations during early pregnancy can influence adequate fetal development in ewes carrying multiple offspring (Gootwine et al. 2007). Reduced body weights of individual lambs born in litters with more than one lamb have features in common with lambs with intra uterine growth restriction born from models such as undernutrition (Gootwine et al., 2007). These features include reduction of total uterine blood flow per fetus and lower metabolite concentration in the maternal and fetal circulations (Gootwine et al., 2007).

The reduction in body weights of individual lambs as litter size increases is associated with a reduction of total energy and protein content in the body of the lamb (Gootwine et al., 2007). Glucose requirements during the last third of gestation are greater in multiple pregnancies than in singleton pregnancies (Gootwine et al., 2007). Prolactin, placental lactogen, and growth hormone levels in maternal blood are directly correlated with litter size (Gootwine et al., 2007). Estrogen and progesterone concentrations are alsogreater in the maternal blood of twin pregnancies (Gootwine et al., 2007).

Possible explanations for decreased birth weights in twins vs singletons can be explained by the inability of the maternal system to fully meet the nutritional demands of multiple fetuses, mutual fetal growth interference (limited uterine space) and lack of adequate placental development because of maternal imprinting genes (Gootwine et al., 2007).

Placenta and blood flow in singletons and twins

Sheep with multiple fetuses have decreased placental and cotyledonary weights and number per fetus when compared to singleton placentas (McCoard et al. 2000a; McCoard et al. 2000b; Gootwine 2005, reviewed in Gootwine et al., 2007). In twin pregnancies overall fetal weights are decreased before day 100 of gestation when compared to singletons (Gootwine et al., 2007). This coincides with the time placental weight reaches its maximum level (Gootwine et al., 2007). Indeed, in twins, reduced growth rates before the period of exponential fetal growth (mid gestation) might be a protective mechanism by which the multiple fetuses are then spared from reduced nutrients during the exponential growth period (late gestation; Gootwine et al., 2007; Muhlhausler et al., 2011). Many studies in sheep have demonstrated the relationship between fetal size and umbilical blood flow (Rigano et al., 2001; Ferrazi et al., 2002). In intrauterine restriction models, decreased fetal sizes and weights concordantly with decreased umbilical blood flow

have been observed (Dwyer et al., 2005; Lemley et al., 2012). Similarly, birth weight and weight of the placenta are often positively correlated in man, pig, guinea pig, rabbit and sheep (Alexander 1964; Vatnick et al., 1991; Vonnahme and Ford 2004; Dwyer et al., 2005). In sheep, positive correlations have also been reported between birth weights and cotyledonary weights (Alexander 1964; Dwyer et al., 2005). In other studies, an experimental reduction of placentome numbers, either through uterine artery ligation or carunclectomy, did not affect placental or fetal size, demonstrating the capacity for compensatory development of the placenta (Reviewed in Gootwine et al. 2007).

The sheep placenta of a single fetus appears to be programmed to involve only about 70% of the total available caruncles in placentome formation (Alexander, 1964). Increasing number of fetuses in a litter increases the occupancy of maternal caruncles and greater aggregate placental weight (Alexander, 1964; Greenwood et al., 2000). However, there is an overall reduction of placental size and fewer placentomes per conceptus (Alexander, 1964; Greenwood et al., 2000). To compensate, cotyledons from pregnancies with greater litters have increased vascularity and increased weights (Gootwine et al., 2007). Nevertheless, these compensations are not enough to reach singleton placental development levels (Gootwine et al., 2007). Placental and fetal weights are poorly correlated in early gestation (Greenwood et al., 2000). However, they are highly correlated in late gestation (Greenwood et al., 2000). Uterine blood flow per fetus is reduced in multiple pregnancies (Gootwine et al., 2007). A reduction in uterine blood flow is associated with an increase in the efficiency of nutrient transport from maternal to fetal blood but with a significant reduction in oxygen delivery and fetal arterial oxygen content (Gootwine et al., 2007).

In twins, increased capillary density in the cotyledons is secondary to a greater VEGF and VEGF receptor synthesis in the placenta (Gootwine et al., 2007). When adjusted for total placental volume per fetus, capillary volume was less in the cotyledon and caruncle in twins compared with singletons (Vonnahme et al., 2008). Vascular endothelial growth factor protein expression decreased in placentomes as number of fetuses increased (Vonnahme et al., 2008).

Nutrition effects in multiple pregnancies

Maternal undernutrition during the majority of pregnancy may affect twin birth weight, but not singleton birth weight (Edwards et al., 2005). In undernourished mothers carrying twins, caruncle vascularity was enhanced while uterine blood flow was decreased only in singleton fetuses (Gootwine et

al., 2007). Overfeeding of ewes that carry multiple fetuses does not promote fetal growth to individual body weights that are similar to those for singleton lambs (Gootwine et al., 2007).

Importance of different maternal adaptations to singleton and twin pregnancies

In women, perinatal mortality rates, preterm delivery rates and very low birth weights are more common in twins than in singletons (Muhlhausler et al., 2011). In sheep and other animals, it seems that intra uterine growth restriction in twins is different than that seen in other models of intra uterine growth restriction in singletons (Muhlhausler et al., 2011). Reduced twin birthweights start during early gestation (Muhlhausler et al., 2011). There are unknown mechanisms of how undernutrition during early pregnancy can program slow fetal growth trajectory during late gestation (Muhlhausler et al., 2011). It has been hypothesized that these mechanisms could be similar to those of reduced growth trajectory in twins before the period of exponential growth (Muhlhausler et al., 2011). These changes have been hypothesized to be epigenetic (Muhlhausler et al., 2011). It is unknown if low birth weight in twins is associated with the same postnatal diseases in singletons with low birth weights (Muhlhausler et al., 2011). Indeed, when one of two twin fetuses is removed from a pregnant ewe, the remaining fetus does not acquire the body weight of a singleton fetus, however it reaches greater birth weight than twin fetuses (Gootwine et al., 2007). Therefore, studies investigating which mechanisms differ between intrauterine growth restriction in nutritional models and intra uterine growth restriction in pregnancies carrying multiple fetuses are necessary.

Statement of the problem

Reproductive physiology in production animals is a key economic component of longevity and profitability of animal farming. There are several components that can benefit or compromise adequate pregnancy periods. Sheep production is not only a very important economic activity for farmers around the United States, but sheep are also an important medical and surgical model to study human diseases. In this thesis, we aim to investigate and answer some of the important questions of sheep reproductive physiology. We designed investigations that targeted the analysis of the origins of adequate plasma volume expansion during pregnancy. Similarly, we investigated if an adequate plasma volume expansion during the time of conception or early gestation can benefit or predict a successful pregnancy. Moreover, we investigated the effects of nutrient restriction during mid gestation in umbilical blood flow of adolescent ewes. We analyzed if a hypothetical decrease in umbilical blood flow upon nutrient restriction can be

rescued after realimentation. Finally, we investigated the differences in umbilical blood flow between singleton and twin pregnancies. Further analyzing if a similar umbilical blood flow, placental development and plasma volume expansion in twins and singleton pregnancies would be enough to obtain similar birthweights in singletons and twins.

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CHAPTER 2. ACUTE EFFECTS OF ESTRADIOL-17β ON PLASMA VOLUME IN SHEEP

Abstract

In women and other species, failure to increase blood volume during pregnancy has been related to fetal growth retardation (Goodlin et al., 1981). Previous work has theorized that estradiol-17β could potentially have an endocrine effect signaling increased plasma volume in pregnant females (Longo, 1983). We hypothesized that a short duration of increased estradiol-17β would increase plasma volume in ovariectomized ewes. Adult non-pregnant Romanov ewes (n = 15) were ovariectomized. After ovariectomy, ewes were individually housed and were offered water for ad libitum intake and were fed a pelletted diet at maintainance once daily according to body weight (NRC, 1985). After at least 30 days post-ovariectomy ewes were fasted and received an implant that contained 100 mg of estradiol-17β (E2; n = 8) or a sham implant (0 mg E2; CON, n = 7). Implants were placed in the axillary region. Ewes were weighed prior to plasma volume measurement procedures. Plasma volume was determined using the Evans blue dye method. Blood samples were taken at times 0 (pre dye injection), 5, 10, 15, 20, 25, 30, 40, 50 and 60 after dye injection. After the final blood collection, ewes were euthanized with an overdose of sodium pentabarbital, and then uterine weights were recorded. Plasma volume tended to be greater in E2 vs CON (2.75 ± 0.11 vs. 2.54 ± 0.12 L, P = 0.07) and uterine weights were greater in E2 vs CON (27.25 ± 2.35 vs. 17.35 ± 2.51 g, P < 0.01). However, water intake after implant placement was not different in E2 vs CON (3.85 vs. 4.87 ± 0.67 L; P = 0.28). Our results have demonstrated that 24 h of E2-treatment tended to increase plasma volume in ewes.

Introduction

In all physiological states an adequate blood volume is necessary for normal nutrient and oxygen delivery to the tissues. During pregnancy, an increase in plasma volume is very important for the physiological adaptation of the dam to the addition of a new vascular component (conceptus) to its own cardiovascular system. There are two main reasons for the importance of increased blood volume increase during pregnancy. The mother needs to compensate for the new metabolic demands of the enlarged uterus (Pritchard, 1965; Torgersen and Curran, 2006) and counteract the blood loss of parturition (Pritchard, 1965; Torgersen and Curran, 2006). An adequate increase in blood volume is also necessary to protect the mother and fetus from the deleterious effects of a reduced venous blood return and cardiac output

(Pritchard, 1965; Torgersen and Curran, 2006). Moreover, failure to increase blood volume during pregnancy could be the cause or the consequence of many fetomaternal disorders (Goodlin et al., 1981). In women, failure to increase blood volume during pregnancy has been related to pregnancy-induced toxemia (preeclampsia), fetal growth retardation, and premature labor (Goodlin et al., 1981).

In women, plasma volume increases rapidly during the first two trimesters and stabilizes in the third, with the principal reason for increased occurring blood volume during the first two thirds of pregnancy (Longo, 1983). The increase in hematocrit is usually the opposite, increasing at a slower rate during the first two trimesters and reaching plasma volume increase rates only prior to parturition (Pritchard, 1965). Previous work has theorized that estradiol-17β (E2) could potentially have an endocrine effect for increasing plasma volume in pregnant females (Longo, 1983). This effect, however, is hypothesized to happen during mid and late gestation, when the fetus is developed enough to stimulate the maternal renin-angiotensin-aldosterone system through its own adrenal gland production of dehydroepiandrosterone (Longo, 1983). Indeed, a previous study conducted in ovariectomized ewes demonstrated that after 3 weeks of exogenous E2 treatement, plasma volume increased (Ueda et al., 1986). However, our lab has recently observed that hematocrit, a measure of plasma volume, at time of fertilization and during very early pregnancy is decreased in pregnant ewes. Estradiol-17β increases during proestrus and estrus in the female plasma. This suggests that perhaps plasma volume expansion may occur early in pregnancy due to high E2 levels from the ovulatory follicle.

We believe that E2 not only increases plasma volume during mid-pregnancy but also an early pregnancy to allow for proper embryonic development allowing for establishment of pregnancy. Therefore, we hypothesized that E2 will acutely (24 h) increase plasma volume in ovariectomized sheep. Since water intake decreases during estrus in dairy cattle (Reith et al., 2014), we monitored water intake to determine if our results would be altered by water intake. Consequently, we also hypothesize that water intake in E2-treated ewes will be decreased, suggesting an early stimulation of E2 to the renin-angiotensin-aldosterone system. The objectives were to measure plasma volume and water intake in ewes treated with a pharmacological dose of E2.

Materials and methods

Animals and experimental design

Animals care and use was approved by the Institutional Animal Care and Use Committee at North Dakota State University (#A17009).

Multiparous Romanov ewes (n = 15) were randomly selected from the Animal Nutrition and Physiology Center (ANPC) flock at NDSU and were individually housed indoors. All ewes received a pelleted diet (Table 1) that provided 100% of their nutritional recommendations as described by NRC (2007). Light-dark cycles were timer controlled and were set to 12h light 12h darkness. After 5 days of indoor housing, ewes were ovariectomized via midventral laparotomy as previously described (O'Neal et al., 2009).

After ovariectomy, ewes were allowed to recover for a minimum of 30 days to ensure that any circulating endogenous estrogens were cleared from the body before treatments were initiated. During this time period, ewes continued to be fed once daily (before 9AM) a pelleted diet that provided 100% of their nutritional recommendations (NRC, 2007) and received water for ad libitum intake. Water intake was monitored daily starting 10 days prior to the plasma volume measurement test and after treatment was applied by individually weighing the amount of water consumed. To adapt and control stress the ewes to a show stand, where the plasma volume measurement test took place, eight to 10 days prior to the measurements, ewes were conditioned to a show stand. For 2 days, ewes were placed in the show stand for a period of 15 minutes each day. The next 2 days, ewes were placed in the show stand for 30 minutes each day. The next 2 days, ewes were placed in the show stand for 1 hr each day. The last 2 days prior to the plasma volume mesurement test, ewes were placed in the show stand for 2 hours. At day 30 after ovariectomies were conducted, ewes were divided into two groups and received either an implant that contained 100 mg of estradiol-17β (E2; n = 8) or a sham implant with 0 mg of estradiol-17β (CON; n = 7). Estradiol-17 β was placed into a silastic capsule (ID x length; 3.35 x 15 mm respectively. A blood sample (3 ml; jugular vein) was obtained before implant placement and hematocrit was immediately analyzed (Unico micro-hematocrit centrifuge, Dayton, NJ).

For implant placement, ewes were placed on their rear. The axillary region was cleansed with Novasan scrub, rinsed with water, sprayed with 70% ethanol and injected with 1 mL of lidocaine. After 1 to

2 minutes, a small incision (~ 2.5 cm) under the skin was made with a sterile scalpel. Using a sterile hemostat, a "pocket" was formed by opening up the area ~2.5 cm deep. The implant was 1.5 cm inches long by 0.5 cm wide. After the implant was inserted the insition was closed with sterile surgical staples. Ewes were weighed the day before and on the day of the plasma measurement test.

Table 2.1. Diet composition

Ingredients	Percentage
Corn	9.3
SBM	4.0
Beet pulp	28.9
Alfalfa meal	33.4
Wheat middlings	24.4
Total	100

Diet per kg: 2.659 Mcal; CP: 169.8 g; MP: 118.86 g; NDF: 369.5 g; Starch: 294.24 g; Ca: 7.33 g; P: 4.084 g; Cu: 104.44 ppm; Se: 3.813 ppm. Diet based on NRC recommendations (NRC, 1985).

Plasma volume measurement method

For plasma volume measurements we utilized the Evan's blue dye method (Zweens and Frankena, 1981; Brown et al., 1992). Evan's blue dye binds to serum albumin, therefore measuring plasma volume indirectly (Zweens and Frankena, 1981; Brown et al., 1992; Farjanel et al., 1997). This method has been widely used to measure plasma volume in ewes and other species.

Plasma volume measurement procedures

Twenty four hours after implants were applied, the jugular regions of the neck of the ewes were shaven and washed with Novasan scrub, rinsed with water, sprayed with 70% ethanol, these steps were repeated two more times. On the left jugular vein a catheter (Approx. 10 inch length) was placed and a 3 ml blood sample was obtained (EDTA containing tubes; Becton Dickinson Vacutainer, Franklin Lakes, NJ). Next, 3 ml of Evans blue dye (5 mg/ml) was injected into the right jugular vein. After the dye injection, the catheter was secured with a veterinary elastic wrap. Thereafter, 3 ml blood samples were collected via the catheter at 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes after time 0. Time 0 is described as the time of the first blood collection, prior to dye injection. After each blood draw, 3 mL of sterile heparinized saline was placed back through the catheter to prevent clotting. Hematocrit was assesed immediately after blood collections.

Blood samples obtained were immediately centrifuged (3010x g for 20 min), plasma was collected and stored at – 20 °C until Evans blue dye concentrations could be estimated. After the last collection, the ewe was euthanized with an overdose of sodium pentabarbital, confirmation of the implant was assessed, and uteri were dissected and weighted.

A spectrophotometer (620 nm absorption) was used to measure the concentration of Evans blue dye in the plasma samples. The concentrations were then compared to a standard curve plotted from concentrations of Evans blue dye that contained dilutions of 15, 7.5, 3.75, 1.875 and 0.938 mg of the dye diluted in a liter of saline solution. Plasma volume per ewe was then calculated using the formula: Plasma volume = mg Evans blue infused/predicted concentration of Evans blue at time 0, through a regression analysis.

Statistical analysis

The study was conducted as a completely randomized design. Plasma volume, hematocrit and uterine weight data were analyzed using the GLM procedure of SAS (SAS software version 9.4, SAS Institute, Cary, NC). Ewe was considered the experimental unit for all variables. The model included the fixed effect of treatment and the random effect of the ewe. Plasma volume and uterine weights were the dependent variables. Ewe weight was included as a covariate. Means were separated using the LSMEANS statement.

For the water intake analyses, water intake was averaged for the 10 previous days before the plasma volume test. These averages were then compared to the water intake after the implant was applied. The influence of the implant applied was also analyzed. For these analyses the MIXED procedure of SAS was used with ewe as the random variable and time (pre or post implant placement) and treatments used as fixed effects. Time was included as a repeated measure and water intake was the dependent variable. Means were separated using the LSMEANS statement. P values ≤ 0.05 were considered significant. Tendencies are described when P values are > 0.05 but ≤ 0.10.

Results

Body weights were similar for both treatment groups when the plasma volume measurement test was performed (P = 0.50; 59.81 vs. 57.21 ± 2.77 kg for E2 and CON, respectively).

For water intake, there was no interaction between treatments and time of implant placement (pre and post). Intake was similar between treatment groups during the 10 days previous to implant placement (P = 0.20; 2.01 vs. 2.56 ± 0.30 L per day for E2 and CON respectively). Water intake was reduced during the 24 hours after implant placement when compared to intake average during the previous 10 days to implant placement for both treatment groups (P = 0.02; 2.28 vs. 1.98 ± 0.21 L per day for pre implant placement and post implant placement, respectively). However, water intake was not different between treatment groups during the 24 hours after the implant was placed (P = 0.28; 1.75 vs 2.21 ± 0.30 L for E2 and CON groups, respectively).

Plasma volume tended to be increased in E2-treated compared with CON ewes (P = 0.07; 2.74 vs. 2.53 ± 0.36 L, for E2 and CON, respectively). Uterine weights were greater in E2 vs CON (P < 0.01; 27.25 vs. 17.35 ± 2.51 g, for E2 and CON, respectively).

Hematocrit was not different between treatments the day before the implant was applied (P = 0.80; 42.04 vs. 40.98 ± 1.52 %, for E2 and CON, respectively and the day of the plasma volume test (P = 0.11; 29.80 vs. 31.38 ± 1.35 %, for E2 and CON, respectively).

Discussion

Plasma concentration of E2 during estrus ranges from 5 to 10 pg/ml (Karsch et al., 1980). Our lab has previously reported E2 plasma concentrations averaging 60 pg/ml 24 hours after a 100 mg implant was applied in ovariectomized ewes (O'Neal et al., 2009). In this study we have demonstrated that an acute exogenous supraphysiological dose of E2 tends to increase plasma volume in ovariectomized sheep in a 24-hour period. It has been reported that the size of the corpus luteum will increase progesterone production in sheep (Gonzalez de Bulnes et al., 2000). This increased progesterone production has then been correlated with a greater fertilization rate (Parr et al., 1987). However, in sheep the size of the CL is determined by the size of the ovulatory follicle and the size of the follicle is usually positively correlated with the amount of E2 secreted into the maternal blood stream (Webb and England, 1992; Souza et al., 1997). This raises the question if an early effect of E2 could be beneficial for fertilization and early pregnancy maintenance. The importance of an adequate plasma volume increase to achieve successful healthy pregnancies has been described previously by many researchers for several species including sheep (Pritchard et al., 1965; Goodlin et al., 1981; Longo, 1983; Daniel et al., 1989). Similarly, it has been

previously reported that several doses of E2 administered chronically to ovariectomized ewes increased plasma volume (Ueda et al., 1986). This study demonstrates that a single pharmacological dose (100 mg) of E2 has similar effects.

Moreover, even though we had hypothesized that water intake would decrease in E2 treated ewes and water intake was not different between treatments we suggest that the observed increase in plasma volume is not due to an increase in water intake but because of an increase in water retention. Indeed, water intake decreased in dairy cows during estrus (Reith et al., 2014). It was hypothesized that this was a consequence of increased movement of the cow within the pen (Reith et al., 2014). In our study water intake decreased both in the control group and the E2-implanted group upon implant placement. However, water intake was not different between E2-treated and control animals. Even though we did not measure urine output in this experiment, the acute increase in plasma volume in E2-treated ewes could be a consequence of decreased sodium excretion and consequential water retention. In fact, in humans, male and female dogs and in ovariectomized rats, different doses of E2 treatments resulted in stimulation of the renin-angiotensin-aldosterone system and water and sodium retention (Thorn and Harrop, 1937; Woolley and Timiras, 1964; Skowsky et al., 1979). A greater formation of metabolic water (water released into the tissues during carbohydrate and fatty acid oxidation) could also be hypothesized, however estrus seemed not to alter body composition nor metabolic water production in rats (Frisch et al., 1977). Therefore, it seems more likely that the acute increase in plasma volume presented in this study is a consequence of an increase in water retention.

Disproportional changes upon different physiological conditions in plasma volume and hematocrit have been reported in humans (Vogt and Johnson, 1967). Plasma volume decreases of around 12.5% have been reported with hematocrit increases of only 7.3% during a 10-day bed-rest study (Vogt and Johnson, 1967). These disproportional variations between the two measurements have been explained as a consequence of plasma protein movements into the cellular space and back depending of certain physiological states (i.e. exercise, recumbency, etc.; Van Beaumont et al., 1972). In this study we were experimenting with supraphysiological doses of E2, therefore it is possible that the physiological hematocrit response of the ewes in this experiment is different than that of ewes during estrus or early pregnancy. Consequently, this could be the reason of why we did not observe a decrease in hematocrit upon acute E2

administration. This is in contrast to the decreased hematocrit that our lab had previously observed during early pregnancy in sheep. Future studies addressing this hypothesis should be undertaken. Hematocrit values vary depending on the site of sample collection (Mchedlishvili and Varazashvili, 1986; Okazawa et al., 1996). In this study, decreased values in hematocrit were observed in the samples taken from both treatment groups after the implants were applied when compared to the day before. However, it is difficult to compare these values, since the day before the implants were applied the samples were taken from the jugular vein with a 1.5-inch needle rather than the catheter.

Greater uterine weight in ovariectomized E2-treated ewes observed in our study were also reported by others (Reynolds et al., 1998). Acute increases in uterine weight upon E2-treatment has been explained as a consequence of a rapid increase in uterine blood flow (Magness et al., 1998), hyperplasia, hypertrophy and an increase in microvascular volume in all the uterine tissue compartments (Reynolds et al., 1998).

Estradiol-17β could potentially have a major role in the early establishment and proper adaptation to pregnancy in ewes. It seems possible that an early increase in plasma volume, may be an important physiological cue for an adequate maternal adaptation to pregnancy. Greater E2 secretion by the ovulatory follicle could influence early plasma volume expansion and therefore result in a better physiological adaptation of the dam to pregnancy. Similarly, ewes that respond with an enhanced increase in plasma volume to E2 could be better prepared for a successful pregnancy. Further studies determining the effect of physiological doses of E2 on plasma volume increase and pregnancy success are necessary. If confirmed, our results could indicate a major role of E2 during fecundation and early pregnancy. Similarly, these results could suggest possible treatments for inadequate plasma volume expansion during fertilization and the beginning of pregnancy.

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CHAPTER 3. TIMING AND DURATION OF NUTRIENT RESTRICTION AND ITS IMPACTS ON PLACENTAL DEVELOPMENT AND UMBILICAL BLOOD FLOW IN ADOLESCENT SHEEP

Abstract

Nutrient-restriction beginning on day 50 of gestation in nulliparous ewe lambs decreases umbilical blood flow by day 80. We hypothesized that ewes would experience a decrease in umbilical blood flow upon nutrient restriction (day 50 to 90) and that blood flow would recover to control values upon realimentation during late gestation (d 90 to 130), or remain reduced in ewes that continued to be nutrient restricted. On d 50 of gestation, young nulliparous white face ewes (6 - 8 mo; n = 41) carrying singletons were randomly assigned to two dietary treatments where ewes received 100% of NRC recommendations (CON) or 60% of CON (RES). On day 90 of gestation, ewes either remained on CON or RES until day 130, or CON ewes were RES from day 90 to 130, or RES ewes were realimented to CON from day 90 to 130. This resulted in 4 treatment groups: CON-CON, CON-RES, RES-RES, RES-CON on day 130. On day 50, and every 10 days until day 110, umbilical blood flow, fetal and placental measurements were obtained via ultrasonography. Non-survival surgeries were performed on days 50, 90 and 130. Fetal and placental weights, uterine artery blood flow and umbilical blood flow were measured during surgery. Placentomes were collected to analyze binucleate cells numbers. The study was conducted as a completely randomized design arrangement with repeated measures. Data were analyzed using the MIXED procedure of SAS. Surgical fetal and placental weights, uterine artery blood flow and umbilical blood flow was analyzed with the GLM procedure of SAS. There was a significant interaction between nutritional treatment and day for period 1 (Day 50 to 90; $P < 0.01$) on umbilical blood flow with CON ewes having greater blood flow compared to RES by day 90. There were nutritional effects on fetal biparietal distance, abdominal width and kidney area in CON-RES with all these measurements increasing ($P \leq 0.05$) during periods of late gestation. We partially accept our hypothesis as nutrient restriction during mid gestation decreased umbilical blood flow however blood flow did not return to control levels upon realimentation. By day 130, fetal and placental weights were not decreased in RES-RES groups when compared with CON-CON groups. Binucleate cell numbers in the fetal trophoblast were not modified by nutritional treatments. Our findings suggest that an adequate placental development during mid gestation could protect the fetus from a decreased umbilical blood flow later on gestation when nutrients were limited by 40%.

Introduction

Seasonal conditions influence forage quality, which in turn influences nutrient availability, to the animal, particularly during pregnancy. Sheep are normally managed within grazing systems. Forages with lower quality have decreased nutrient components particularly protein and energy (Milchunas et al., 2004). During times of drought, ewes may experience deficient total energy requirements. Furthermore, during different stages of conceptus development, ewes may need additional nutritional inputs. Therefore, it is important for producers to know when during gestation to supplement animals with additional feed, especially in growing pregnant animals. Indeed, adolescent pregnant ewes compete with the fetus for nutrients (Redmer et al., 2004; Wallace et al., 2019). Consequently, conceptus from growing ewes are more susceptible to nutritional challenges than adult ewes (Redmer et al., 2004; Wallace et al., 2019).

The placenta is a temporary organ that allows for oxygen, nutrient and waste exchange between mother and fetus. In ruminants, this nutrient exchange occurs at specialized structures called placentomes. Nutrition during pregnancy affects fetal and placental development (Wu et al., 2004; Andrade et al., 2013; Lekatz et al., 2013). The majority of placental growth happens in the first two-thirds of pregnancy, with the placenta reaching its maximum weight by day 80 to 90 in sheep (Stegeman, 1974; Redmer et al., 2004). Impairing placental growth or utero placental blood supply affects fetal growth trajectory (Reynolds et al., 2005; Vonnahme et al., 2014). In humans and sheep, abnormal umbilical blood flows (UmbBF) are the earliest abnormalities observed through Doppler ultrasonography that could be detected in intrauterine growth restriction (Rigano et al., 2001; Ferrazzi et al., 2002). Our laboratory has demonstrated that in young primiparous ewes (6 to 12 months of age), a 40% nutrient restriction during mid to late gestation had reduced UmbBF from day 80 until day 130 of gestation (Lemley et al., 2012). Similarly, we have reported that uterine artery blood flow (UtBF) decreases on day 130 of gestation after 80 days of nutrient restriction (Lemley et al., 2012).

Limited studies have been done analyzing the effects of realimentation in pregnant animals. In beef cows, a study from our group demonstrated that a 40% nutrient restriction applied from day 30 to 140 of gestation did not change UtBF (Camacho et al., 2014). Upon realimentation (day 140 to 198 of gestation), uterine ipsilateral blood flow was increased in the previously restricted cows, compared to cows that never

experienced a nutrient restriction (Camacho et al., 2014). To our knowledge there is limited information investigating if realimentation rescues decreased UmbBF in the ewe.

In a similar nutritional model, our laboratory has demonstrated that hematocrit values were greater in multiparous pregnant ewes compared to during nutrient restriction (Vasquez-Hidalgo, 2016). The same study showed that nutrient restriction does not affect UmbBF in adult ewes (Vasquez-Hidalgo, 2016). Similarly, we showed that hematocrit values recover to control values by day 90 of gestation, the peak time of placental growth (Vasquez-Hidalgo, 2016). Our hypothesis is that maternal hematocrit increases due to nutrient restriction as a compensatory mechanism. Greater hematocrit increases shear stress, therefore potentially driving UtBF, thus stimulating UmbBF (Birchard, 1997; Salazar-Vazquez et al., 2010).

In sheep, placentomes are formed by multinuclear trophoblastic giant cells, trophoblastic binuclear cells (BNC), trophoblastic mononuclear cells, epithelial cells, endothelial cells, fibroblasts, immune cells and others. After implantation (about day 16), binucleate cells are found in placental tissues in all ruminants throughout normal pregnancy (Wooding, 1982). Binucleate cells contribute to the overall function of the placenta, namely in allowing the placenta to be more efficient in nutrient delivery to the fetus. In rodents, angiogenic and vasoactive factor production by trophoblast giant cells (similar to BNCs in sheep) is critical for maternal vascular development in the pregnant uterus (Cross et al., 2002). In sheep, important molecules, such as somatomammotropin, are produced by the BNCs (Baker et al., 2016). Moreover, a knockdown of placental lactogen in BNCs causes intrauterine growth restriction in ewes (Baker et al., 2016). Additionally, our lab has shown that BNC numbers in ovine placenta are affected by nutritional changes during pregnancy (Vasquez et al., 2016).

The objective of the present study was to determine if realimenting previously mid-gestation restricted pregnant adolescent ewes would restore UmbBF to control levels. We hypothesized that during restriction, UmbBF, UtBF and fetal and placentome measurements would be lower as compared to adequately fed ewes, but upon realimentation, ewes would have similar UmbBF, UtBF, fetal and placentome measurements as ewes that were never restricted. Similarly, we hypothesized that hematocrit would increase in restricted ewes and would normalize after realimentation. Finally, we investigated the changes in BNCs during the different nutritional challenges, hypothesizing that numbers would decrease during nutrient restriction periods and would be positively related to fetal weights.

Materials and methods

Animals and experimental design

Animal care and use were according to protocols approved by the North Dakota State University Animal Care and Use Committee (#A18009).

Ninety western white face ewe lambs were transported to the Animal Nutrition and Physiology Center (ANPC; Fargo, ND) in July of 2017, placed on pasture, and provided hay and corn to meet or exceed maintenance nutrient requirements (NRC, 1985). Water was provided for *ad libitum* intake. On September 2017, three rams of similar breed fitted with crayon marking harnesses were placed with the ewes. Mating was recorded every 12 h. Pregnancy diagnosis and fetal enumeration were performed via ultrasonography at days 30 to 40 of gestation. Forty-eight dams carrying singletons were transported inside into a temperature-controlled facility (14°C; ANPC) with a 12:12-h light-dark cycle for the remainder of the study. Breeding dates ranged from September 20, 2017 to February 19, 2018. At day 40 of gestation, confirmation of fetal enumeration via ultrasonography (Aloka alpha 6; Aloka America, Wallingford, CT) was performed inside ANPC and ewes were housed in individual pens (0.91 x 1.2 m). From days 40 to 50 of gestation, ewes were adapted to an entirely pelleted diet in a stepwise manner and were fed once a day in the morning to meet maintenance nutrient requirements (Table 1; NRC, 1985). Water was provided for *ad libitum* intake for the remaining of the study. At day 50 of gestation, after ultrasonography measurements were obtained, 7 ewes were euthanized after surgical data was collected and tissues were collected as described below. From days 50 to 90 of gestation ewes were fed either 100% of nutrient requirements (CON; n = 20), or 60% of CON (RES; n = 21). On day 90 of gestation, after ultrasonography measurements were obtained, 14 ewes were euthanized after surgical information was collected and tissues were collected as described below. From days 90 to 130 of gestation the remaining CON ewes either continued to be fed 100% of nutrient requirements (CON-CON; n = 6) or were fed 60% of CON (CON-RES; n=7), the remaining RES ewes either continued to be fed 60% of CON (RES-RES; n = 7) or were fed 100% of nutrient requirements (RES-CON; n = 7). On day 130 of gestation, after ultrasonography measurements were obtained, all remaining ewes were euthanized after surgical data was obtained and tissues were collected as described below. Ewes were weighed every 10 days from day 50 to day 130 of gestation. Nutrient requirements were adjusted relative to body weight to provide the nutritional treatments described above. Maternal body

condition was scored (1 to 5 scale, with 1 = emaciated and 5 = obese) by one experienced evaluator on days 50, 80, 110 and 130 of gestation.

Table 3.1. Diet composition

Ingredients	Percentage
Corn	9.3
SBM	4.0
Beet pulp	28.9
Alfalfa meal	33.4
Wheat middlings	24.4
Total	100

Diet per kg: 2.659 Mcal; CP: 169.8 g; MP: 118.86 g; NDF: 369.5 g; Starch: 294.24 g; Ca: 7.33 g; P: 4.084 g; Cu: 104.44 ppm; Se: 3.813 ppm. Diet based on NRC recommendations (NRC, 1985).

Ultrasonography measurements

Beginning on day 50, and every 10 days until day 110, ewes were restrained in an elevated cart so that conceptus measurements and umbilical hemodynamics could be obtained as previously described (Lemley et al. 2012; Prosound Alpha 6, Hitachi Aloka, Wallingford, CT). Ultrasonography examinations started at 7 AM each day and ended before 11 AM. Ewes were fed after ultrasound evaluations took place. Conceptus measurements included the length and width from 10 random placentomes, fetal biparietal and abdominal lengths, and kidney length and width. Placentome area was calculated by multiplying width by height measurements for each placentome. For umbilical hemodynamic measurements, Doppler mode was used to obtain UmbBF, pulsatility index (PI) and resistance index (RI, an indirect measure of vessel wall resistance to dilation that is negatively correlated with placental flow; Mari and Hanif 2008) as previously described (Lemley et al. 2012). Maternal jugular vein samples were collected via jugular venipuncture in the morning before ultrasound measurements on days 50, 60, 70, 80, 90, 100, 110, 120 and 130 of gestation. Hematocrit was measured immediately after blood collection (Unico micro-hematocrit centrifuge, Dayton, NJ).

Gestational days 50, 90 and 130 intraoperative ultrasonography measurements and tissue collection.

On days 50, 90 and 130 of gestation, dams were weighed and anesthetized with sodium pentobarbital 50 mg/ml per 20 kg of body weight. All surgeries took place before 11 AM. Anesthesia was maintained via a jugular catheter. The abdomen was sheared and cleaned with Betadine scrub. The following surgery procedures were non-survival; however, aseptic techniques were used to preserve blood and tissues for further analysis. Maternal heart rate and breathing periodicity were assessed through a veterinary phonendoscope. The uterus was exposed via a midventral laparotomy, covered with warm, moistened surgical towels, and liberal amounts of physiologic saline (37°C) were applied to the uterus every 5 min. The gravid uterine artery was located and hemodynamics (blood flow, PI, RI) were assessed by color Doppler ultrasonography as previously described (Lemley et al., 2012). Intraoperative measurements were recorded by placing the finger transducer next to the uterine artery and prior to the first bifurcation of the uterine artery. Intraoperative measurements of umbilical artery hemodynamics were recorded by scanning the gravid uterine horn until a clear image of the umbilical artery was located (Lemley et al., 2012). After ultrasonography measurements were recorded, dams were euthanized with an overdose of sodium pentobarbital (50 mg/ml). Ceasing of heart beats and respiratory movements was assessed through a veterinary phonendoscope. Fetal and placental weights were recorded. Placentomes were collected from the placenta and were fixed for immunohistochemistry.

Placentome immunohistochemistry and binucleate cells (BNC) image analysis

Placentome sections (3 μm thickness) were mounted onto slides and de-paraffinized in xylene and graded alcohol. Tissue sections underwent antigen recovery by boiling in 0.01 M citric buffer followed by cooling to room temperature and followed by overnight incubation at 4ºC with a specific primary antibody to detect target proteins. Tissue sections were stained sequentially with biotinylated DBA lectin, Texas red avidin and fluorescein BSL I lectin, all diluted in 10% normal goat serum. DAPI mounting media was used to visualize the nuclei of the cells.

Images from random placentome areas were generated using an Axio Imager microscope (Zeiss Inc., Thornwood, NY). Image analysis of the slides was performed using Axio vision rel 4.8 software. Five

representative images per sample were obtained at magnification of 20x. Binucleate cell number within the fetal trophoblast was averaged per sample for statistical analyses.

Statistical analyses

The research was conducted as a completely randomized factorial arrangement with repeated measures. Nutritional treatments (CON, RES) from day 50 to 90 of gestation was designated as factor 1 (Period 1), nutritional treatments (CON, RES) from day 90 to 130 was designated as factor 2 (Period 2). Repeated data were analyzed using the MIXED procedure of SAS (SAS software version 9.4, SAS Institute, Cary, NC). Ewe was treated as a random independent variable; treatment and day were treated as fixed effects. Umbilical blood flow, PI, RI, placentome area, fetal biparietal and abdominal lengths, kidney area, ewe body weight and hematocrit were the dependent variables. Coding included ewe, treatment and day in the class statement; day was included in the repeated statement, dependent variables (treatment, day and their interaction) were included in the model statement, LSmeans were separated using the PDIFF option of the LSMEANS statement.

Data collected at surgery and postmortem were analyzed using the GLM procedure of SAS, class statement included dietary treatment and the model statement included UtBF, UmbBF, fetal and placental weights and BNC numbers. Data were analyzed by day of collection, interactions between day and diet and by the main effect of day. Since day 50 data had no dietary treatment it is included only in day analyses. Means were separated using the LSMEANS statement.

For all data collected, P values ≤ 0.05 are considered significant. Tendencies are described when P values are > 0.05 but ≤ 0.10.

Results

Ewe body weights and body condition score

A treatment by day interaction was observed for period 1 ($P < 0.01$; figure 1) and period 2 ($P < 0.01$; figure 1) for maternal body weight. Maternal body weights were similar ($P \geq 0.61$) between all dams on day 50 of gestation (Figure 1). Maternal body weights of CON-CON and CON-RES were similar between each other from day 50 of gestation until day 120 of gestation (Figure 1). On day 130 of gestation, CON-CON had a greater body weight when compared to CON-RES ($P = 0.05$; 52.16 vs 47.24 $\pm$ 1.77 kg; figure 1). From day 50 of gestation until day 90 of gestation RES-CON and RES-RES dams had similar body

weight (Figure 1), and both groups tended (P = 0.08) to have decreased body weight and had (P = 0.05) a decreased body weight on days 80 and 90 of gestation respectively when compared to CON-CON and CON-RES (Figure 1). On day 100 of gestation RES-CON tended to have an increased body weight when compared to RES-RES (P = 0.10; 47.17 vs 43.29 $\pm$ 1.64 kg; figure 1). From day 110 to 130 of gestation body weight of RES-CON dams was greater when compared to RES-RES (Figure 1). From day 100 to 130 of gestation RES-CON dams had similar weights to CON-CON and CON-RES (Figure 1). However, CON-RES did not reach RES-RES body weights by day 130 of gestation (P = 0.07; 47.24 vs 42.96 $\pm$ 1.64 kg, respectively; figure 1). Body condition score (BCS) was not affected by nutritional treatment and/or by day interactions for period 1 (Days 50 and 80 of gestation; P = 0.91; figure 2). However, there was a treatment by day interaction for period 2 (Days 100 and 130 of gestation; P = 0.02; figure 2). On days 110 and 130 of gestation, CON-CON and RES-CON ewes tended (P = 0.08) to have greater and had greater (P = 0.02) BCS than CON-RES and RES-RES ewes respectively (Figure 2). Finally, for all ewes BCS decreased as day of gestation advanced (Figure 2).

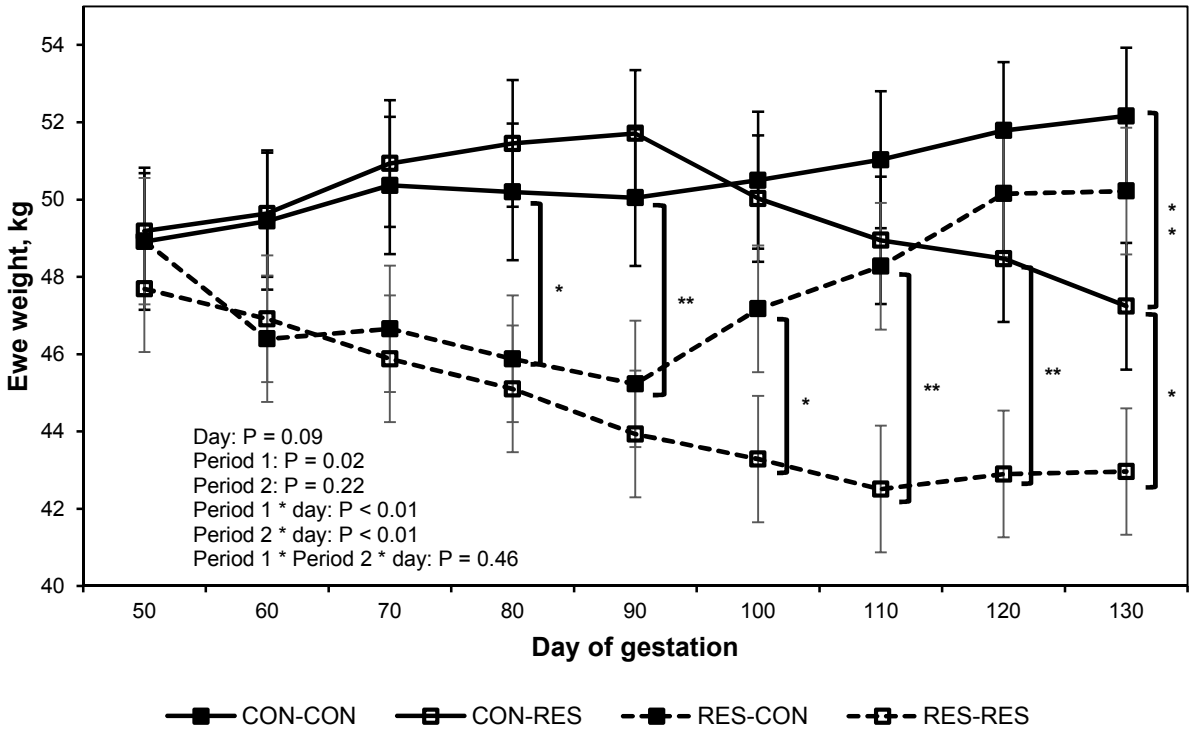

Figure 3.1. Impacts of maternal nutrition on ewe weight from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). Differences between CON-CON, CON-RES, RES-CON and RES-RES are denoted by ** P ≤ 0.05, * P ≤ 0.10 within a day.

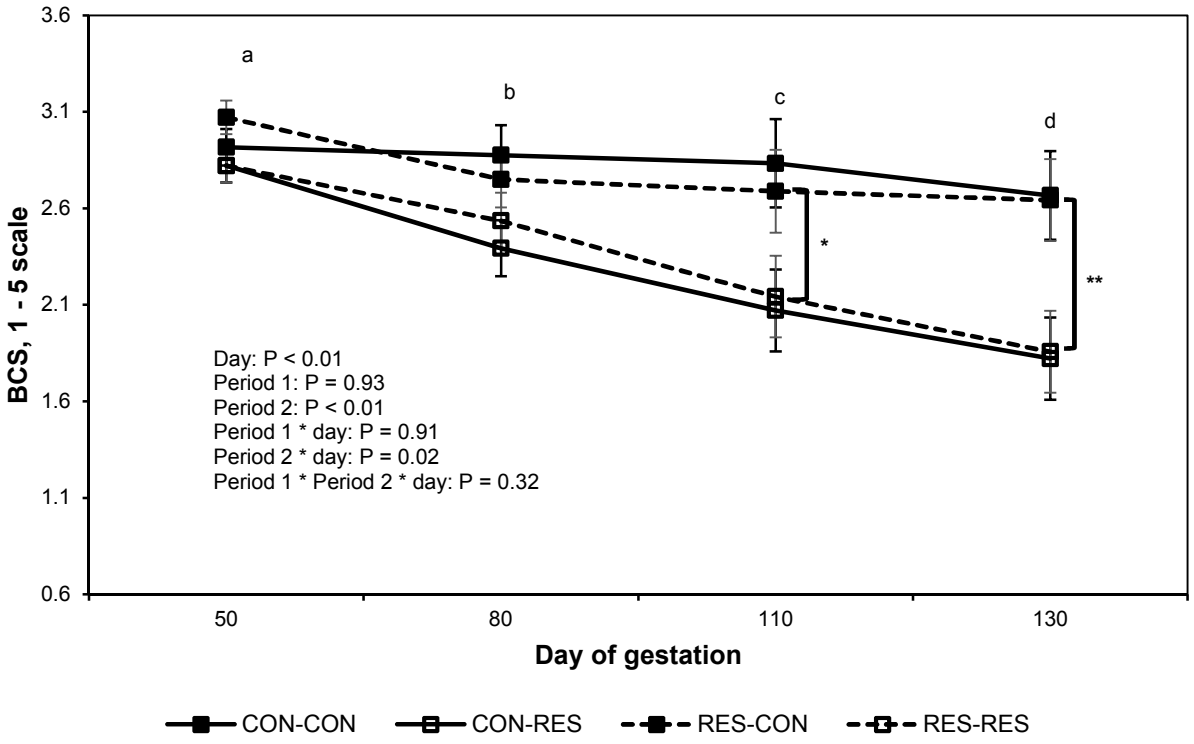

Figure 3.2. Impacts of maternal nutrition on ewe body condition score from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 and 80) and/or period 2 (day 110 and 130). [abcd]LSMEANS ± SEM between day differ P ≤ 0.05. Differences between CON-CON, CON-RES, RES-CON and RES-RES are denoted by ** P ≤ 0.05, * P ≤ 0.10 within a day.

Ultrasonography measurements and hematocrit

There was a treatment by day interaction for period 1 (P < 0.01; figure 3) and no treatment by day interaction for period 2 (P = 0.86; figure 3) in UmbBF. Dams of CON-CON and CON-RES tended to be greater (P ≤ 0.09) and were greater (P ≤ 0.04) than RES-CON and RES-RES in days 90 and 110 of gestation respectively (Figure 3). For all ewes UmbBF increased as gestation advanced (Figure 3). Treatment by day tended to have an interaction for period 1 (P = 0.06; figure 4), with no treatment by day interaction for period 2 (P = 0.55; figure 4) in PI. All ewes were similar (P ≥ 0.80; figure 4) between them on day 50 of gestation. Dams of CON-RES, RES-CON and RES-RES had similar (P ≥ 0.23; figure 4) PI on days 60 and 70 of gestation. Ewes of CON-CON had decreased (P ≤ 0.05; figure 4) PI than CON-RES, RES-CON and RES-RES on day 60 of gestation. On day 80 of gestation, CON-RES ewes tended to have greater PI than RES-RES (P = 0.08; 1.16 vs 1.03 ± 0.05; figure 4). On day 100 of gestation, CON-RES

tended to have greater PI than RES-CON (P = 0.10; 0.96 vs 0.83 ± 0.05; figure 4). On days 90 and 110, PI was similar between all groups (Figure 4). For all ewes PI was greatest on day 80 and lowest on days 50, 100 and 110 of gestation with days 60, 70 and 90 being intermediate (Figure 4). Resistance index was not affected by treatment and day of gestation for period 1 (P = 0.26) or period 2 (P = 0.27), nor a main effect of day of gestation (P = 0.17).

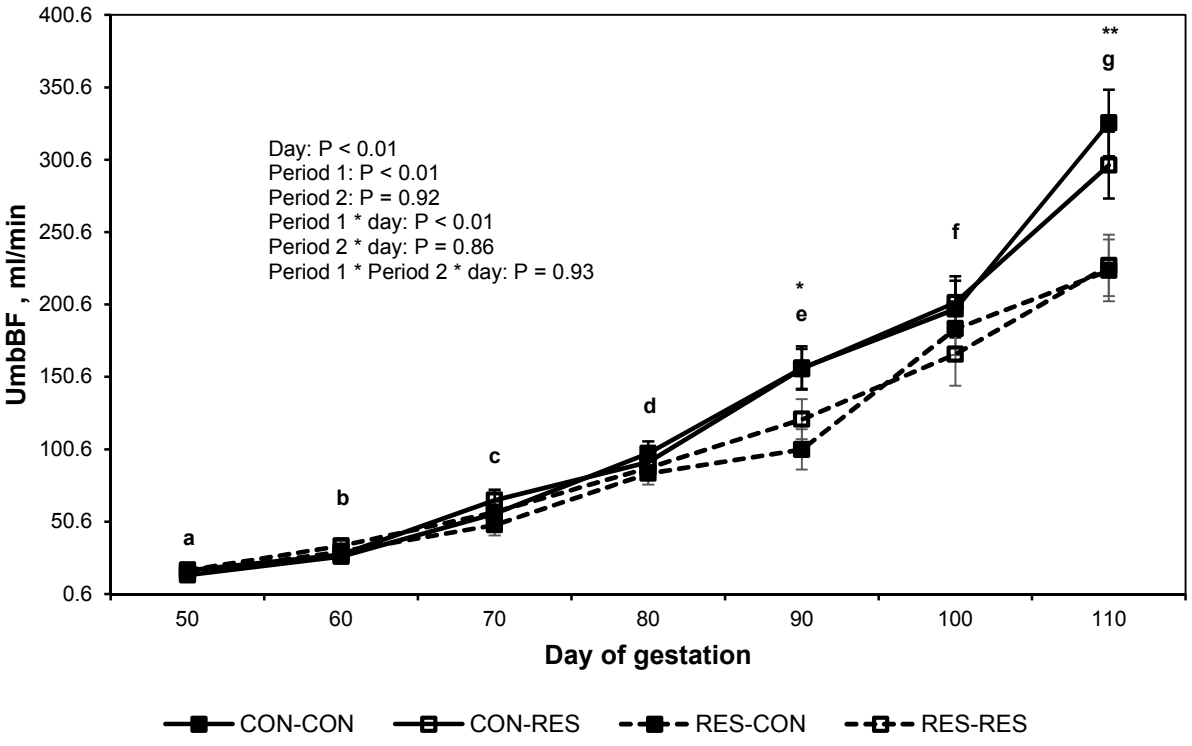

Figure 3.3. Impacts of maternal nutrition on ewe umbilical blood flow from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). ^{abcdefg}LSMEANS ± SEM between day differ P ≤ 0.05. Differences between CON-CON, CON-RES, RES-CON and RES-RES are denoted by ** P ≤ 0.05, * P ≤ 0.10 within a day.

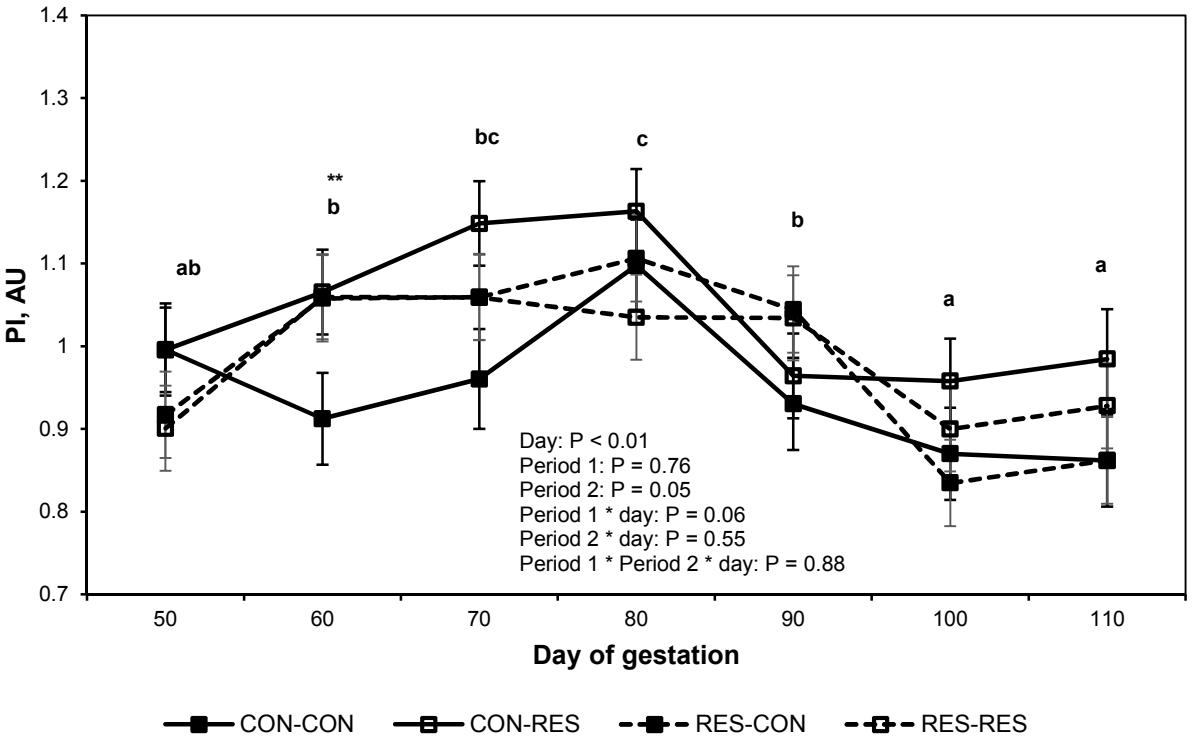

Figure 3.4. Impacts of maternal nutrition on ewe umbilical pulsatility index arbitraty units (AU) from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). [abc]LSMEANS ± SEM between day differ P ≤ 0.05. Differences between CON-CON, CON-RES, RES-CON and RES-RES are denoted by ** P ≤ 0.05 within a day.

There was a treatment by day interaction for period 1 (P < 0.01; figure 5) and no treatment by day interaction for period 2 (P = 0.86; figure 5) in biparietal distance. On day 80 of gestation, CON-RES fetuses had greater (P ≤ 0.05; figure 5) biparietal distances than CON-CON, RES-CON and RES-RES fetuses. On day 110 of gestation, fetal biparietal distances were greater in CON-RES than RES-RES (P < 0.01; 4.96 vs 4.56 ± 0.13 cm; figure 5). On days 50, 60, 70, 90 and 100 of gestation, biparietal distances were similar in all groups (Figure 5). Biparietal distances increased in all groups as gestation advanced (Figure 5). There were no treatment by day interactions for period 1 (P = 0.39; figure 6) or period 2 (P = 0.51; figure 6) for abdominal distance. However, there was a main nutritional effect for period 1 (P = 0.04; figure 6). On day 110 of gestation, CON-RES fetuses tended (P = 0.08; figure 6) to have greater abdominal distances than RES-CON and RES-RES fetuses (Figure 6). On days 50, 60, 70, 80, 90 and 100, abdominal distances were similar between all groups (Figure 6). Abdominal distances increased in all groups as gestation advanced (Figure 6). There was a three-way interaction of nutritional treatments of period 1, period 2 and

day of gestation (P = 0.04; figure 7) for kidney area. On day 80 of gestation, RES-CON fetuses tended to have greater kidney area than CON-CON fetuses (P = 0.10; 1.86 vs 1.32 ± 0.24 cm^2). On day 90 of gestation, kidney area from CON-RES fetuses was greater than from CON-CON fetuses (P = 0.03; 3.23 vs 2.53 ± 0.24 cm^2). On day 100 of gestation, fetuses from CON-RES and RES-CON had greater (P < 0.01) kidney areas than fetuses from CON-CON and RES-RES groups (Figure 7). On day 110 of gestation, fetuses from RES-CON tended to have greater kidney areas than CON-RES (P = 0.07; 5.62 vs 5.05 ± 0.22 cm^2). On days 50, 60 and 70, kidney areas were similar in fetuses between all groups (Figure 7). Kidney areas increased as gestation advanced in fetuses from all groups (Figure 7).

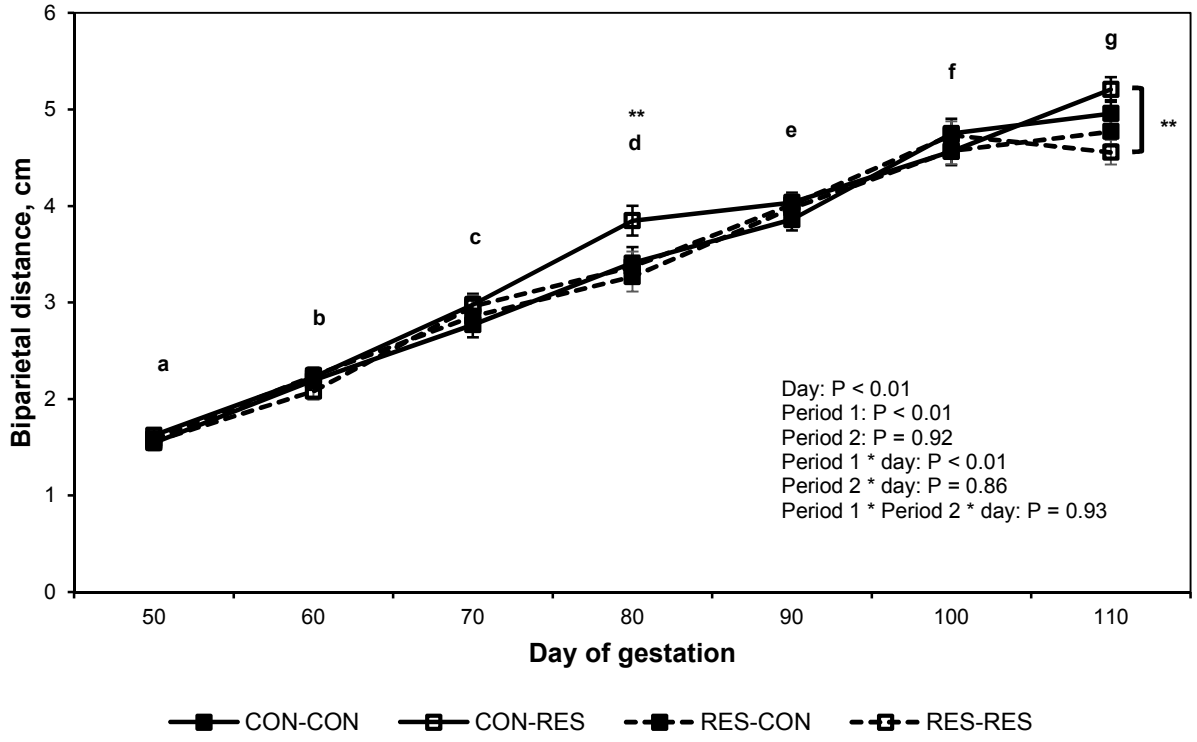

Figure 3.5. Impacts of maternal nutrition on fetal biparietal distance from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). [abcdefg]LSMEANS ± SEM between day differ P ≤ 0.05. Differences between CON-CON, CON-RES, RES-CON and RES-RES are denoted by ** P ≤ 0.05 within a day.

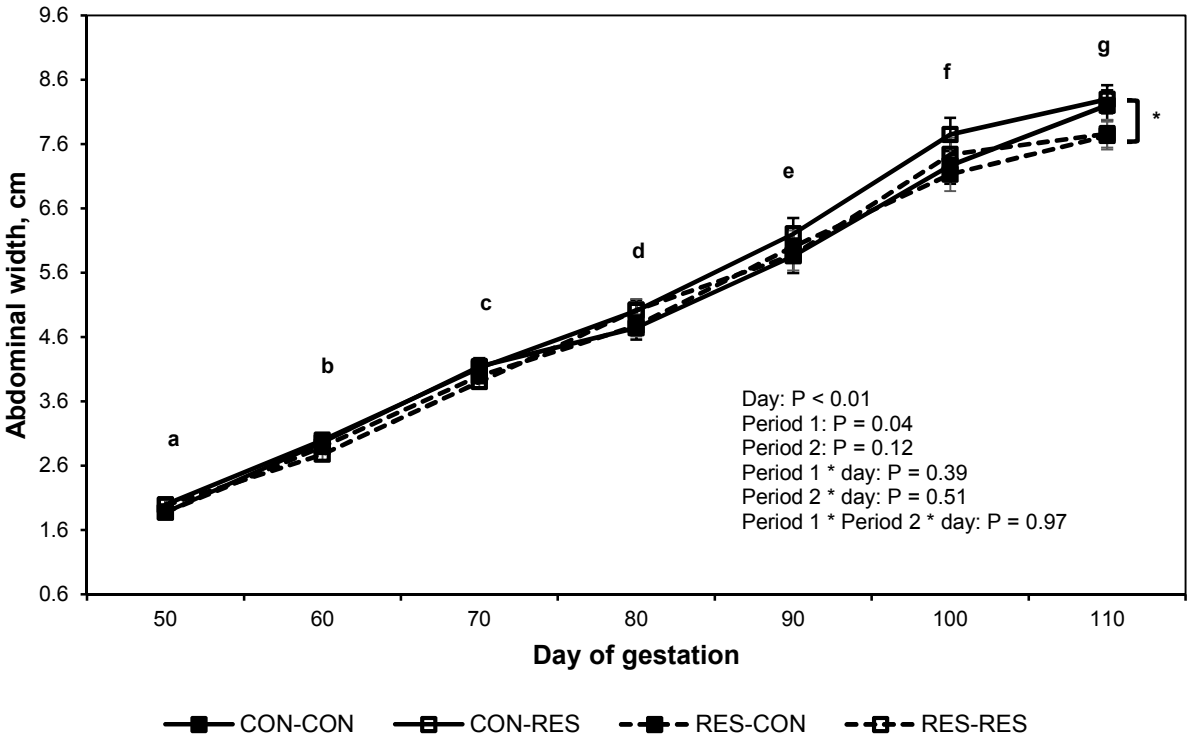

Figure 3.6. Impacts of maternal nutrition on fetal abdominal width from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). [abcdefg]LSMEANS ± SEM between day differ P ≤ 0.05. Differences between CON-CON, CON-RES, RES-CON and RES-RES are denoted by * P ≤ 0.10 within a day.

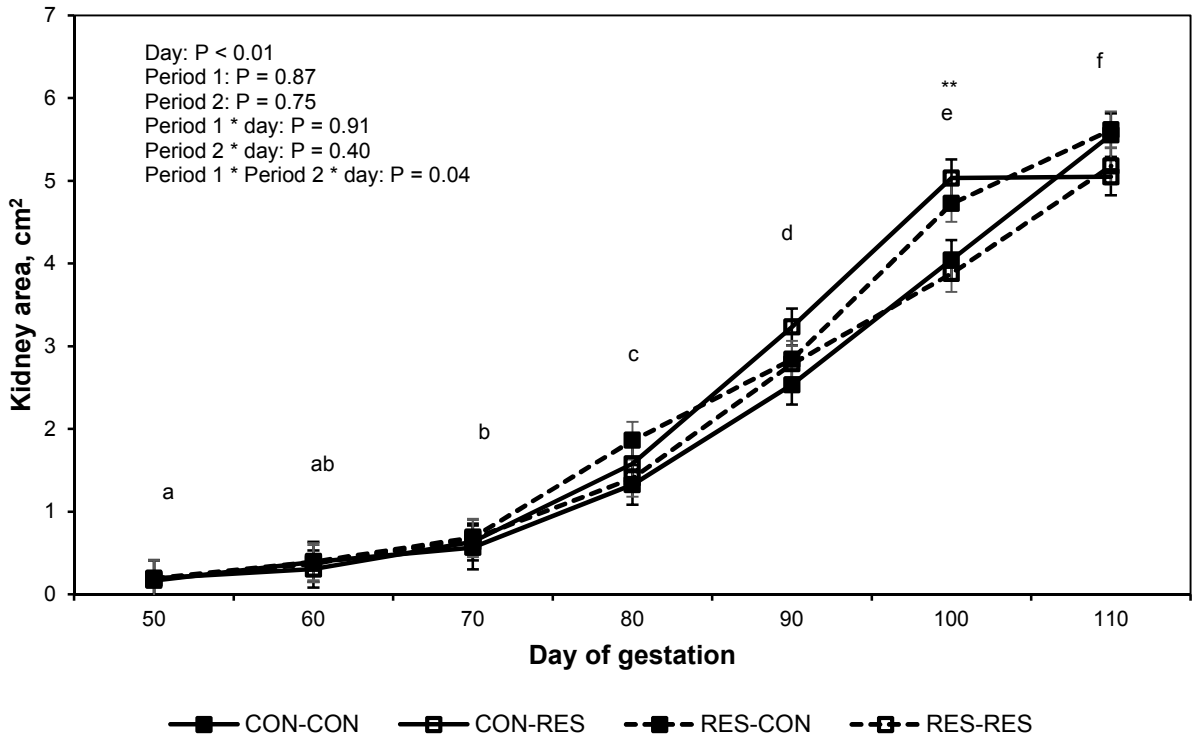

Figure 3.7. Impacts of maternal nutrition on fetal kidney area from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). [abcdef]LSMEANS ± SEM between day differ P ≤ 0.05. Differences between CON-CON, CON-RES, RES-CON and RES-RES are denoted by ** P ≤ 0.05 within a day.

There were no interactions of treatment by day for period 1 (P = 0.11; figure 8) or period 2 (P = 0.16; figure 8) for placentome area. Similarly, there were no nutritional main effects on period 1 (P = 0.25; figure 8) or period 2 (P = 0.72; figure 8). Placentome area increased during days 50 and 60 of gestation and reached their greatest area during days 70, 80 and 90 of gestation (Figure 8). During days 100 and 110 of gestation, placentome area were greater than those of day 60, but smaller than days 70, 80 and 90 of gestation (Figure 8).

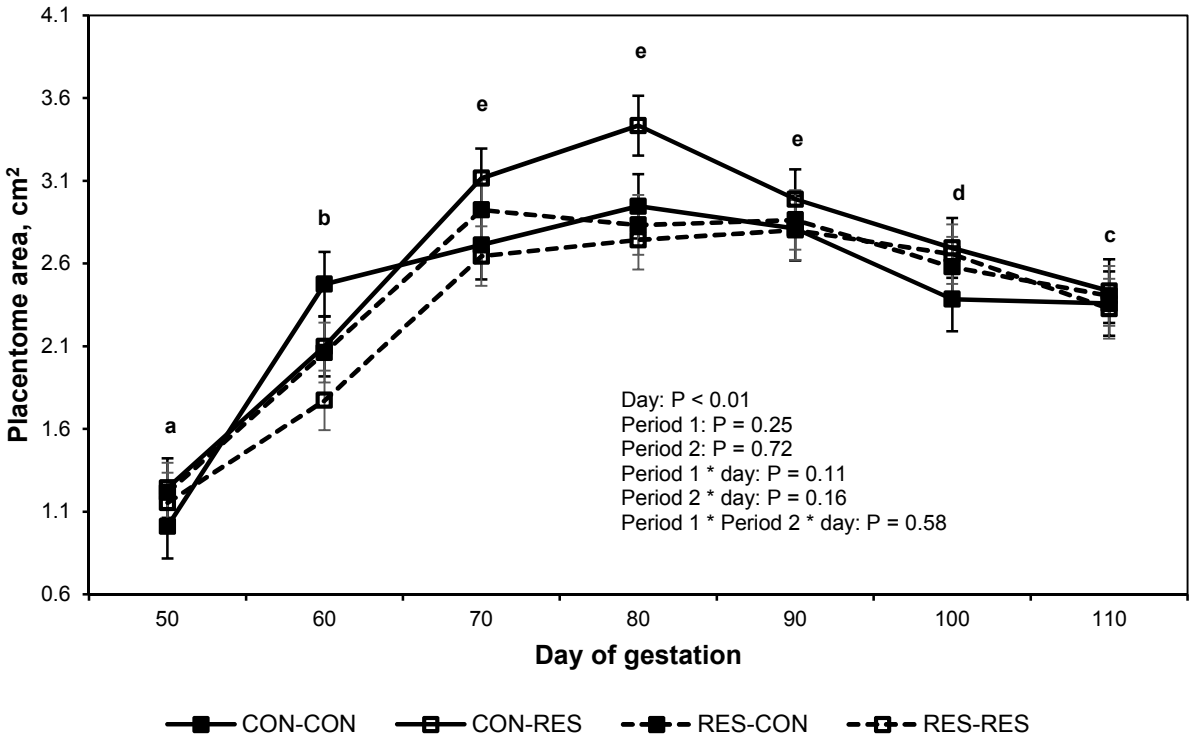

Figure 3.8. Impacts of maternal nutrition on placentome area from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). [abcde]LSMEANS ± SEM between day differ $P \leq 0.05$.

There were no interactions of treatment by day for period 1 (P = 0.32; figure 9) or period 2 (P = 0.22; figure 9) for maternal hematocrit. Similarly, there were no nutritional main effects on period 1 (P = 0.76; figure 9) or period 2 (P = 0.89; figure 9). Hematocrit decreased in all groups as gestation advanced (Figure 9).

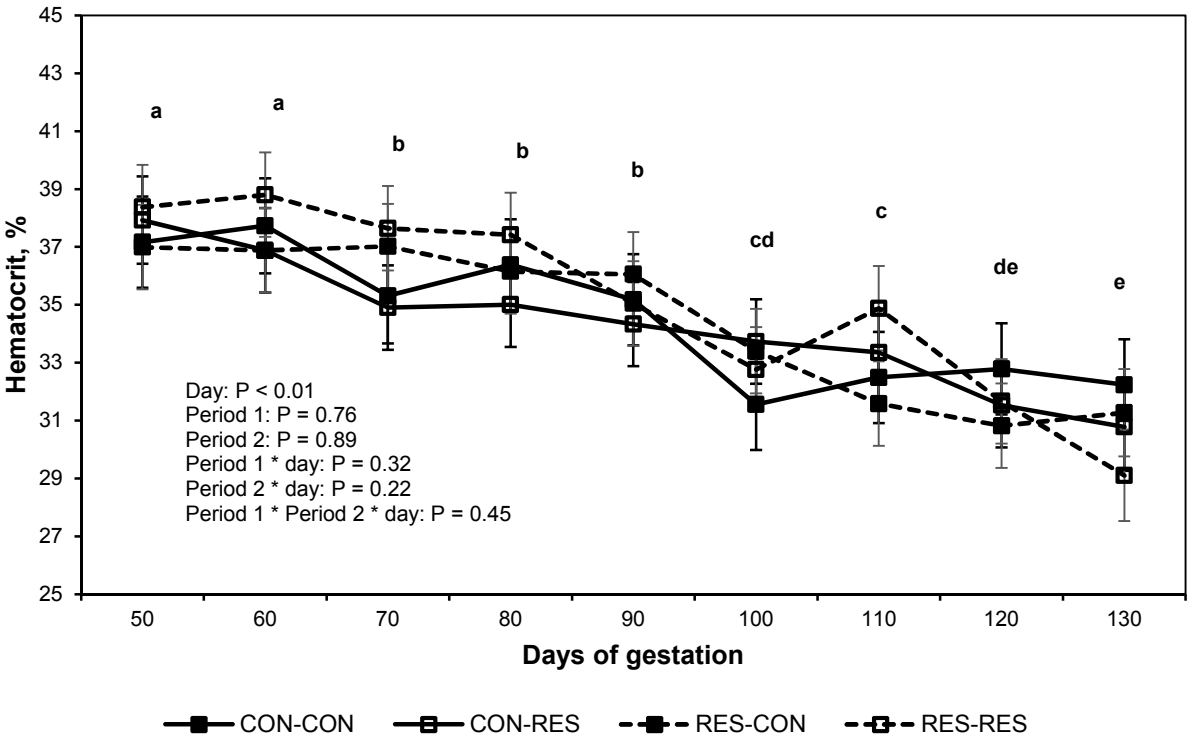

Figure 3.9. Impacts of maternal nutrition on maternal hematocrit from d 50 to 130 of gestation. CON = 100% of NRC recommendations. RES = 60% of CON. Restriction was divided into period 1 (day 50 to 90) and/or period 2 (day 90 to 130). [abcde]LSMEANS ± SEM between day differ $P \leq 0.05$.

Gestational days 50, 90 and 130 intraoperative ultrasonography measurements, placental and fetal weights and binucleate cell number.

Fetal weight, uterine blood flow and umbilical blood flow all increased as gestation advanced (Table 2). Placental weights were greater on days 90 and 130 of gestation when compared to day 50 of gestation (Table 2). Binucleate cell numbers within the fetal trophoblast were greatest on day 90 of gestation, intermediate on day 50 of gestation and smallest on day 130 of gestation (Table 2).

Table 3.2. Binucleate cell and surgical measurements obtained on days 50, 90 and 130 of gestation

	Day 50	Day 90	Day 130	SEM	*P* value
Placental weight, g	112.31[a]	598.87[b]	508.56[b]	39.21	< 0.01
Fetal weight, g	18.89[a]	613.24[b]	3488.78[c]	40.89	< 0.01
UtBF, ml/min	255.26[a]	464.79[a]	1218.2[b]	125.54	< 0.01
UmbBF, ml/min	12.58[a]	134.50[b]	431.37[c]	29.81	< 0.01
BNC, No. per mf	17.72[ab]	19.78[a]	15.56[b]	1.12	< 0.01

UtBF = Uterine artery blood flow; UmbBF = Umbilical artery blood flow; BNC = Binucleate cells; mf = microscopic field.

Length of nutrient restriction had a nutritional treatment by day interaction for UBF (Table 3). Greatest UmbBF was detected in RES-RES ewes on day 130 of gestation, intermittent in CON-CON values

on day 130 of gestation, and lowest in CON and RES ewes on day 90 of gestation (Table 3). There were no nutritional treatment by day interactions for length of nutrient restriction on placental weight, fetal weight, uterine artery blood flow and BNC numbers (Table 3).

Table 3.3. Length of nutrient restriction effects on BNC number and surgical measurements

	Day 90		Day 130		SEM	P value		
	CON	RES	CON-CON	RES-RES		Trt	Day	Trt* Day
Placental weight, g	598.87	572.25	508.56	515.79	63.82	0.86	0.19	0.76
Fetal weight, g	613.24	567.46	3488.78	3424.93	79.74	0.43	< 0.01	0.90
UtBF, ml/min	508.34	420.17	1163.42	796.18	211.60	0.17	0.01	0.44
UmbBF, ml/min	134.43[a]	98.79[a]	431.37[b]	684.87[c]	72.66	0.1	< 0.01	0.03
BNC, No. per mf	20.65	20.15	16.73	14.67	1.05	0.24	< 0.01	0.48

UtBF = Uterine artery blood flow; UmbBF = Umbilical artery blood flow; BNC = Binucleate cells; mf = microscopic field.

There were no effects of nutrient restriction on placental weight, fetal weight, uterine artery blood flow, UBF or BNC numbers on day 90 of gestation (Table 4).

Table 3.4. Nutrient restriction effects on BNC number and surgical measurements, day 90

	CON	RES	SEM	P value
Placental weight, g	598.87	572.25	51.25	0.72
Fetal weight, g	613.24	567.46	27.76	0.27
UtBF, ml/min	464.79	376.60	81.50	0.46
UmbBF, ml/min	134.43	98.79	17.25	0.17
BNC, No. per mf	18.62	20.36	0.96	0.21

UtBF = Uterine artery blood flow; UmbBF = Umbilical artery blood flow; BNC = Binucleate cells; mf = microscopic field.

There was a tendency for nutritional treatments to have an effect on BNC numbers on day 130 of gestation (Table 5). Nevertheless, there were no effects of nutritional treatments on placental weight, fetal weight, uterine artery blood flow and UBF on day 130 of gestation (Table 5).

Table 3.5. Nutrient restriction effects on BNC number and surgical measurements, day 130

	CON-CON	CON-RES	RES-CON	RES-RES	SEM	P value
Placental weight, g	508.56	487.39	523.02	515.79	55.75	0.89
Fetal weight, g	3547.70	3644.86	3216.38	3160.34	246.03	0.67
UtBF, ml/min	1216.37	1524.17	985.58	1068.12	175.21	0.14
UmbBF, ml/min	443.56	757.53	415.22	498.59	118.39	0.17
BNC, No. per mf	17.04	14.50	16.23	15.51	1.41	0.06

UtBF = Uterine artery blood flow; UmbBF = Umbilical artery blood flow; BNC = Binucleate cells; mf = microscopic field.

Discussion

Our lab has previously reported that a 40% nutrient restriction decreases UmbBF during mid-gestation (day 80; Lemley et al., 2012). However, we have also reported that a 40% nutrient restriction

during mid-gestation does not decrease UmbBF in adult multiparous or adult nulliparous ewes (Vasquez-Hidalgo, 2016). In this study we confirm that nutrient restriction during mid-gestation decreases UmbBF in young nulliparous ewes. We also report that refeeding nutrient restricted ewes, after placental growth ends, does not rescue UmbBF values to control levels. Placental growth in sheep ceases on days 80 to 90 of gestation (Redmer et al., 2004; Vasquez-Hidalgo, 2016). In sheep, after day 90, among other characteristics, placental development is focused on vascular expansion, adequate vasodilation and blood flow (Vonnahme, 2012; Vonnahme, 2013). Therefore, in sheep it is crucial to achieve and adequate placental growth during mid-gestation. Since our observations demonstrate that UmbBF cannot be rescued by realimentation after day 90 of gestation, we suggest that the inherited ability of the placenta to provide an adequate UmbBF to the fetus is hindered by nutrient restriction during mid-gestation. Indeed, many studies have shown the negative effects of undernutrition on angiogenesis and blood vessels vasodilation in the sheep placenta (Lekatz et al., 2010; Reviewed in Wallace 2019). Similarly, refeeding undernourished ewes to control values does not rescue blood vessel formation (Reviewed in Wallace et al., 2019). In this study, placental weights were not reduced, and placentome area was not decreased in nutrient restricted ewes. However, analyzing placentome area only by the effect of nutrient restriction during mid-gestation (period 1; data not shown) we observe a decrease on placentome area in nutrient restricted dams on days 60 and 80 of gestation. These observations confirm the negative effect of nutrient restriction during mid-gestation on placental growth.

Pulsatility and resistance indexes were used in the onset of Doppler ultrasonography routines in hemodynamic assessments of pregnancy (Galan et al., 1998). These indices were used as an indirect measurement for blood flow (Mari and Hanif; 2008). Indeed, greater PI and RI are expected with decreased blood flow and vice versa (Galan et al., 1998). We report no effect of nutrient restriction on RI and a slight effect of nutrient restriction on increasing umbilical artery PI during mid-gestation. Since in this and previous studies (Lemley et al., 2012) we have demonstrated clear effects of nutrient restriction in UBF along with slight effects on PI and RI in adolescent sheep, we conclude that, in this model, changes in UBF are not entirely mirrored by changes in PI and RI.

Our lab previously observed decreased fetal weights and UtBF in nutrient restricted ewes on day 130 of pregnancy (Lemley et al., 2012 In this study, we did not observe the same effects after an 80-day

nutrient restriction. However, we report here that UtBF increased in ewes after 80 days of nutrient restriction compared to control animals. Moreover, at day 130, UmbBF in restricted animals was not different than controls. The recovery of uterine and umbilical blood flows by day 130 in restricted animals is interesting and might be related to unaffected fetal weights in this study. Nutritional insults in sheep have been shown to decrease maternal metabolic growth factors (De Brun et al., 2015; Reviewed in Wallace, 2019), modify maternal steroidal hormone concentration (Lekatz et al., 2010; Satterfield et al., 2011; Reviewed in Vonnahme et al., 2013; Reviewed in Wallace et al., 2019) and amino acid and glucose transport (Bell et al., 1999). Further analyses investigating these factors will help elucidate the observations found in this study.

Fetal biparietal, abdominal and kidney dimensions displayed increased values during the initial portion of late gestation (days 80 to 110) in CON-RES animals compared to the other nutritional treatment groups. Similarly, although not significant, surgical UtBF and UmbBF values were numerically the greatest in the same group on day 130 of gestation. The importance of adequate nutrition during mid gestation on placental development has been previously discussed. We suggest that upon an adequate nutrition during mid gestation, placental tissues are able to resist and compensate a nutritional challenge during late gestation. Moreover, we have previously reported that BNC numbers seem to be more affected with mid-gestation undernutrition than late-gestation undernutrition (Vasquez et al., 2016). However, in this study BNC number was not related to uterine and umbilical blood flows or fetal sizes as it was hypothesized. Nevertheless, it would be interesting to measure growth factors that are secreted by BNCs upon nutritional insults during the different periods of gestation. In fact, our lab has already reported different gene expressions in BNCs during different days of gestation (Vasquez-Hidalgo et al., 2019). Whether nutritional insults will affect those expressions remains to be investigated.

Previous observations from our lab had shown that hematocrit increases in pregnant nutrient restricted ewes (Vasquez-Hidalgo, 2016). In this study we do not observe any effect on hematocrit upon nutrient restriction or realimentation. However, our previous studies were performed in adult ewes, while this study is performed in young, growing animals. Moreover, in our previous studies (Vasquez-Hidalgo, 2016) we did not observe any effects of nutrient restriction on umbilical hemodynamics. Therefore, it is possible that an increase in hematocrit is a compensatory response to undernutrition in fully developed

adult animals. Indeed, in women anemia is related with preterm birth and low birth weight (Zhou et al., 1998; Xiong et al., 2000). Moreover, in adolescent pregnant women nutrient deficiencies limit erythropoietic capacity (Alfonso Maia et al., 2007). Further analyses investigating the effects of undernutrition in erythrocyte formation of adolescent pregnant ewes would be interesting.

In this study, maternal weights and BCS decreased with nutrient restriction as was previously reported (Lemley et al., 2012; Vasquez-Hidalgo 2016). This suggests that nutrient restriction had the desired effect of producing nutritional and metabolic stress in the ewes. Opposed to what we have reported in adult ewes (Vasquez-Hidalgo, 2016), this stress seems sufficient in young ewes to observe the effects of nutrient restriction on UmbBF. Indeed, lamb birth weights increase as age at gestation advances (Gootwine et al., 2007). Additionally, a nutritional conflict between the growing pregnant ewe and the developing fetus has been hypothesized as the cause for an increased susceptibility of ewe lambs to negative environmental factors (Gootwine et al., 2007).

In summary, nutrient restriction during mid-gestation affects UmbBF in adolescent ewes with the inability of refeeding to rescue those values in a 20 day period. Moreover, we confirm the importance of an adequate placental development during mid-gestation. The fact that fetal and placental weights were not affected demonstrates the plasticity of the placenta to adapt to gestational stresses in ewes. However, further analyses in areas such as genetics, epigenetics, fetal and maternal hormones and placental cellular characteristics remain to be performed to fully understand the effects of nutrient restriction in adolescent ewes. Similarly, further fetal analyses are needed to assess possible effects on the normal development of the offspring during late gestation and post-natal life.

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CHAPTER 4. UMBILICAL HEMODYNAMICS AND CONCEPTUS MEASUREMENTS IN SINGLETON AND TWIN PREGNANCIES IN EWES

Abstract

In sheep, adequate physiological maternal adaptations to twin fetuses are key for their prenatal development. We hypothesized that there is a relationship between maternal circulating steroid hormone concentration, plasma volume, and patterns of conceptus growth and umbilical hemodynamics in twin vs singleton pregnancies. Western white face adult ewes carrying singletons (n=6) or twins (n=7) were selected. Hematocrit, progesterone (P4) and estradiol-17β (E2) concentrations were analyzed. Umbilical blood flow (UBF) was assessed through Doppler ultrasonography. Ewes carrying twins had a decreased ($P = 0.03$) hematocrit. There was a tendency ($P = 0.08$) for ewes carrying twins to have increased P4 from day 20 to 40. From day 50 to 70, P4 tended to increase ($P = 0.07$) and E2 was increased ($P = 0.01$) in ewes carrying twins. From day 90 to 120, ewes carrying twins had greater ($P \leq 0.04$) P4. Ewes carrying twins had increased ($P < 0.01$) P4 and tended to have ($P = 0.06$) increased E2. From day 60 until 110, placentomes from twins were larger ($P \leq 0.05$). On day 50, UBF was greater ($P = 0.04$; 27.38 vs. 20.95 ± 1.99 ml/min) in ewes carrying twins. Physiological adaptations including increased UBF prior to peak placentome area, increased maternal P4 and E2, and increased plasma volume could explain the similarity of birth weights in twin and singleton lambs.

Introduction

In eutherian mammals, adequate physiological maternal adaptations when carrying multiple fetuses are key for the prenatal development for a viable and thrifty birth weight. Important adaptations include sufficient placental development for each fetus, adequate nutrient exchange between mother and fetuses, and an appropriate increase in plasma volume during pregnancy. In sheep, placentomes increase in size until d 80 of gestation (Redmer et al., 2004; gestation length: 143 to 147 days). After d 80, placentomes discontinue growing, however fetal growth is exponential during the last 50 days of gestation (Redmer et al., 2004). Placentomes increase their efficiency of nutrient and oxygen transport by increasing their vascularity after d 80 (Redmer et al., 2004). This is especially important in twin pregnancies since placentome sizes and weights are reduced per fetus compared to singleton pregnancies (McCoard et al., 2000a; McCoard et al., 2000b; Gootwine et al., 2007; Grazul-Bilska et al., 2006). Similarly, twin calves and

lambs are usually born lighter than their singleton counterparts (McCoard et al., 2000a; McCoard et al., 2000b; Dwyer et al., 2005; Gootwine 2005; Echternkamp et al., 2006; Grazul Bilska et al., 2006). Therefore, it is hypothesized that if each twin had a similar placental size, or similar nutrient exchange across their placentas as singleton lambs, birth weights would be similar given adequate uterine space.

Doppler ultrasonography has been used successfully to assess umbilical artery and vein hemodynamics (Ferrell and Reynolds 1992; Reinsch, Murphy et al., 1994; Galan et al., 1998; Gamez et al., 2015). In humans, fetal aortic isthmus pulsatility index (PI, an indirect measure of vessel wall resistance to dilation that is negatively correlated with umbilical blood flow; Mari and Hanif 2008) is similar between healthy singleton and twin babies (Gamez et al., 2015). While total utero-ovarian blood flow was greater in ewes carrying multiples compared to singletons during late gestation, blood flow per fetal weight remained constant as gestation advanced (Christenson and Prior 1978). Furthermore, indices of umbilical vascular resistance were decreased in twin vs. singleton lambs on d 40 of gestation, resulting in larger twin fetuses during mid to late gestation that had increased umbilical peak systolic and end diastolic velocities near term (Erdogan et al., 2016).

In sheep, the expansion of blood volume is greater in ewes with multiple fetuses compared to ewes carrying singletons (reviewed in Gootwine et al., 2007). Estradiol-17β (E2) is thought to have an endocrine effect to increase maternal plasma volume in pregnant females (Longo 1983). Our laboratory has previously reported that circulating progesterone (P4) and E2 are increased in ewes carrying twins vs. singletons during the last third of pregnancy (Swanson et al., 2017). We hypothesized that the pattern of steroid hormones observed by Swanson et al. (2017) would be similar for a greater proportion of pregnancy. Moreover, we hypothesized that there is a relationship between maternal circulating steroid hormone concentrations and patterns of conceptus growth and umbilical hemodynamics in twin vs singleton pregnancies in sheep during gestation. Our objectives were to measure and compare umbilical hemodynamics, conceptus growth patterns, and maternal hormone concentrations, as well as hematocrit (as a proxy for plasma volume) in ewes with singletons and twins throughout gestation and relate them with lamb and placental weights obtained at birth.

Materials and methods

Animals and experimental design

Animal care and use were according to protocols approved by the North Dakota State University Animal Care and Use Committee (IACUC #A15076). Multiparous Dorset ewes (n = 13) were randomly selected from the NDSU Sheep Unit. Estrus was synchronized using progesterone containing Controlled Internal Drug Release (CIDR) devices (Zoetis, Parsippany, NJ). After synchronization, ewes were transported 1.5 miles to the NDSU Animal Nutrition and Physiology Center where ewes were housed in a common pen and fed for ad libitum intake pellets (Table 1) and hay that met or exceeded NRC recommendations. Ewes also had *ad libitum* access to water. All ewes were bred to one ram and breeding dates were recorded. Pregnancy diagnosis and fetal enumeration were performed on d 30 of gestation via ultrasonography (Prosound Alpha 6, Hitachi Aloka, Wallingford, CT). Ewes carrying singleton (n = 6) or twins (n = 7) were randomly selected to have conceptus measurements assessed throughout pregnancy. Ewes were weighed every 10 days from d 20 until d 130 of gestation. Similarly, 20 mL of maternal blood was collected every 10 days from d 20 to 130 via jugular venipuncture (EDTA containing tubes; Becton Dickinson Vacutainer, Franklin Lakes, NJ). Hematocrit was measured immediately after collection (Unico micro-hematocrit centrifuge, Dayton, NJ). After centrifugation (3010 g for 20 min), plasma was collected and stored at – 20 °C until progesterone (P4) and estradiol-17β (E2) concentrations were analyzed by Radioimmunoassay. Radioimmunoassays to determine estradiol-17β concentrations were conducted using methods described by Perry and Perry (2008), and to determine concentrations of progesterone using methods described by Engel et al. (2008). Inter- and intra-assay CVs for P4 were 9.6% and 4.5%, respectively and assay sensitivity was 0.4 ng/mL, and inter- and intra-assay CVs for E2 were 14.97% and 4.72%, respectively and assay sensitivity was 0.5 pg/mL.

On d 130 of gestation, ewes were transported back to the NDSU Sheep Unit and received hay and water for *ad libitum intake*. Birth was monitored and placentas were collected. Lamb birth weights, placental weights, and number of cotyledons were recorded.

Table 4.1. Diet composition

Ingredients	Percentage
Corn	9.3
SBM	4.0
Beet pulp	28.9
Alfalfa meal	33.4
Wheat middlings	24.4
Total	100

Diet per kg: 2.659 Mcal; CP: 169.8 g; MP: 118.86 g; NDF: 369.5 g; Starch: 294.24 g; Ca: 7.33 g; P: 4.084 g; Cu: 104.44 ppm; Se: 3.813 ppm. Diet based on NRC recommendations (NRC, 1985).

Ultrasonography measurements

Beginning on day 50, and every 10 days until day 110, ewes were restrained in an elevated cart so that conceptus measurements and umbilical hemodynamics could be obtained as previously described (Lemley et al., 2012). All measurements were obtained before 10 AM. Conceptus measurements included the length and width from 10 random placentomes, fetal biparietal and abdominal lengths, and kidney length and width. Placentome area was calculated by multiplying width by height measurements for each placentome. For umbilical hemodynamic measurements, Doppler mode was used to obtain umbilical blood flow, PI and resistance index (RI, an indirect measure of vessel wall resistance to dilation that is negatively correlated with placental flow; Mari and Hanif 2008) as previously described (Lemley et al., 2012).

Statistical analyses

To assess conceptus development and maternal hormone concentrations relative to placentome growth, variables were assessed in 3 stages: Day 20 to 50 (prior to placentome measurements), day 50 to 70 (growth of placentomes) and day 80 to 110 (after peak placentome growth). The study was conducted as a completely randomized design with repeated measures. Repeated data were analyzed using the MIXED procedure of SAS (SAS software version 9.4, SAS Institute, Cary, NC). Ewe was the random independent variable; fetal number and day were fixed effects. Placentome area, umbilical blood flow, PI and RI, fetal biparietal and abdominal lengths, kidney length and width, ewe body weight, hematocrit, P4 and E2 were the dependent variables. Least squares means were separated using the PDIFF option of the LSMEANS statement. Birth was analyzed using the GLM procedure. LSMeans were separated using the LSMEANS statement. Correlations were analyzed using the PROC CORR procedure. In addition,

hematocrit and hormone data were further analyzed by calculating area under the curve (AUC) with the use of SigmaPlot 8.0 (Aspire Software International, Ashburn, VA, USA). Area under the curve data were tested with the GLM procedure of SAS. P values ≤ 0.05 are considered significant. Tendencies are described when P values are > 0.05 but ≤ 0.10.

Results

Ewe body weights and blood analyses

Body weight was similar (P = 0.69; 68.78 vs. 67.38 ± 2.52 kg for ewes carrying singletons and twins, respectively) at the initiation of the study (day 20) and remained similar on d 130 (P = 0.28; 70.43 vs. 76.58 ± 4.08 kg for ewes carrying singletons and twins, respectively).

There were no fetal number by day interactions, nor main effects on maternal hematocrit (Figures 3.1 – 3.3). Of interest was that during late gestation, maternal hematocrit values tended to (P = 0.08) be greater and were greater (P = 0.03) in ewes carrying singletons than in ewes carrying twins on days 100 and 130 of gestation, respectively (unprotected *F*-test; figure 3.3). When area under the curve was calculated throughout gestation, ewes carrying singleton fetuses had a greater (P = 0.03) hematocrit compared to ewes carrying twins (Figure 3.4).

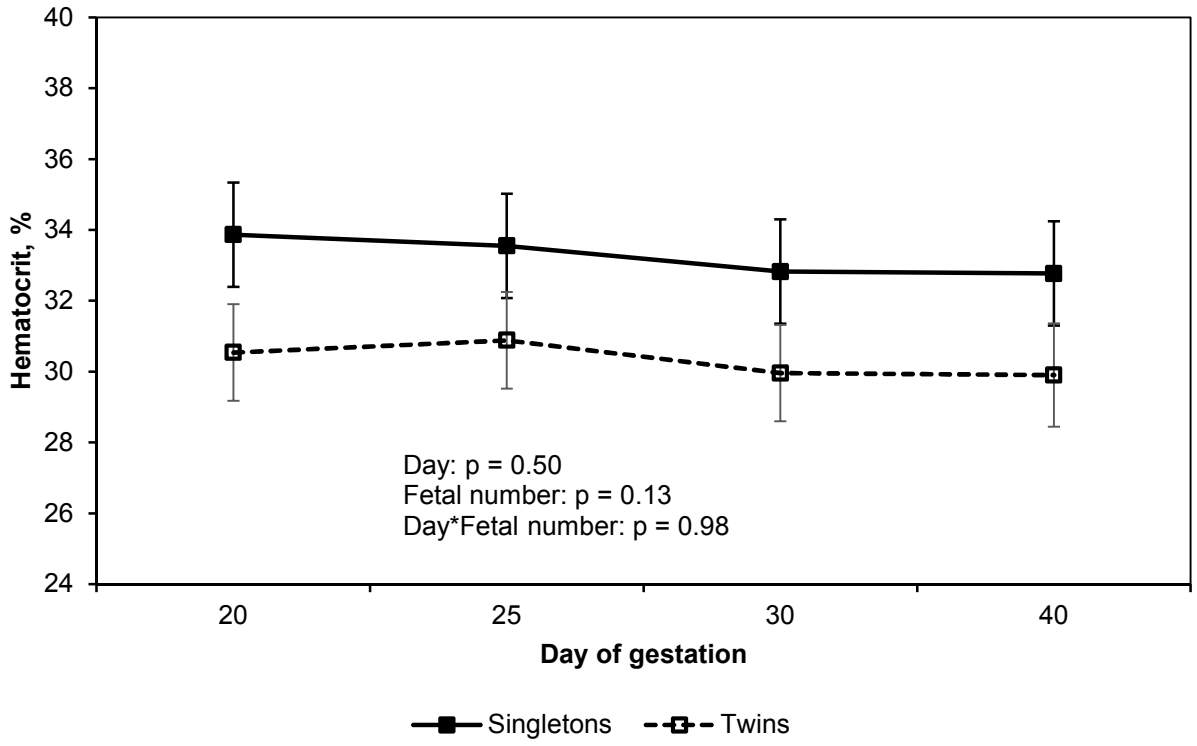

Figure 4.1. Fetal number effects on early gestation (d 20 – 40) on hematocrit.

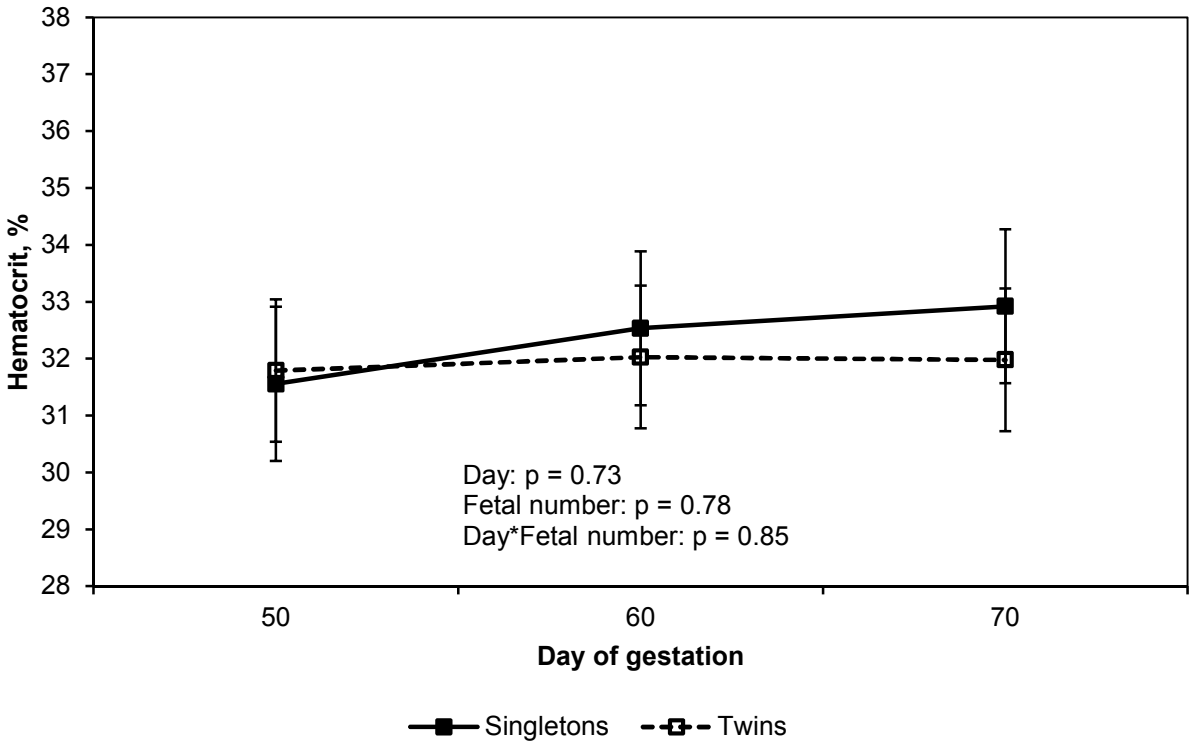

Figure 4.2. Fetal number effects on mid gestation (d 50 – 70) on hematocrit.

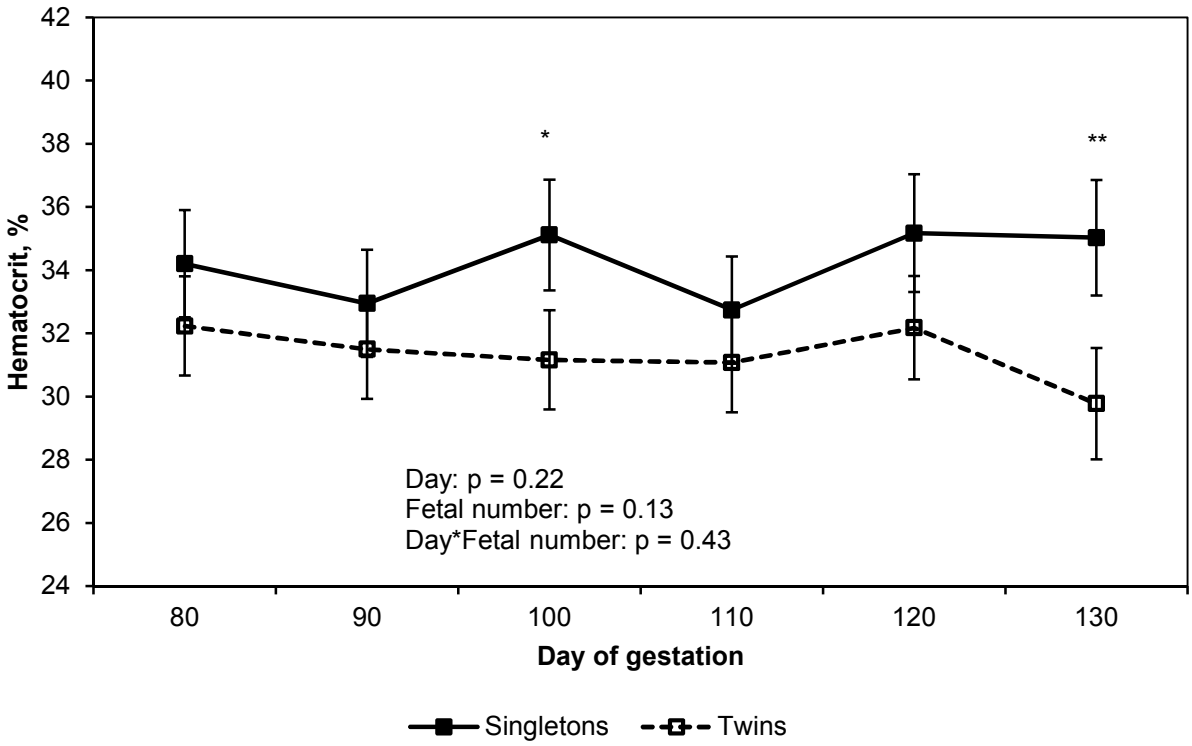

Figure 4.3. Fetal number effects on late gestation (d 80 – 130) on hematocrit. Differences between singletons and twins are denoted by ** P ≤ 0.05, tendencies are denoted by * P ≤ 0.10

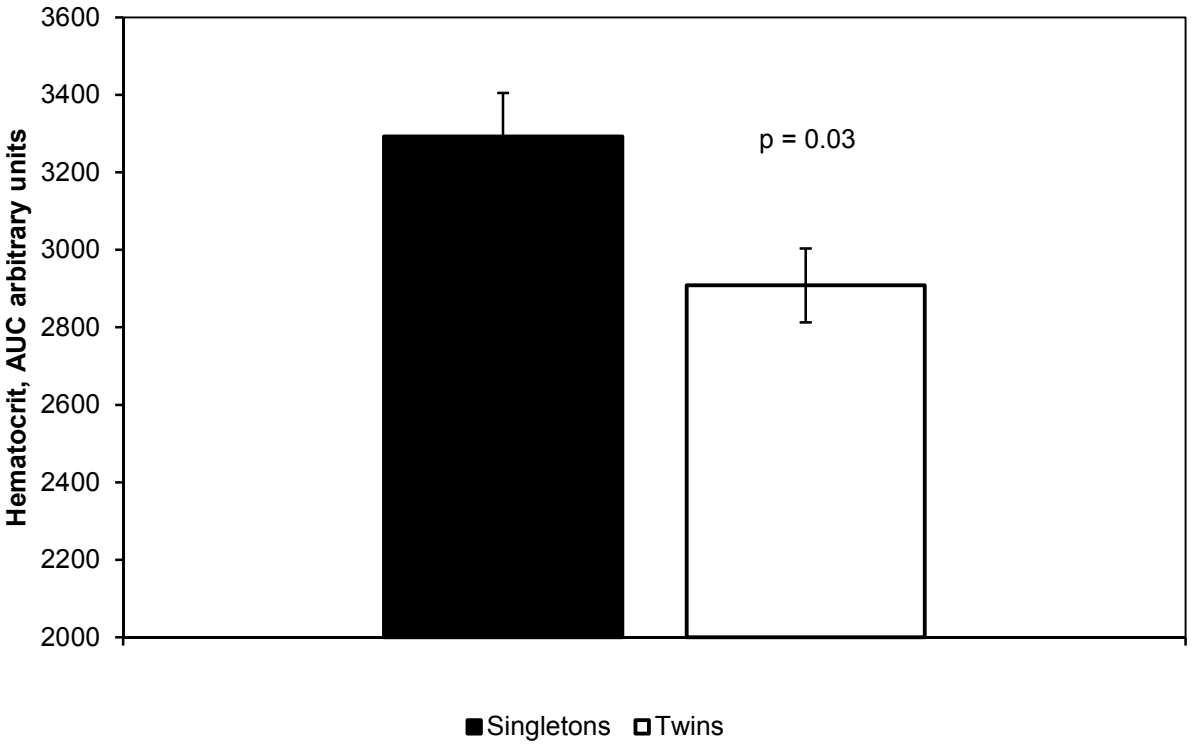

Figure 4.4. Fetal number effects on area under the curve (AUC) of hematocrit.

From day 20 to 70, there were no fetal number by day interactions for P4 and E2 (Figures 3.5 – 3.8). There was a tendency (P = 0.08) for ewes carrying twins to have increased P4 from day 20 to 40 with P4 being greater (P = 0.03) on day 40 compared to ewes carrying singletons. There was no impact of fetal number or day (P ≥ 0.41; figure 3.6) on E2 concentrations in ewes from d 20 to 40. From day 50 to 70 there was no effect of fetal number (P = 0.89; figure 3.8) in E2 concentrations, however, P4 tended (P = 0.07; figure 3.7) to be elevated in ewes carrying twins vs singletons. By late gestation, there was only a day effect (P = 0.01; figure 3.10) on E2 concentrations with peak E2 occurring day 130. For P4, there was a tendency (P = 0.10; figure 3.9) for a fetal number by day interaction. From day 90 to 120, ewes carrying twins had greater (P ≤ 0.04) P4 concentrations compared to ewes carrying singletons (Figure 3.9). When area under the curve was calculated for E2 and P4 throughout gestation, ewes carrying twins had greater (P < 0.01) P4 and tended to have (P = 0.06) greater E2 (Figures 3.11 and 3.12) compared to ewes carrying singletons.

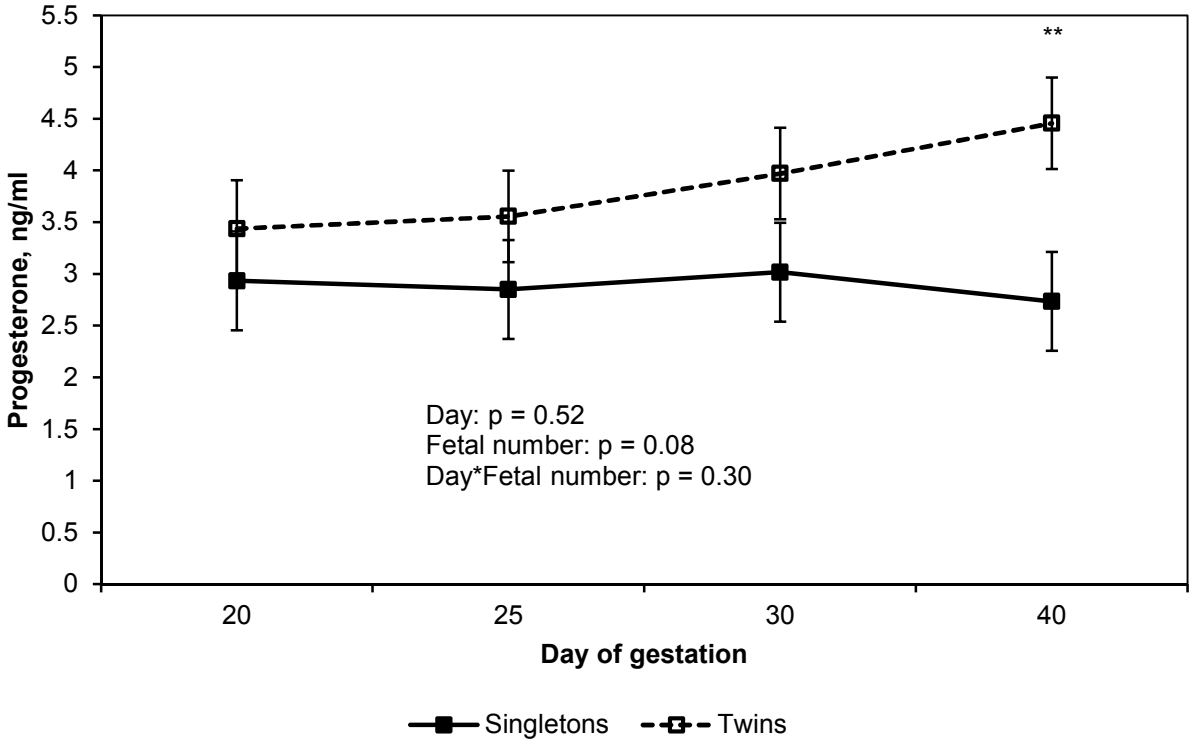

Figure 4.5. Fetal number effects on early gestation (d 20 – 40) on progesterone. Differences between singletons and twins are denoted by ** P ≤ 0.05, tendencies are denoted by * P ≤ 0.10

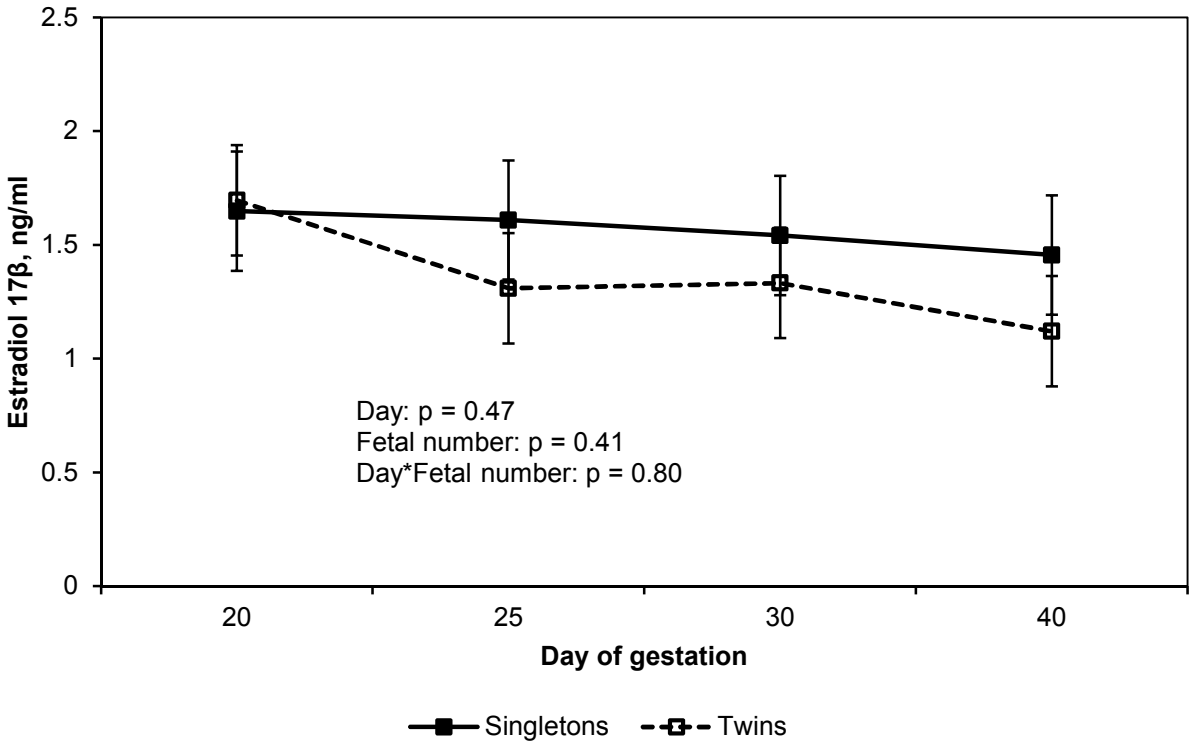

Figure 4.6. Fetal number effects on early gestation (d 20 – 40) on estradiol-17β.

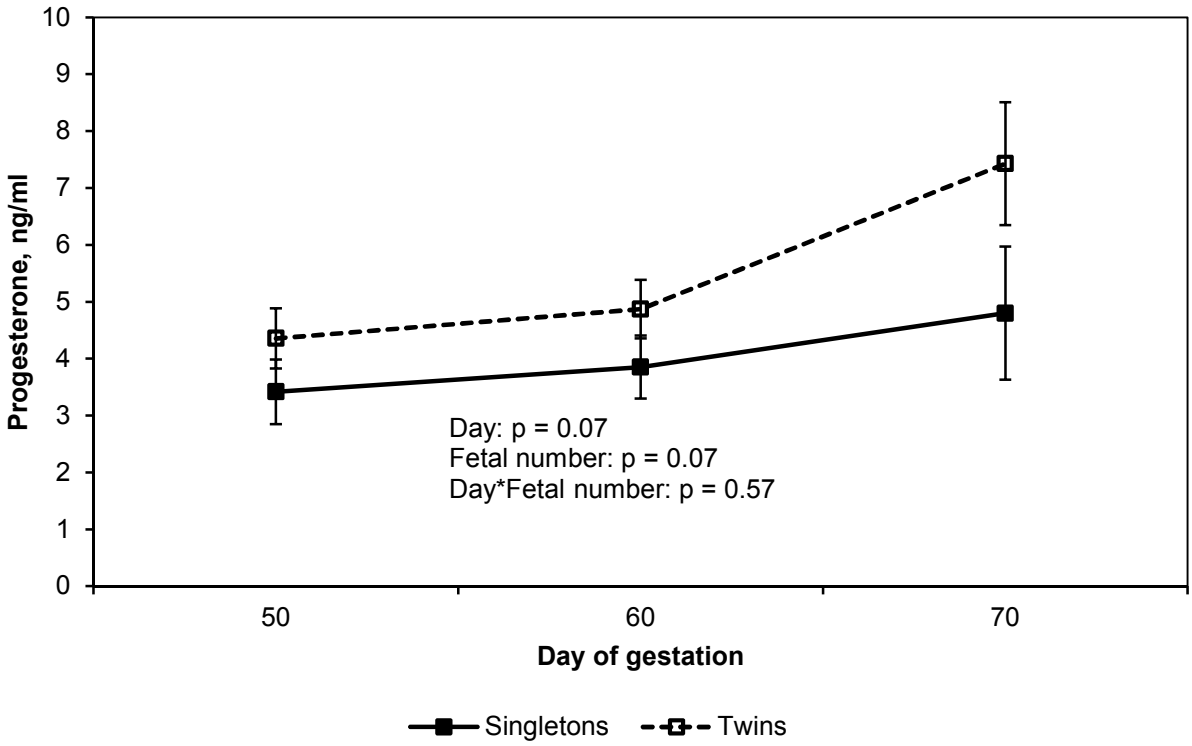

Figure 4.7. Fetal number effects on mid gestation (d 50 – 70) on maternal progesterone.

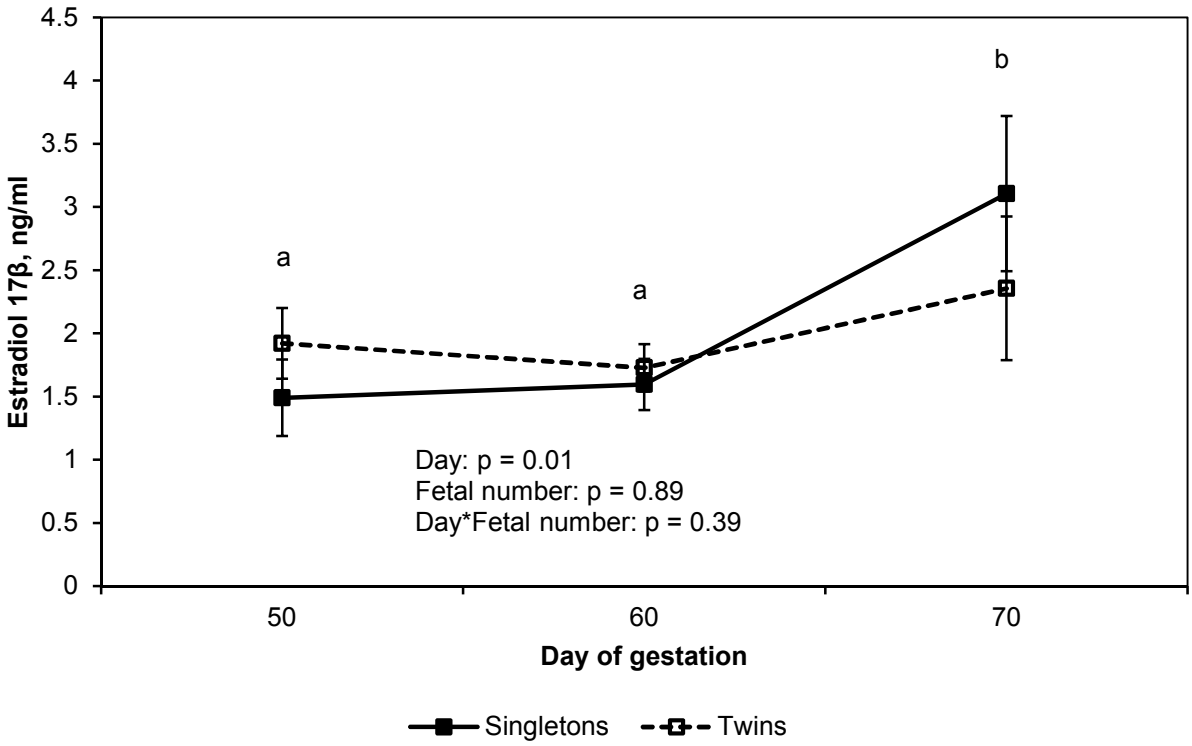

Figure 4.8. Fetal number effects on mid gestation (d 50 – 70) on maternal estradiol-17β. [abcdefg]LSMeans ± SEM by day differ P < 0.05.

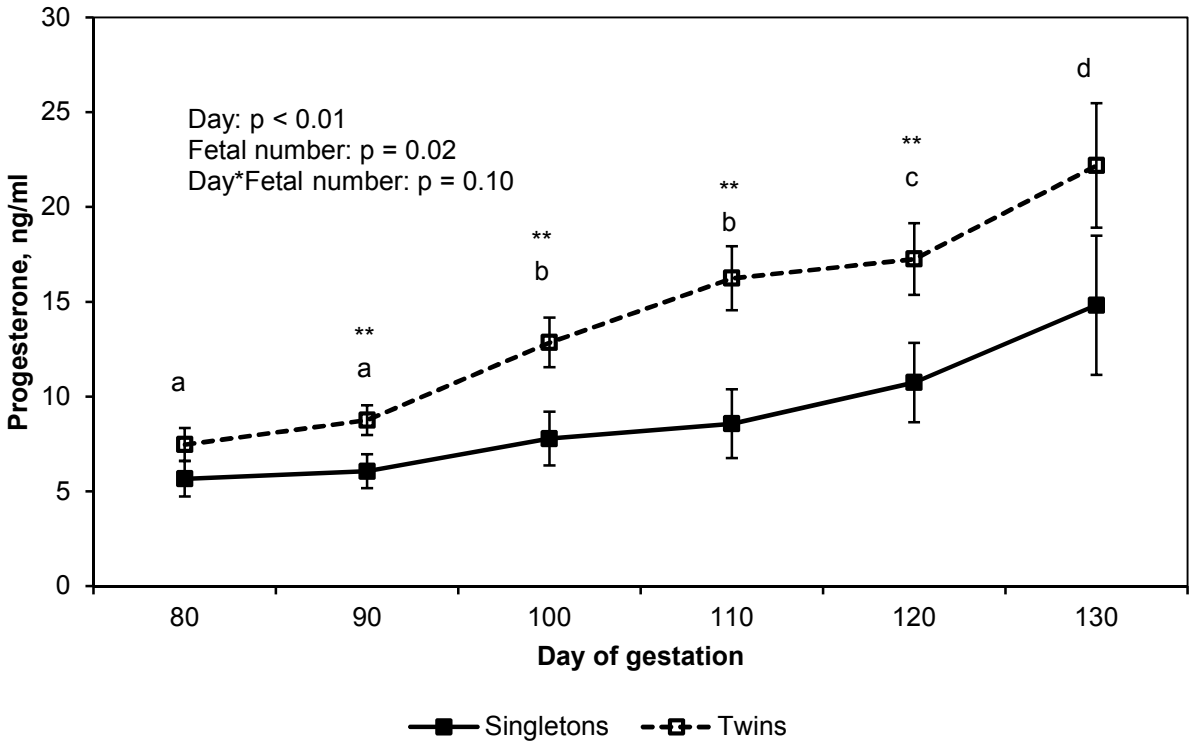

Figure 4.9. Fetal number effects on late gestation (d 80 – 130) on maternal progesterone. [abcdefg]LSMeans ± SEM by day differ P < 0.05. Differences between singletons and twins are denoted by ** P ≤ 0.05, tendencies are denoted by * P ≤ 0.10

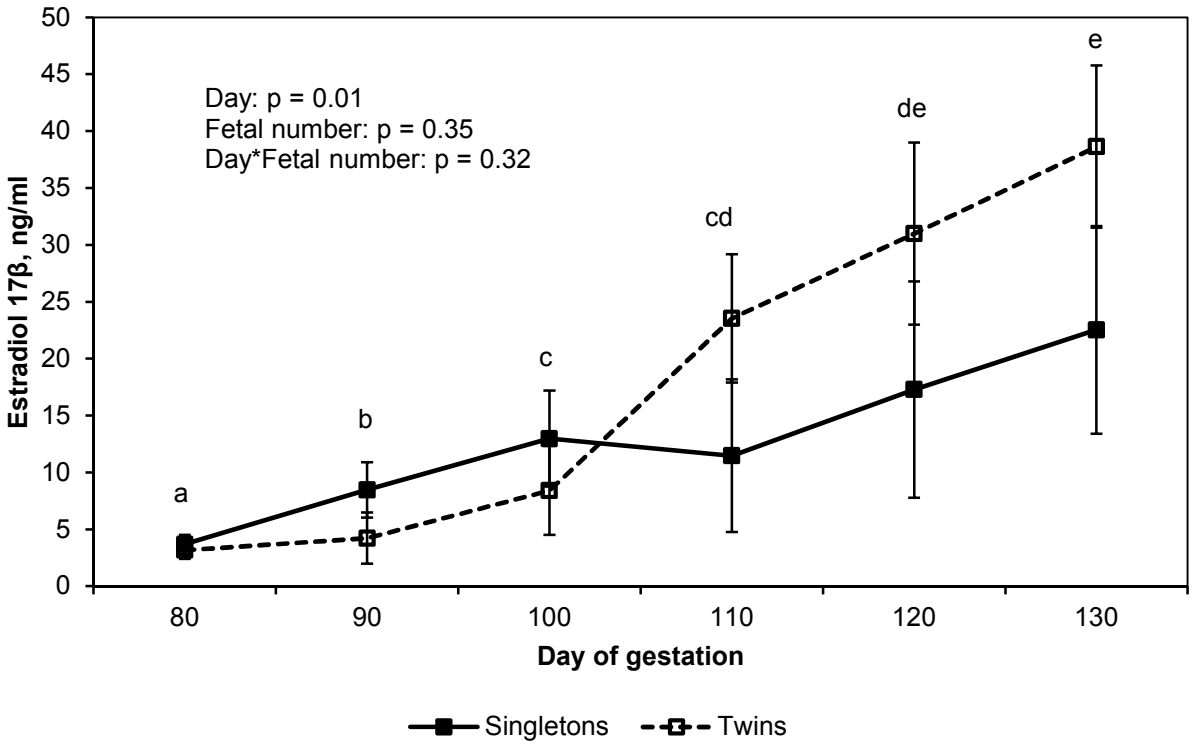

Figure 4.10. Fetal number effects on late gestation (d 80 – 130) on maternal progesterone. [abcde]LSMeans ± SEM by day differ P < 0.05.

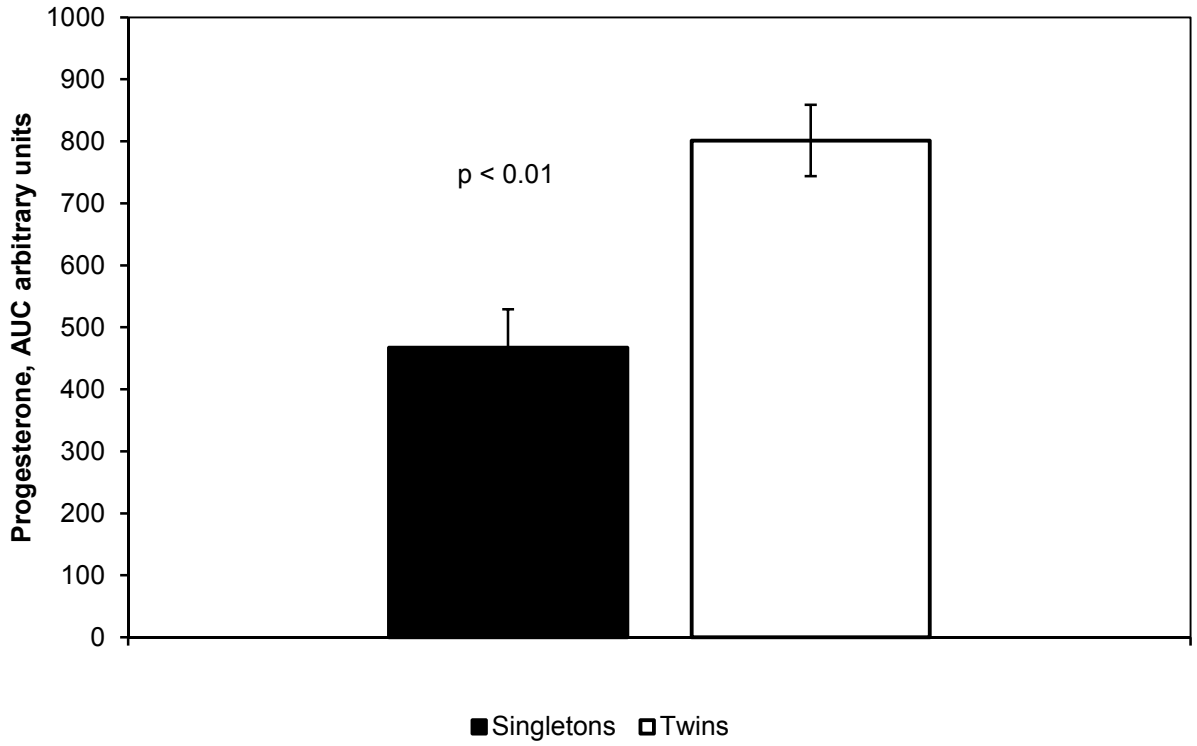

Figure 4.11. Fetal number effects on area under the curve (AUC) of progesterone.

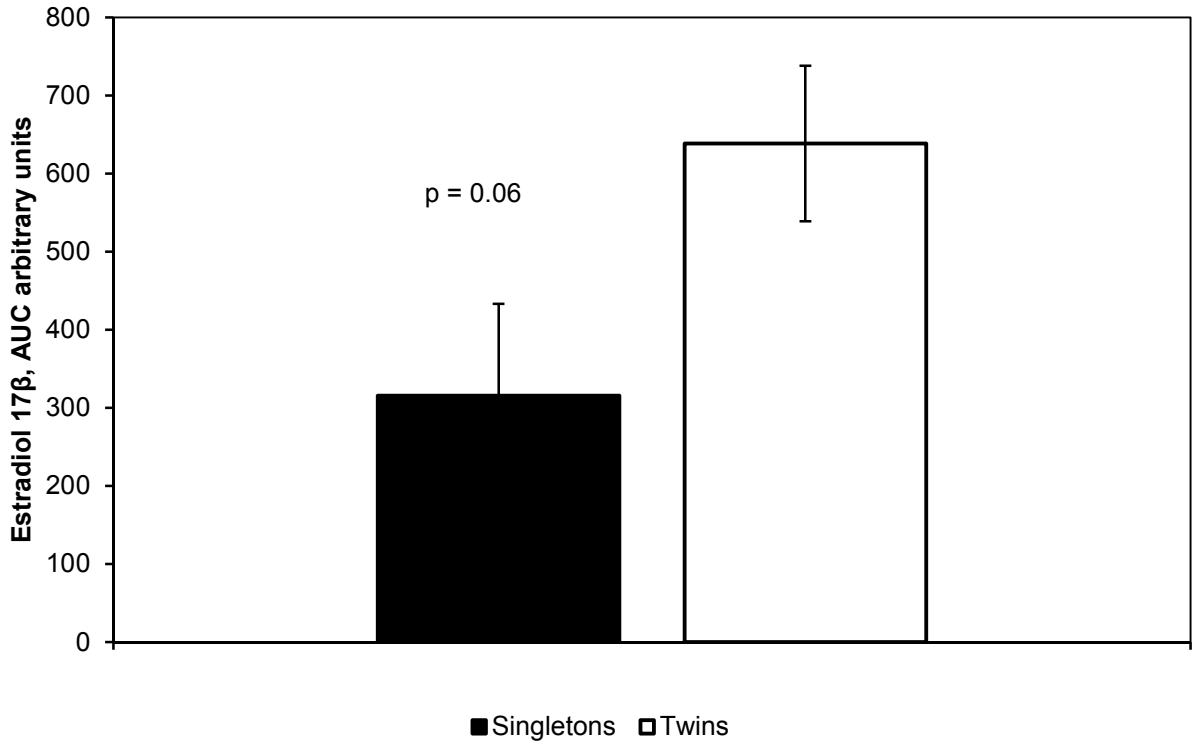

Figure 4.12. Fetal number effects on area under the curve (AUC) of estradiol-17β.

Conceptus measurements

There was no fetal number by day interaction (P = 0.53) and no main effect of fetal number (P = 0.18) for fetal biparietal distance (Table 2). A fetal number by day interaction (P = 0.05) was observed for fetal abdominal distance with the interaction being driven by a tendency (P = 0.07) for twins to be thinner on d 80 of gestation (Table 2). There was no fetal number by day interaction nor main effect of fetal number (P ≥ 0.26) for kidney length and width throughout the experiment (data not shown). As expected, all fetal measurements increased (P < 0.01), as gestation advanced.

Table 4.2. Fetal measurements from singleton and twin fetuses obtained by ultrasonography during mid to late pregnancy

Day	Singletons	Twins[1]	S.E.M.	Day	Fetal number	Day*Fetal number
Fetal biparietal distance, cm				<0.01	0.18	0.53
50	1.59	1.68	0.04			
60	2.29	2.38	0.04			
70	3.04	3.11	0.12			
80	3.61	3.51	0.14			
90	4.17	4.24	0.16			
100	4.74	4.60	0.26			
110	4.93	5.53	0.19			
Fetal abdominal width, cm				<0.01	0.42	0.05
50	1.97	2.10	0.05			
60	2.85	2.83	0.06			
70	4.07	3.78	0.13			
80	5.00	4.78*	0.08			
90	5.95	5.70	0.24			
100	7.11	6.60	0.33			
110	7.55	7.99	0.40			

[1]Measurements were obtained from both twin fetuses and averaged for these calculations.
*There was a tendency (P = 0.07) for twin fetuses to be thinner on day 80 vs. singletons.

There was a tendency for a fetal number by day interaction (P = 0.07) on placentome area. Area of placentomes were similar (P = 0.19) on d 50 (Figure 3.13), but by d 60 until 110, placentomes from twins were larger (P ≤ 0.05) than placentomes from singletons (Figure 3.13).

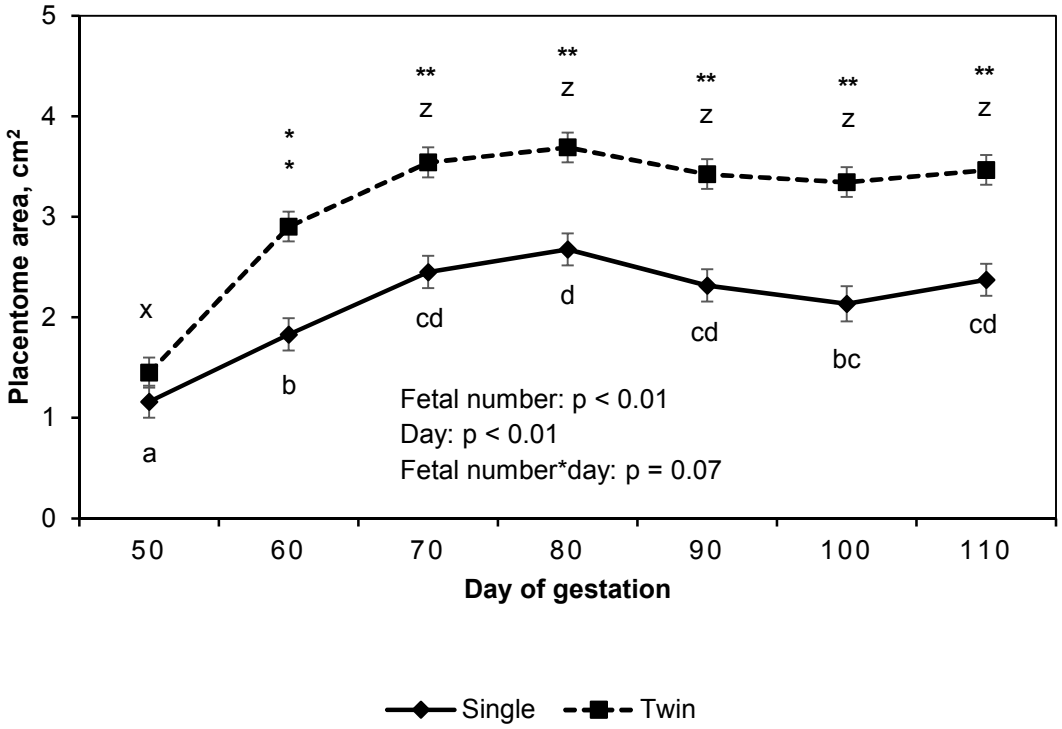

Figure 4.13. Fetal number effect on placentome size from d 50 to 110 of gestation. [abcd]LSMeans ± SEM within singleton differ P < 0.05; [xyz]LSMeans ± SEM within twins differ P < 0.05. Differences between singletons and twins are denoted by ** P ≤ 0.05

Hemodynamics

From d 50 to 70, there were no fetal number by day interactions for PI, RI, and umbilical blood flow (Figures 3.14 – 3.16). Of interest was that on day 50, umbilical blood flow values were greater (P = 0.04; figure 3.16) in twins when compared to singletons (unprotected F-test). All hemodynamic values greater from day 50 to 70 (P ≤ 0.02; figures 3.14 – 3.16).

From d 80 to 110 there was a fetal number by day interaction (P = 0.02) and a tendency for a fetal number by day interaction (P = 0.07) interaction for PI and RI respectively (Figures 3.14 and 3.15). Days 80 and 100 tended to be greater (P = 0.06) and day 110 was greater (P = 0.02) for PI while days 100 and 110 tended to be greater for RI (P = 0.06; Figures 3.14 and 3.15) in twins when compared to singletons. There was no fetal number by day interaction (P = 0.37) nor main effect of fetal number (P = 0.52; Figure 3.16) on umbilical blood flow from day 80 to 110.

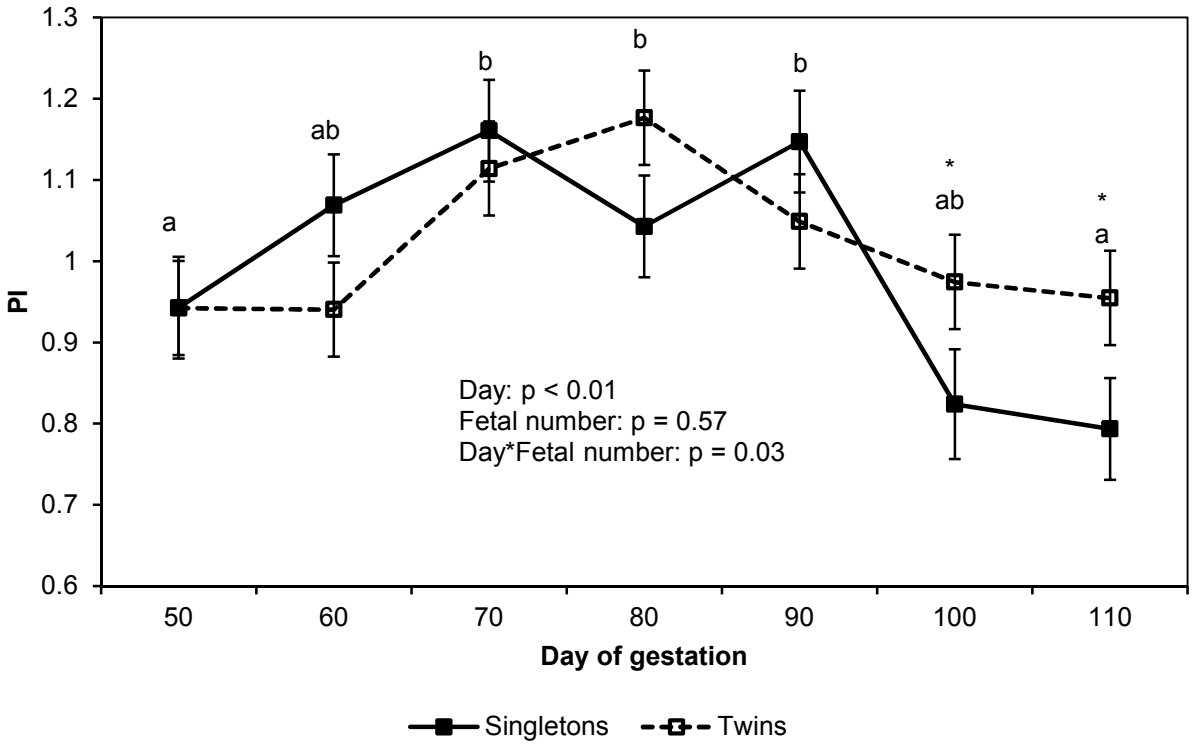

Figure 4.14. Fetal number effect on pulsatility index from d 50 to 110 of gestation. [ab]LSMeans ± SEM within singleton differ P < 0.05; [xyz]LSMeans ± SEM within twins differ P < 0.05. Differences between singletons and twins are denoted by ** P ≤ 0.05

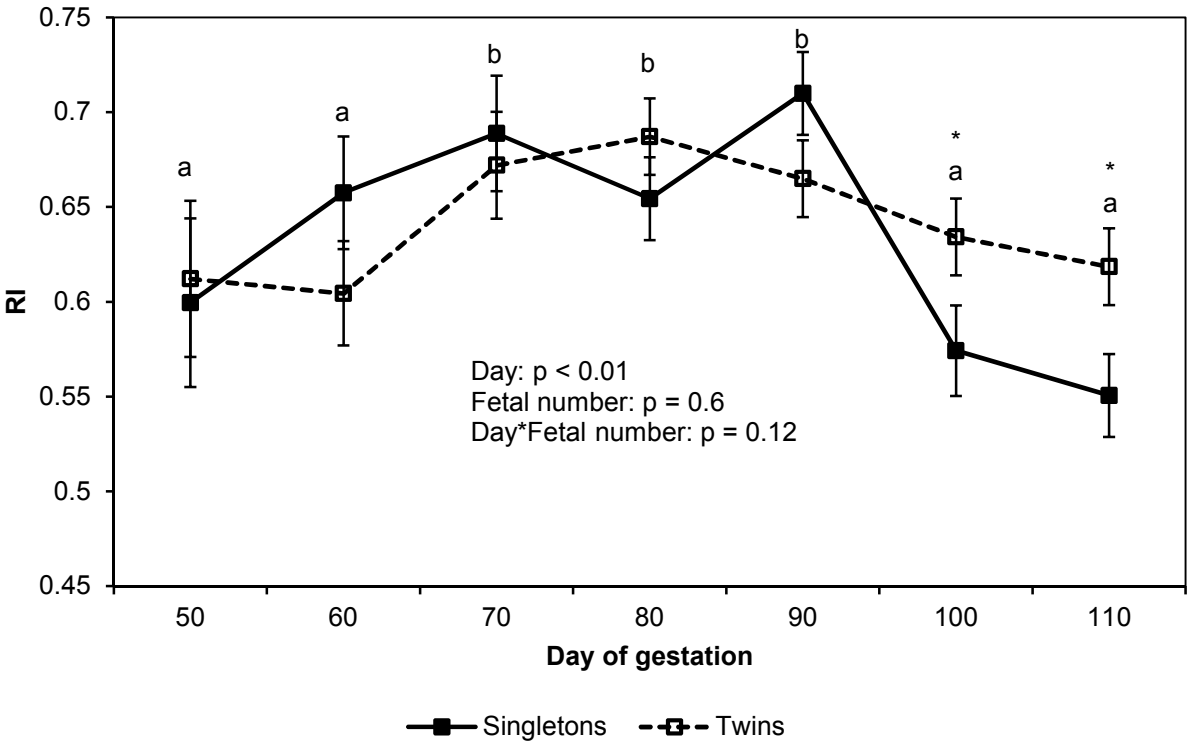

Figure 4.15. Fetal number effect on resistance index from d 50 to 110 of gestation. [ab]LSMeans ± SEM within singleton differ P < 0.05; [xyz]LSMeans ± SEM within twins differ P < 0.05. Differences between singletons and twins are denoted by ** P ≤ 0.05

Hemodynamic values (day 50 to 110) demonstrated an interaction (P = 0.03) for fetal number by day for PI with twin measurements tending to be greater (P ≤ 0.10) during day 100 and 110 (Figure 3.14). Resistance index and umbilical blood flow presented no fetal by day interactions or main effect of fetal number (Figure 3.15). Resistance index increased from day 50 to 70 and decreased from day 90 to 110 (Day: P < 0.01; Figure 3.15), while umbilical blood flow increased as gestation advanced (Day: P < 0.01, Figure 3.16).

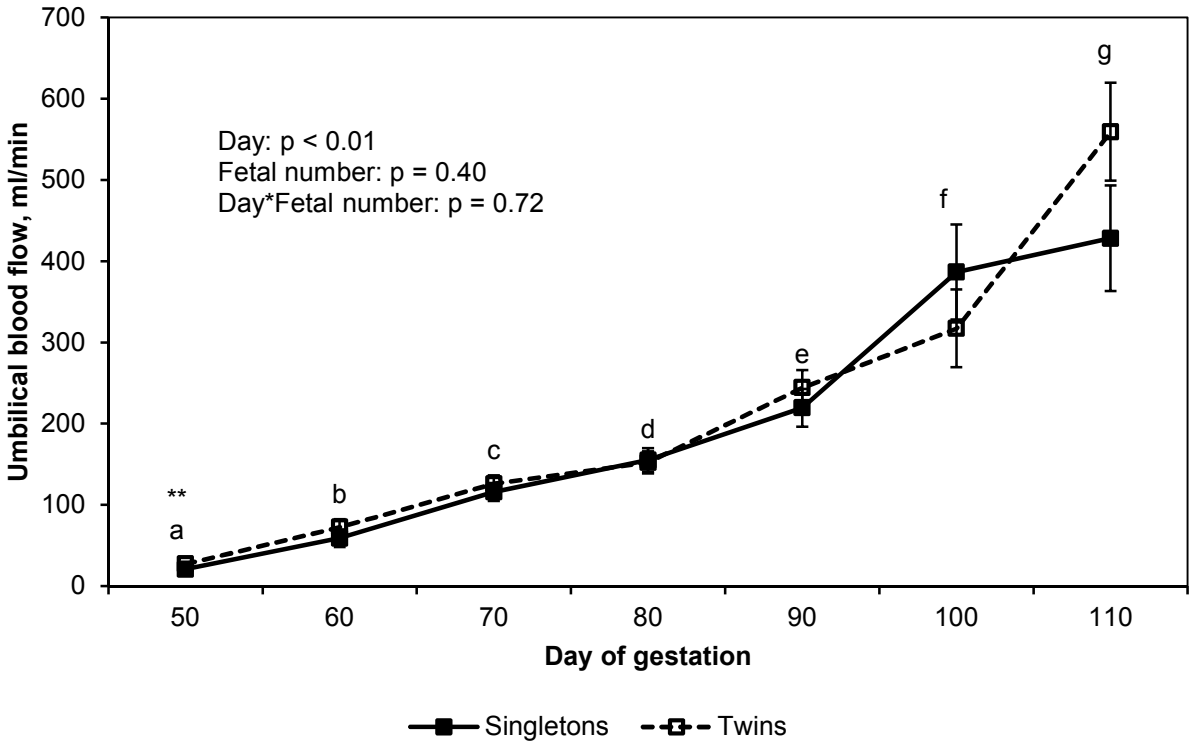

Figure 4.16. Fetal number effect on umbilical blood flow from d 50 to 110 of gestation. ^{abcdefg}LSMeans ± SEM within singleton differ P < 0.05; ^{xyz}LSMeans ± SEM within twins differ P < 0.05. Differences between singletons and twins are denoted by ****** P ≤ 0.05

Birth measurements

Total lamb birth weights were greater (P = 0.01) in twins vs singletons (Table 3). However, average lamb birth weights and total cotyledon numbers were similar (P ≥ 0.40; Table 3) in singleton and twin pregnancies. Nevertheless, total number of cotyledons by lamb birth weight were greater (P = 0.02) in singletons vs twins (Table 3). Total placental, cotyledon and fetal membrane weights were greater (P < 0.01) in twins vs. singletons (Table 3). However, total placental, cotyledon and fetal membrane weights by lamb birth weight were not different (P ≥ 0.44) between the groups (Table 3).

Table 4.3. Offspring and placental lambing data

	Singleton	Twin	SEM	P
Total lamb weight, kg	4.25	8.76	1.04	**0.01**
Average lamb weight, kg	4.25	4.38	0.64	0.88
Total placental weight, g	310.7	738.1	56.9	**< 0.01**
Placental weight/lamb weight*, g/kg	79.72	86.50	9.05	0.58
Total cotyledon weight, g	80.9	199.6	21.9	**< 0.01**
Cotyledon weight/lamb weight**, g/kg	21.08	22.99	2.8	0.61
Total cotyledon number	96.8	104.2	5.9	0.40
Total fetal membrane weight, g	203.9	493.9	33.2	**< 0.01**

* Calculated by dividing total placental weight by total lamb weight;
** Calculated by dividing total cotyledon weight by total lamb weight

Discussion

During late gestation placental weight, and cotyledonary weight and number per fetus are usually greater in singleton compared to twin pregnancies in sheep (McCoard et al., 2000a; McCoard et al., 2000b; Gootwine 2005, reviewed in Gootwine et al., 2007; Grazul-Bilska et al., 2006). Our results do not show decreased cotyledonary weights per fetus in twin vs singleton pregnancies at birth. Similarly, our results do not show decreased birthweights in twin lambs when compared to singletons. Moreover, umbilical blood flow was not different between singleton and twin fetuses. While we realize our study was not powered to full assess conceptus growth, we do have confidence that the physiological parameters assessed may explain the lack of differences in this study. Many studies have demonstrated the relationship between fetal size and umbilical blood flow (Rigano et al., 2001; Ferrazi et al., 2002). Intrauterine restriction models report decreased fetal sizes and weights concordantly with decreased umbilical blood flow (Dwyer et al., 2005; Lemley et al., 2012). Similarly, birth weight and weight of the placenta are often positively correlated in man, pig, guinea pig, rabbit and sheep (Alexander 1964; Vatnick et al., 1991; Vonnahme and Ford 2004; Dwyer et al., 2005). In sheep, positive correlations have also been reported between birth weights and cotyledonary weights (Alexander 1964; Dwyer et al., 2005). We report that total placental weights, fetal membrane weights and cotyledon weights by lamb birth weights are not different. Moreover, results from this study demonstrating cotyledon numbers were greater when divided by lamb birth weight in singleton vs twins and the fact that umbilical blood flow was not different between singletons and twins is suggestive of a full placental efficiency compensation in twin pregnancies. Indeed, in other studies, an experimental reduction of placentome numbers, either through uterine artery ligation or carunclectomy did not affect

placental or fetal size, demonstrating the amazing capacity for compensatory development of the placenta (Reviewed in Gootwine et al., 2007).

Placentome area as seen by ultrasonography were increased in twin placentas and they reached a peak size at day 80 of gestation. However, increased pulsatility and resistance indices in day 80 to 100 in twins vs singletons suggest different vascular adaptations of the placenta in twin vs singleton pregnancies once peak placentome sizes are reached. Nevertheless, the fact that greater PI and RI values in twins did not translate into a decrease in umbilical blood flow during this period of gestation could suggest other compensatory mechanisms in the twin placental vascular system, such as increase nutrient perfusion (Reynolds et al., 2005; Vonnahme et al., 2005; Vonnahme et al., 2008).

Our laboratory has previously reported greater P4 and E2 levels in ewes carrying twins compared to those carrying singletons (Swanson et al., 2017). Moreover, prolonged estrogen infusion increases blood flow to the female reproductive and non-reproductive tissues (Magness et al., 1998). Our lab has observed that a supraphysiological dose of E2 tends to cause an acute plasma volume increase in ewes (Vasquez-Hidalgo et al., 2019). Similarly, as plasma volume increases during early pregnancy, hematocrit decreases, allowing hematocrit to be a marker for plasma volume (Longo 1983). Moreover, P4 administration increases blood flow to the caruncles and reduces blood flow to the myometrium and the cervix (Anderson et al., 1973). Additionally, antiprogestins have been shown to decrease uterine artery blood flow in humans (Wilkens et al., 2008). In this study, we report an increased P4 and an increased E2 area under the curve as well as a decreased hematocrit value in ewes carrying twins when compared with ewes carrying singletons. We also report that umbilical blood flow was greater in twins compared to singletons on day 50. Physiological adaptations during early pregnancy can impact adequate fetal development in ewes carrying multiple offspring (reviewed in Gootwine et al., 2005; reviewed in Gootwine et al., 2007). Our findings suggest that an increased umbilical blood flow early (day 50), along with an increased maternal concentration of P4 and E2, and an increased plasma volume (decreased hematocrit) could be some of those adaptations. Further studies analyzing differences in blood flow distribution to the uterus and the placenta of singleton and twin pregnancies would be interesting.

While placentome area likely peaked between day 80 and 90, the majority of fetal weight gain happens after d 90 (Redmer et al., 2004). The fact that abdominal girth was greater for singleton fetuses

on day 80 of gestation and fetal biparietal distance was greater for twin fetuses on d 110 of gestation could mean that twin fetuses were smaller than singleton fetuses earlier in gestation and that twin fetuses recovered and surpassed singletons during later gestation. Similarly, this could be hypothesized as a consequence of increased placentome size, and the proposed increased nutrient perfusion in twins vs. singletons during the second half of gestation. However, the lack of other fetal measurements (kidney area) that concur with these data as well as more solid statistical values for these findings restrain us from making such assumptions.

It has been suggested that physiological adaptations during early pregnancy can significantly impact fetal development in multiple offspring carrying ewes (Oliver et al., 2007). Our findings suggest that increased umbilical blood flow early in gestation, associated with increased maternal concentrations of P4 and E2, and an increased plasma volume (decreased hematocrit) could contribute to these adaptations. Twins are commonly lighter than singleton lambs (McCoard et al., 2000a; McCoard et al., 2000b; Gootwine 2005, reviewed in Gootwine et al., 2007; Grazul-Bilska et al., 2006). Average umbilical blood flow, placental and cotyledonary weights are also commonly decreased in twins. Our reduced ewe numbers could be a contributor of why these differences were not observed in this study. However, similar umbilical blood flow, placental growth and placental weights per fetal unit seem necessary in twins in order to attain similar birthweights as singletons.

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CHAPTER 5. GENERAL CONCLUSION AND FUTURE DIRECTIONS

Results found in chapter 2 suggest that estradiol-17β could potentially have a major role in the early establishment and proper adaptation to pregnancy in ewes. It seems possible that an early increase in plasma volume, determined by a rapid increase in estrogens may be an important physiological cue for an adequate maternal adaptation to pregnancy. Greater estradiol-17β secretion by the ovulatory follicle could determine early plasma volume expansion and therefore a better physiological adaptation of the dam to pregnancy. Similarly, ewes that respond with an enhanced increase in plasma volume to estradiol-17β could be better prepared for a successful pregnancy. Further studies determining the effect of physiological doses of estradiol in acute plasma volume increase and pregnancy success are necessary.

Repeated studies during several years have enabled us to suggest that in the NDSU model of nutrient restriction umbilical blood flow is affected in adolescent but not adult ewes. Similarly, there is an inability of refeeding to rescue decreased blood flow values back to control levels values in a 20 day period. We confirm the importance of an adequate placental development during mid-gestation. However, further analyses in areas such as genetics, epigenetics, fetal and maternal hormones and placental cellular characteristics remain to be performed to fully understand the effects of nutrient restriction in adolescent ewes. Analyses on the effects that undernutrition might have in the expression of proteins being produced by binucleate cells remain very important in the future. Similarly, the effects of long term impacts of undernutrition in postnatal performance have been only slightly studied. Future analysis regarding these effects remain to be analyzed.

Finally, it has been suggested that physiological adaptations during early pregnancy can significantly impact fetal development in multiple offspring carrying ewes. Our findings indicate that increased umbilical blood flow early in gestation, associated with increased maternal concentrations of progesterone and estradiol-17β, and an increased plasma volume could contribute to those adaptations. Similar umbilical blood flow, placental growth and placental weights per fetal unit seem necessary in twins in order to attain similar birthweights as singletons.

This thesis answers questions that arose from our previous investigations. Here we report that age at gestation plus nutrient restriction during mid gestation decreases umbilical blood flow as it was hypothesized by our lab during previous work (Chapter 2). We report different variations in hematocrit upon

nutrient restriction in young ewes compared to what we observed in adult ewes (Chapter 2) and hypothesize that the hematopoietic capacity of growing compared with adult animals during pregnancy is decreased. Further studies investigating spleen and bone marrow erythropoietic abilities in adolescent vs adult pregnant ewes will be interesting. These analyses will also answer some of the remaining questions regarding blood volume expansion during early pregnancy and if there is any difference between young and adult animals in blood volume increase.

If confirmed, our results could determine a major role of estradiol-17β during fecundation and early pregnancy. Estradiol-17β seems to have a major role not only in plasma volume expansion, but also in reproductive behavior and BNC migration. Future studies addressing these roles are important.