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The Effects of Poor Maternal Nutrition on Offspring Growth and Maternal and Offspring Inflammatory Status During Gestation

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ABSTRACT

Breeding and management of pregnant females are essential components of livestock production. Improper gestational management creates maternal stressors which negatively affect prenatal and postnatal offspring development. We hypothesized that 1) using transabdominal ultrasound, pregnancy diagnosis would be accurate during early gestation in ewes and measure fetal growth patterns during mid-gestation, and 2) inflammation may be a mechanism contributing to suboptimal offspring performance when maternal nutrition is poor during gestation. To investigate, 99 ewes were scanned three times per wk between d26 and d40 of gestation. The sensitivity and specificity of pregnancy diagnoses were >90% from d33 onward, coinciding with visualization of fetal and placental development. This demonstrated that transabdominal ultrasound can be accurately integrated during early gestation for proactive flock management. Ewes determined to be pregnant (n=82) were randomly assigned to diets of 100% (CON), 60% (RES) or 140% (OVER) of NRC from d30 through parturition. Ultrasounds continued weekly between d45 and d90 to monitor growth of the fetal heart width (HW), umbilical diam., (UMB) and rib width (RW). At d45 and d90, ewes (n=20 or 21) were euthanized and fetuses were obtained for comparative gross measurements. As gestation advanced, measurement of the HW and UMB increased (P<0.001) but neither were affected

by maternal diet ($P \geq 0.12$). Interactions of maternal diet and litter size with gestation were observed for the RW; however, the effects were inconsistent and explained by Bland-Altman analysis that demonstrated measurement bias on ultrasound. At d45 ultrasound measurement underestimated RW by 7.7% but overestimated RW by 23.8% at d90. To determine how gestational diet affected the inflammatory status of dams (n=5 to 7 per diet) and offspring (n=6 per diet), RT-PCR arrays were utilized. RES and OVER promoted differing maternal systemic pro-inflammatory gene expression, with RES linked to increased NEFA concentrations. At birth, offspring systemic *C-C motif chemokine ligand 8* increased in RES and OVER, and hepatic *tumor necrosis factor* increased in RES. Pro-inflammatory cytokines induced by maternal diet may antagonize offspring growth and performance. Future research may address anti-inflammatory interventions which can be implemented according to gestational information gained via ultrasound.

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The Effect of Poor Maternal Nutrition on Offspring Growth and Maternal and Offspring Inflammatory Status During Gestation

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DEDICATION

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INTRODUCTION

The livestock sector is an essential component of global society because it provides a nutritional protein source to the human diet (Thornton, 2010; Turk, 2016). Recent modeling of global demographics projects that the world population will increase from 7.3 billion people in 2015 to 9.7 billion people in 2050 (United Nations, 2015). Low-income regions, such as Africa are projected to increase in population by 108%, whereas the population of developed regions such as Northern America will remain more stagnant, with a projected increase of 17% (United Nations, 2015). In response to the increasing global population, the livestock industry must continually improve production practices to meet growing consumer demands and ensure worldwide food security, while maintaining nutritious and affordable outputs for consumption.

According to the 2016 Global Agricultural Productivity Index (GAP), agricultural production must improve by an average rate of 1.75% annually to achieve the output necessary for 2050 (Global Harvest Initiative, 2016). Currently, global agricultural productivity is approaching this rate; however, low income countries are struggling to contribute to the necessary agricultural production rate (Global GAP = 1.73%; Low income GAP = 1.31; [Global harvest initiative, 2016]). Additionally, low-income regions such as Africa rely on imports to meet their regions' food demands, while developed nations such as the United States and Canada produce food products in excess for export (FAO, 2017). This demonstrates that increasing outputs to secure the world food source is largely the responsibility of developed nations (Turk, 2016; FAO, 2017). However, increasing production to reach the necessary GAP must be achieved while inputs, including land, feed, and labor, are maintained or reduced (Global Harvest Initiative, 2016). This must be accomplished by improving current management tools and practices to raise animals in an efficient manner, as well as understanding variables that

reduce livestock output so that these situations can be mitigated (Thornton, 2010). Likewise, the transfer and adaptation of management tools and practices to low-income nations may improve livestock production globally, and begin to alleviate the production burden in developed nations.

One factor that can influence livestock productivity is maternal programming, the phenomena whereby maternal stressors and/or the intrauterine environment encountered during gestation can alter the growth trajectory of offspring and impair the function of organs postnatally (Nathanielsz et al., 2007). In livestock, maternal programming has the potential to reduce postnatal growth rates, reduce lean carcass, and impair the health of offspring, reducing productivity of the livestock sector (Wu et al., 2006). Therefore, it is necessary to investigate strategies to improve gestational management of breeding females, as well as understand mechanisms by which maternal programing reduces productivity, to improve global food security.

REVIEW OF LITERATURE

Gestational management

Management of livestock species depends on the life stage of an animal, and differs during growth, maintenance, pregnancy, lactation, and geriatric. Because breeding is an essential component of livestock production, the breeding and gestational management of pregnant females is important for production efficiency and profitability. Nutrition is an essential component of gestational management, as both maternal restricted- and over-nutrition during gestation result in unfavorable offspring performance postnatally (Wu et al., 2006; Hoffman et al., 2017). During gestation, the nutritional needs of pregnant ewes change to accommodate the needs of the fetus; however, these needs are dependent on the stage of gestation and number of fertilized embryos. From conception through mid-gestation, the nutritional requirements of a pregnant ewe are equivalent to maintenance (National Research Council, 1985), as only 10% of fetal growth occurs before day (d) 90 of gestation. The last 6 weeks (wk) of gestation correspond with rapid fetal growth, and therefore the maternal nutritional requirements increase accordingly (National Research Council, 1985). However, nutrient requirements during late gestation are also dependent on the number of fetuses developing, and classified based on anticipated litter size (National Research Council, 1985).

To ensure that ewe nutrition is managed appropriately, producers must have access to accurate information on the date of conception and litter size. Recording conception dates is typically accomplished by monitoring the breeding activity of rams. Rams are fitted with raddle or marking harnesses that leave a colored mark on the rump of the ewe after mounting to breed. Acquiring accurate conception dates allows producers to forward calculate when bred ewes will advance to late gestation as well as estimate parturition dates. However, management of ram

activity is tedious, and it may be difficult to maintain accurate breeding records for ewes requiring multiple ram services. Further, ram service does not confirm pregnancy. Ultrasonography is one tool that livestock producers commonly utilize to diagnose pregnancy, confirm fetal viability, and estimate conception dates of breeding females.

Litter size information is also valuable to sheep producers because managing breeding females according to the number of fetuses that ewes are carrying is critical during late gestation. In ewes, the risk of negative energy balance is increased during the last 6 wk of gestation because of the rapid increase in fetal demands (Andrews, 1997; Rook, 2000). Further, multiple fetuses crowd the abdomen of the ewe, compromising space in the rumen for nutrient ingestion (Andrew 1997, Rook, 2000). These situations can independently or synergistically result in ketosis, commonly known as pregnancy toxemia, in ewes. Ketosis is a metabolic disease that commonly affects small ruminants during late gestation. The pathogenesis of ketosis is characterized by mobilization of maternal ketone reserves to support energy demands during negative energy balance (Bergman, 1971; Rook, 2000). Thus, identifying ewes in late gestation, providing appropriate nutrition based on litter size, and monitoring for symptoms of ketosis are important components of proper gestational management of a breeding flock. Obtaining information related to gestational age and litter size precedes these management actions and is commonly accomplished by performing ultrasonography during gestation.

Integration of ultrasonography for gestational management

Ultrasonography is commonly integrated into the management of livestock operations to diagnose pregnancy because of its user-friendly technique, low cost per animal, fast scanning time (1 to 2 min per animal with an experienced technician), and immediate results. Recent

advances in ultrasound technology have improved both the image clarity and the portability of ultrasound machines, improving usefulness of this tool for livestock production and research. Applying ultrasonography during an optimal time-frame in ewes can improve flock gestational management, and therefore the health of the offspring. For example, detecting a fetus to diagnose pregnancy during the first 40 d of gestation allows for fetal counting and improves rebreeding and culling decisions (Ishwar, 1995; Jones and Reed, 2017). Scanning throughout mid- and late-gestation allows technicians and producers to monitor growth patterns of the offspring, with the potential to identify abnormalities in growth before parturition (Ishwar, 1995; Jones and Reed, 2017). Additionally, gestational age can be determined based on information acquired via ultrasound, rather than only relying on ram activity (Jones and Reed, 2017). This is important for producers to provide appropriate nutrition to a breeding flock, by increasing feed during late gestation and monitoring ewe needs as gestation advances. This also allows for the estimation of parturition dates, improves late gestational management even if breeding dates are unknown, and increases the likelihood of supervised lambing to decrease neonatal mortality. These factors all contribute to proper gestational management decisions to ultimately improve the health of the animals and reduce input costs for the producer.

History of ultrasound use

Ultrasonography was pioneered in the 1950s for body imaging by John Wild and later for human obstetrics by Ian Donald (Donald et al., 1958; Campbell, 2013) Early A-mode (amplitude mode) ultrasound provided a one-dimensional image of the uterus, reflecting objects as a function of depth (Campbell, 2013). However, interpretation of A-mode for pregnancy diagnosis was difficult and resulted in image artifacts, leading to the development of a 2D contact scanning

machine by Ian Donald and Tom Brown that produced static, bistable (purely black and white) images (Donald et al., 1958; Campbell, 2013). Although use in human obstetrics began with the work of Ian Donald in the 1950s, application of ultrasound in livestock reproductive management did not begin until the 1980s, with the advent of real-time B-mode (brightness mode) imaging. Since that time, the use of ultrasound has become well integrated into reproductive management of the horse (Ginther, 2014) and the cow (Quintela et al., 2012). In sheep and goats, ultrasound was first employed for pregnancy diagnosis in the early 1980s (Buckrell, 1988); however, widespread adoption in small ruminants has been slower due to extensive management practices. As technologies have improved, the information available from ultrasonography has become more useful for sheep and goat management. This includes detection of fetal number and sexing, and estimating gestational age through observed placental and fetal development during early gestation or measurement of fetal organs during mid- and late- gestation. Additionally, sheep have become a popular biomedical research model for pregnancy, and therefore application of ultrasound to track fetal growth during gestation in multiple experimental conditions is also relevant. As such, ultrasound has become an extremely useful tool for researchers, clinicians, and producers to gain valuable information during gestation, improve management decisions, and provide prenatal fetal growth measurements using a non-invasive approach in small ruminants.

Ultrasound technique in small ruminants

To visualize the uterus in the small ruminant, ultrasound can be performed transrectally or transabdominally. For transrectal scanning, the female is restrained manually in dorsal recumbence to position the uterus and fetus within the pelvic canal (Ishwar, 1995; Dinc et al.,

2001). The probe is lubricated and inserted into the rectum for dorsal visualization of the uterus. From this position, both uterine horns can be identified on either side of the bladder, which is advantageous for counting the number of fetuses implanted (Dinc et al., 2001). Epidemiological data in the ewe has indicated that performing ultrasound transrectally with such restraint does not decrease lambing rate or prolificacy (Wurst et al., 2007), and this approach allows pregnancy to be diagnosed as early as d 17 of gestation in ewes (Garcia et al., 1993). However, scanning transrectally can be stressful for the animal and handler, and increases the risk of rectal perforation. This approach can also require more time per animal due to the restraint necessary, and involves the challenge of obtaining optimal probe contact in the rectum without the technicians hand for direct manipulation or pressure on the probe. Thus, contrary to large ruminants, the transabdominal approach is commonly considered for small ruminants.

For the transabdominal approach, the animal is restrained in a standing position and the lubricated probe is applied in the right inguinal pit. The absence of hair in this area improves probe contact. During early pregnancy, the gravid uterus fills with fluid and becomes displaced by the rumen towards the right of the abdomen, making scanning on the right side of the animal ideal (Buckrell et al., 1986). As gestation advances, the uterus moves deeper into the abdomen and closer to the abdominal wall (Buckrell et al., 1986). Depending on the wool-line of the breed being scanned, shaving of abdominal wool may be considered to facilitate transabdominal scanning during mid to late gestation when the probe is placed on the abdomen rather than in the inguinal pit. Scanning transabdominally may be less stressful and less invasive for the animal, and requires less time per animal because minimal restraint is required.

Pregnancy diagnosis during early gestation

Early pregnancy diagnosis is possible in sheep by detecting uterine characteristics indicative of implantation and a fetal heartbeat, indicating fetal viability. During the first 20 d of gestation, the uterus is situated within the pelvic canal, the same position as observed during non-pregnancy, making it difficult to determine the pregnancy status of ewes (Buckrell, 1988; Garcia et al., 1993). As such, the sensitivity of pregnancy diagnosis in ewes via ultrasound is low (< 80 %) before d 25 of gestation, but increases significantly once the fetus can be consistently detected (Buckrell et al., 1986; Gearhart et al., 1988; Garcia et al., 1993; Medan et al., 2004; Romano and Christians, 2008; Ganaie et al., 2009; Karen et al., 2014). Due to the initial location of the uterus, the transrectal approach is well suited for early scanning (before d 30) because of the close proximity of the ultrasound probe to the uterine wall (Garcia et al., 1993; Amer, 2008). As gestation advances and the uterus is displaced toward the abdominal wall within the imaging limits of transabdominal ultrasound, this approach also becomes suitable for pregnancy diagnosis (Kandiel et al., 2015). Recent advances in imaging technology have improved scanning penetration, allowing greater depth of the abdominal cavity to be scanned, and thus early pregnancy diagnosis is now possible from both the transabdominal and transrectal approach.

The same fetal developmental stages have been observed from both transrectal and transabdominal scanning before d 40 of gestation in sheep. The first detectable characteristic of pregnancy is the formation and enlargement of circular and elongated uterine cross-sections which distinguish the gravid uterus from the bladder on ultrasound and can be viewed transrectally in ewes as early as d 17 to 19 of gestation (Garcia et al., 1993; Doize et al., 1997; Padilla-Rivas et al., 2005; Aly and Hayder 2007; Amer, 2010), or transabdominally between d 25 and 28 of gestation (Padilla-Rivas et al., 2005). However, it is notable that uterine fluid is not a

sole indicator of pregnancy, as uterine fluid is also associated with estrus and uterine abnormalities. Accompaniment of uterine fluid by the amniotic vesicle and presence of the fetus with a heartbeat is imperative for confirming a viable pregnancy (Padilla-Rivas et al., 2005; Aly and Hayder 2007; Amer, 2010).

Reports of fetal detection and the corresponding pregnancy diagnosis are variable between d 15 and 30 of gestation in sheep, and are likely due to differences in frequency of scanning, breed, litter size, and skill of the technician (Fridlund et al., 2013). However, a consistent trend of earlier pregnancy diagnosis is reported using the transrectal approach. In ewes scanned transrectally, the fetal heart beat has been detected as early as d 16 of gestation (Romano and Christians, 2008) with > 90 % sensitivity after d 24 to 26 (Garcia et al., 1993). Using a transabdominal approach, the fetal heart beat can be identified between d 27 to 30 in ewes; however, inconsistencies exist (Padilla-Rivas et al., 2005; Amer, 2008; Karen et al., 2009; Amer, 2010). Development of placentome structures accompany fetal detection, which support the pregnancy and establish the maternal-fetal interface (Doize et al., 1997; Ganaie et al., 2009). In sheep, placentome units can be visualized transabdominally or transrectally during early gestation, initially appearing as irregular shapes on the uterine wall, and maturing into hollow hemispherical structures around d 40 of gestation (Doize et al., 1997).

In addition to pregnancy diagnosis, determination of gestational age aids in management of ewes as gestation advances. In the absence of breeding information, fetal and placental development is the only alternative source of information to determine gestational age; however, a detailed timeline of fetal development that corresponds with gestational age is lacking in the sheep. In goats, a fetal developmental timeline has been described by Kumar et al. (2015) which describes that the umbilicus can be visualized beginning on d 39 with the limbs extending from

the abdomen at d 42 and skeletal structures including the rib cage, spinal cord and skull visualized from d 48 onward (Kumar et al., 2015). This detailed timeline allows producers to obtain more accurate information regarding gestational age, and distinguish between multiple breeding dates in ewes. Currently, limited information exists about a fetal developmental timeline that can be observed via ultrasound in sheep during early gestation. Such information would improve gestational management and calculation of parturition dates.

Ultrasonographic measures of fetal growth during gestation

Measures of fetal growth during gestation can be useful in determining gestational age and deviations from normal growth patterns. During early gestation, the entire fetus can be visualized on the same ultrasound screen, aiding in whole body measurements such as crown rump length (CRL). In sheep, visualization of the entire fetus becomes progressively difficult after d 40 of gestation, and thus, measurement of specific fetal organs or tissues is more commonly performed. During this time, the transabdominal approach becomes favored, and almost exclusively reported, due to the close proximity of the uterus to the abdominal wall (Erdogan, 2012). A variety of organs, tissues and bone structures have been investigated during mid-gestation that correspond with fetal development, and have been used to generate multiple equations to estimate gestational age. In cases where the conception dates are unknown, predictive equations allow for estimation of gestational age and parturition dates (Jones and Reed, 2017). In cases where fetal age is known, monitoring fetal growth curves allows detection of asymmetrical fetal growth. In either case, this information allows the producer and clinician to determine the best course of action to improve the health and well-being of the dam and offspring through the most appropriate management plan.

In the small ruminant, the most common ultrasound variable for monitoring fetal growth during early gestation is the CRL. In the sheep, CRL has been measured at 1 cm as early as d 25 transrectally (Santiago-Moreno et al., 2005; Aly and Hayder 2007). During early gestation, CRL consistently exhibits a strong relationship (R^2 = 0.94 to 0.95) with gestational age, regardless of the scanning approach (Martinez et al., 1998; Santiago-Moreno et al., 2005; Karen et al., 2009). Differences in the CRL of singleton and multiple pregnancies are also evident between d 32 and 42 in sheep (Godfrey et al., 2010), demonstrating that multiples are shorter than singletons, and thus fetal growth can be compromised by litter size as early as the first trimester of gestation. After d 40, CRL measurement becomes difficult in sheep because the length of the fetus exceeds the ultrasound screen.

The biparietal diam. (BPD) is a symmetrical measurement of the skull that can be measured as early as d 35 in the sheep (Santiago-Moreno et al., 2005). The BPD can be measured throughout mid-gestation, with reports as late as d 115 of gestation in Mouflon sheep (Santiago-Moreno et al., 2005). A strong relationship of BPD with gestational age is evident with measurements acquired both transabdominally and transrectally in sheep (R^2 = 0.96 and 0.98, respectively; Petrujkic et al., 2016). In Suffolk sheep fetuses, the BPD increased with gestational age from d 43 to 96 of gestation (R^2 = 0.98) and in Finn sheep fetuses from d 35 to 95 of gestation (R^2 = 0.98; Haibel and Perkins, 1989); however, BPD measurement in the third trimester is typically limited due to the position of the fetus. Together, these data demonstrate that BPD is a useful measurement to estimate gestational age in sheep, using either ultrasound approach, throughout the first two-thirds of gestation.

Additional skull measurements have been used to estimate gestational age in sheep. During mid-gestation (d 49 to 119), skull diam. was positively correlated with gestational age (r

= 0.96), regardless of litter size (Sergeev et al., 1990). There are also significant correlations between the occipito-nasal diam. and gestational age when ewes were scanned transrectally (R^2 = 0.98) and transabdominally (R^2 = 0.95) between d 46 and 63 of gestation (Petrujkic et al., 2016). Diameter of the fetal eye (orbit) has been detected transrectally, and was highly correlated with gestational age from d 46 to 63 in sheep (R^2 = 0.95; Petrujkic et al., 2016).

Organs and other easily identifiable structural features are also commonly measured and evaluated for their potential of estimating gestational age, especially during mid- to late-gestation, as they generally increase in size with fetal development. Measurements of thoracic diam., kidney length, femur length, stomach diam., umbilical cord, and thoracic and cervical vertebrae are all significantly correlated with gestational age in the sheep (R^2 = 0.65 to 0.98). For example, the fetal abdominal diam. increases linearly from mid- to late-gestation, and is highly correlated (R^2 = 0.96) with gestational age (Lekatz 2013; Rurak and Wittman, 2013). During mid-gestation (d 49 to 119) in sheep, thoracic depth is also positively correlated with gestational age (r = 0.95), regardless of litter size (Sergeev et al., 1990).

Placentome units can be observed on ultrasound during early gestation and their morphology evolves from protrusions of the uterus into hollow, cup-like shapes as gestation advances. Placentome units increase in diam. between during early gestation and mature in size by mid-gestation, with the largest diam. reported between d 74 and 80 of gestation. This growth pattern is consistent with placental development, but causes a weak association of placental diam. to predict gestational age in sheep (Doize et al., 1997; Lekatz 2013).

Determining fetal number using ultrasonography

Determining the number of fetuses carried by a ewe is important for gestational management and economic return of livestock operations. Producers can use litter size information to feed dams to the appropriate nutritional requirements, providing suitably for the fetuses and saving on excessive feed costs. Knowing the fetal number in conjunction with estimated parturition dates allows producers to monitor dams during late gestation for risk factors or symptoms of pregnancy toxemia, and allows timely supervision of dams as they approach parturition. Importantly, integrating these practices into small ruminant operations can improve the lambing survival rate and postnatal growth of offspring, ensuring high quality replacement and market animals for economic return.

In general, accuracy of determining litter size decreases with increasing fetal number and stage of gestation, with similar results obtained scanning transrectally and transabdominally (Fridlund et al., 2013). During early gestation, identification of one fetus corresponds with early pregnancy diagnosis, while detecting multiple fetuses may be performed consistently after d 31, with > 80 % accuracy of determining fetal number (Taverne et al., 1985; Gearhart et al., 1988; Medan et al., 2004; Karen et al., 2014). However, accuracy of determining fetal number decreases after d 70 of gestation, as it becomes difficult to discriminate between fetuses on the ultrasound (Gearhart et al., 1988).

Interpretation of expected fetal number can be complicated by fetal loss rates in small ruminants causing technicians to overestimate predictions, and should be considered when implementing this information for management. In ewes, the greatest embryonic and fetal loss rates (approximately 20%) are observed before d 25 of gestation, with this loss rate decreasing between d 25 and parturition (Dixon et al., 2007; Samir et al., 2016). Additionally, after d 25,

most fetal losses result in smaller litter sizes, rather than complete loss of the pregnancy (Dixon et al., 2007). Overestimating fetal number is also possible by recounting of the same fetus. This error is common when performing transabdominal ultrasound due to the viewpoint of uterine cross sections rather than each horn. This source of error may be addressed in the future by performing fetal predictions with the criteria of counting only the number of fetuses which can be viewed on the same ultrasound screen. However, sources of error with this criterion may result in predicting fewer fetuses than the ewe is actually carrying, creating the opposite management challenge and the need to rescan and confirm fetal number. With recent advancements in ultrasound technology, portable ultrasounds are equipped with high-quality imaging, creating the opportunity to easily recheck or verify the number of fetuses a dam is carrying.

Collectively, incorporation of ultrasonography into the breeding and gestational management of ewes provides numerous opportunities to improve production. This includes early pregnancy diagnosis that results in pro-active gestational management, determining gestational age and litter size that results in management appropriate to gestational needs, and obtaining accurate fetal growth measurements that results in monitoring prenatal fetal performance. This information is a vital component of livestock management because improper gestational management and stress can negatively affect offspring survival, growth and health postnatally.

Maternal Programming

Maternal programming is defined as changes to the intrauterine environment during gestation that alter the growth trajectory and/or organ function of offspring postnatally

(Nathanielsz et al., 2007). The effects of maternal programming can positively or negatively affect offspring performance, and often persist throughout adulthood (Nathanielsz et al., 2007). There is also evidence that the effects of maternal programming may be multigenerational, transmitting to subsequent generations when offspring reproduce (Blair et al., 2010; Shasa et al., 2015). Factors that contribute to maternal programming in the livestock sector can be intrinsic or extrinsic, and occur frequently during production (Wu et al., 2006). These factors may include litter size, uterine capacity, placental transfer, and nutrient availability, among others (Wu et al., 2006). While intrinsic factors may be caused by physiological limitations of the mother, extrinsic factors are caused by environmental stressors that may be mitigated by management practices if better understood.

Intrinsic factors contributing to maternal programming

To improve profitability, producers are interested in increasing the number of healthy offspring produced from each breeding female as a way of reducing input costs but improving output. However, increasing litter size can compromise the growth of offspring (Gootwine, 2005; Wu et al., 2006). Sheep commonly carry one, two, or three offspring, with distinct birth weights and growth rates observed between singleton and multiple pregnancies (Gootwine, 2005; Dixon et al., 2007). During gestation, embryonic and fetal mortality are more common in sheep litters with multiples compared with singletons (Dixon et al., 2007). Additionally, fetal loss patterns in sheep are most often characterized by loss of one fetus of a multiple pregnancy, rather than loss of an entire pregnancy (Dixon et al., 2007). This demonstrates that despite breeding ewes being capable of carrying a pregnancy to term, support for multiple fetuses is often insufficient during gestation (Dixon et al., 2007). In successful litters, reduced growth and development is observed

in lambs born in twin and triplet litters, and neonates of multiple litters exhibit greater morbidity and mortality at birth than singletons (Gootwine, 2005; Dixon et al., 2007). Furthermore, postnatal performance of twin or triplet offspring is suboptimal, demonstrated by reduced feed utilization and growth rates compared with singletons (Wu et al., 2006). The poor performance of twins and triplets is associated with compromised uterine capacity, or the physiological and biochemical limitations imposed on fetal growth and development by the uterus (Bazer et al., 1969). In livestock, limitations of uterine capacity are most often observed in pigs because this species commonly produces litters of eight or more piglets per sow (Bazer et al., 1969). In pigs, runts are associated with the least placental contact and low nutrient transfer, exhibit low birth weight, and demonstrate reduced muscle fiber number and fiber size postnatally (Wigmore and Stickland, 1983). However, the effect of uterine size on fetal growth is also understood by embryo transfer studies in sheep, cattle, and horses which have demonstrated that embryo growth is proportional to the uterine size of the recipient dam, rather than a donor dam (Dickinson et al., 1962; Ferrell, 1991; Allen et al., 2004). Placental transfer is another intrinsic factor causing programming of offspring. During pregnancy, the placenta is responsible for facilitating exchange of nutrients, oxygen, and waste between the mother and fetus (Vonnahme and Lemley, 2012; Vonnahme et al., 2013a). In response to maternal stressors, placental transport can be compromised by improper establishment or adaptations that reduce uteroplacental blood flow, thus reducing nutrient and oxygen delivery to the intrauterine environment (Vonnahme and Lemley, 2012; Vonnahme et al., 2013a). In severe cases, compromised placental transport results in pre-term delivery or abortion because of insufficient nutrient and oxygen delivery to the fetus (Vonnahme and Lemley, 2012).

Extrinsic factors contributing to maternal programming

The maternal diet is an essential component of gestational management because nutrient intake by the dam provides for both the mother and developing fetus. During gestation, the mother partitions nutrients towards the fetus to support fetal growth and development (Redmer et al., 2004). Yet, in many livestock production situations, nutrient availability may be altered by land limitations, seasonal weather fluctuations, or production practices that alter the quality and/or quantity of feed intake (Thomas and Kott, 1995; Wu et al., 2006). Thus, changes to the macro- or micro-nutrients available to breeding females may occur during any time period beginning before conception throughout parturition, and result in poor maternal nutrition (Wu et al., 2006; Hoffman et al., 2017). Many ruminant livestock systems are forage based, and therefore situations of limited or excess nutrients are common. In sheep, the breeding season corresponds with seasonal fluctuations in forage quality and quantity. Ewes are short-day breeders, and therefore typically begin pregnancy in the fall, with the gestational period coinciding with winter when forage quality and abundance is limited on the range (Wu et al., 2006). Ewes reared on range are therefore susceptible to reduced forage intake and quality, often experiencing less than 50% of NRC nutritional requirements during the breeding month in the Western United States (Thomas and Kott, 1995). Alternatively, at intensive sheep production systems located throughout the Northeast United States and at land grant universities it is speculated that ewes are provided more than adequate nutrients, resulting in over-conditioning during breeding and/or gestation (Jobgen et al., 2008). Additionally, sheep producers who are interested in increasing outputs may rely on the practice of flushing, or increasing the feeding level before conception to encourage superovulation in ewes (National Research Council, 1985).

Effect of maternal diet on offspring

Both excessive and limited nutrient availability to the mother during gestation can negatively affect the prenatal and postnatal development of offspring; however, these effects are dependent on the diet type, and the duration and stage of gestation during which the nutrient insult occurred (Wu et al., 2006). During pregnancy, nutrients are partitioned in a hierarchy to support maintenance of maternal tissues and metabolic needs of the fetus, which change as gestation advances (Redmer et al., 2004). Early gestation (< d 50 in sheep) facilitates rudimentary organ development as well as establishment of the placenta (Redmer et al., 2004). Therefore, nutrient insults before conception and during early gestation have negative consequences on the formation, growth, and function of major organ systems, including the nervous, hepatic, and cardiac systems (Edwards and McMillen, 2002; Redmer et al., 2004). In addition, restricted peri-conceptional nutrition is associated with premature activation of the hypothalamic-pituitary axis, resulting in non-infectious preterm deliveries in sheep (Edwards and McMillen, 2002). During early gestation, both maternal restricted- and over-nutrition cause inadequate placental formation which negatively affects placentome function and placental transfer throughout the duration of gestation (Redmer et al., 2004; Vonnahme et al., 2007, 2013a). The placenta is completely established during the first two-thirds of pregnancy, reaching maximum size by d 90 in the sheep, whereas the last third of gestation facilitates 90% of fetal growth (Redmer et al., 2004). Thus, exposure to maternal restricted- or over-nutrition during mid- to late- gestation can cause alterations in offspring body size and composition, organ mass, and metabolism (Ford et al., 2007; Vonnahme et al., 2013b; McGovern et al., 2015; Mois et al., 2016). Additionally, placental adaptations in blood flow and hemodynamics may be observed

when dams experience restricted- or over-nutrition during mid- to late- gestation (Lekatz et al., 2013; Vonnahme et al., 2013a).

In general, offspring exposed to poor maternal nutrition exhibit an unfavorable postnatal phenotype for livestock production. Postnatal growth of livestock is rapid and characterized by maturity at 2 yr of age. Birth weight is an important indicator of offspring survival and postnatal growth because both increased and decreased birth weight correlate with increased mortality in lambs (Dwyer et al., 2016). Offspring born to nutrient-restricted mothers may exhibit reduced birth weight, which is often accompanied by compensatory ADG postnatally (George et al., 2012; Hoffman et al., 2014). Compensatory ADG, commonly known as 'catch-up growth', results in offspring who achieve normal body size at maturity, but their body composition exhibits increased white adipose deposition at the cost of muscle growth. Alternatively, offspring born to over-fed mothers may exhibit increased birth weight, but reduced ADG postnatally (Hoffman et al., 2016). Importantly, many studies also report no difference in birth weight of offspring born to restricted- or over-nourished mothers (Hoffman et al., 2014, 2016; Van Emon et al., 2015; Kleemann et al., 2015; Long et al., 2015; Shasa et al., 2015). Yet, in the absence of birth weight differences, offspring exposed to poor maternal nutrition during gestation still exhibit alterations in postnatal growth, body composition, whole-body and tissue metabolism, and immunity (Ford et al., 2007; Van Emon et al., 2015; Kleemann et al., 2015). This includes reduced muscularity, increased adiposity, reduced insulin and leptin sensitivity, cardiac remodeling, hepatic dysfunction, and muted innate immunity (Ramsay et al., 2002; Vonnahme et al., 2003; Ford et al., 2007; Yan et al., 2011a; Huang et al., 2012; He et al., 2014; Du et al., 2015; Shasa et al., 2015). Consequently, maternal nutrient restriction and over-nutrition have the potential to reduce productivity in livestock by increasing loss of young stock, increasing input

costs to slow growing animals, and reducing carcass quality and quantity in animals raised for consumption. However, the mechanisms by which maternal diet influences offspring postnatal performance remain poorly understood.

Inflammation as a mechanism of fetal programming

Inflammation has been proposed as a mechanism contributing to fetal programming because inflammation has the capacity to alter the maternal and intrauterine environment by interacting with the endocrine and metabolic systems (Hotamisligil, 2006). Inflammation has been reported to persist postnatally in offspring in a tissue-specific manner, which contributes to unfavorable growth, tissue composition, and metabolism in offspring exposed to maternal nutrient restriction or over-nutrition during gestation (Ramsay et al., 2002; Du et al., 2010a; Heerwagen et al., 2010; Hyatt et al., 2011; He et al., 2014). Thus, inflammation may underlie the physiological and pathological processes commonly associated with long-term consequences of fetal programming. Yet, how inflammation changes during situations of maternal restricted- or over-nutrition during gestation remains poorly understood, and further investigation is necessary in both the mother and offspring.

Inflammation

Inflammation is a response of the innate immune system that acts as an immediate defense mechanism in mammals to protect from infection and tissue injury (Medzhitov, 2008). Contrary to adaptive immunity, which is slow, mediated by antibodies, and antigen specific, the innate immune system provides an immediate response to threat that is nonspecific and mediated by leukocytes (Medzhitov, 2008). Typically, inflammatory responses are acute, induced by tissue

trauma, tissue stress, or microbial infection, and involve coordinated communication of plasma and leukocyte cell types throughout the body (Medzhitov, 2008).

The acute inflammatory response occurs in four phases (Soehnlein and Lindbom, 2010). First, disturbances to tissue homeostasis are detected by patrolling monocytes and tissue resident macrophages (Soehnlein and Lindbom, 2010). In response to disturbance, these cell types produce chemotactic signals that activate the innate immune system and recruit neutrophils (Soehnlein and Lindbom, 2010). Next, neutrophils rapidly emigrate to the site of disturbance and release granular contents that recruit, anchor, and influx new monocytes at the site of disturbance (Soehnlein and Lindbom, 2010). These monocytes will differentiate into classical macrophages based on the direction of local signals (Soehnlein and Lindbom, 2010). Next, neutrophils undergo apoptosis at the site of disturbance because of their short lifespan (Soehnlein and Lindbom, 2010). Apoptotic neutrophils release 'find me, eat me' signals that attract and activate classical macrophages to phagocytose dead cells, tissue debris, or foreign molecules from the site of disturbance (Soehnlein and Lindbom, 2010). Finally, tissue homeostasis is restored when dead cells, tissue debris, and foreign molecules have been removed and tissue-resident macrophages resume residence at the site of disturbance (Soehnlein and Lindbom, 2010). Together, the sequence of events involved in the acute inflammatory response are vital for the basic defense of the body.

Inflammatory responses can also be chronic, involving a prolonged inflammatory response that persists at low levels (Medzhitov, 2008). Chronic inflammation can result from failure to resolve an acute inflammatory response, causing persistent tissue damage (Medzhitov, 2008). However, chronic inflammation is also promoted by sustained tissue malfunction that is not associated with injury or microbial invasion (Medzhitov, 2008). Rather, chronic

inflammation induced by tissue malfunction is commonly associated with dysregulated metabolism such as insulin and leptin resistance, and accompanies chronic metabolic diseases such as type 2 diabetes, atherosclerosis, and hepatic steatosis in humans (Hotamisligil, 2006; Medzhitov, 2008). Unfortunately, the phenotype of chronic, low-grade inflammation involves pro-inflammatory molecules, and thus causes unnecessary, persistent harm to the body.

Inflammatory phenotypes

Macrophages are key modulators of the innate immune response and therefore inflammatory phenotypes are classified by the dominant macrophage type that is present (Medzhitov and Horng, 2009; Martinez and Gordon, 2014). Two dichotomous macrophage types have consistently been described in literature: classical or alternative, commonly known as M1 or M2, respectively (Martinez and Gordon, 2014). During differentiation, monocytes are polarized towards an M1 or M2 macrophage phenotype based on the presence of local inflammatory mediators such as complementary components, lipid mediators, cytokines, and chemokines (Medzhitov, 2008; Martinez and Gordon, 2014). M1 macrophages promote inflammation and are associated with molecules produced by Type 1 T-helper cells (Th1) of cell-mediated immunity (Martinez and Gordon, 2014). In contrast, M2 macrophages are responsible for tissue resident and pro-resolving activities that maintain homeostasis. M2 macrophages are associated with molecules produced by Type 2 T-helper cells (Th2; Martinez and Gordon, 2014). During homeostasis, the tissue-resident and whole-body macrophage population should exhibit an M1-M2 balance, or slightly favor the M2 phenotype (Galván-Peña and O'Neill, 2014; Martinez and Gordon, 2014). A shift towards an M1 phenotype results in chronic inflammation, and persistent tissue and metabolic dysfunction (Galván-Peña and O'Neill, 2014; Martinez and Gordon, 2014). Discussion of mediators involved in each phenotype is based on the prominent

cell types involved, with M1 versus M2 discussed when macrophages are dominant or Th1 versus Th2 discussed when leukocytes are dominant. Mediators associated with the M1/Th1 phenotype include, but are not limited to, lipopolysaccharide (LPS), interleukin (IL) 1 beta (IL1B), IL6, IL8, interferon gamma (IFNG), C-C motif chemokine ligand 2 (CCL2, also known as monocyte chemoattractant protein-1 alpha [MCP1A]), reactive oxygen species, and tumor necrosis factor (TNF; Martinez and Gordon, 2014). On the other hand, mediators associated with the M2/Th2 phenotype include, but are not limited to, glucocorticoids, IL4, IL10, IL13, resolvins, and transforming growth factor beta (TGFβ; Martinez and Gordon, 2014). These inflammatory mediators not only contribute to the innate immune response, but also interact with other systems in the body.

Inflammation and metabolism

Chronic inflammation commonly accompanies metabolic disorders in the absence of injury or foreign antigens (Hotamisligil, 2006). Simply put, inflammation disrupts metabolism, and dysregulated metabolism promotes an M1-type inflammatory phenotype. This is partly because the metabolic and immune systems share overlapping molecular and signaling systems (Figure 1). For example, the innate immune system is typically activated by danger- or pathogen-associated molecular patterns (DAMP or PAMP, respectively) recognized through toll-like receptors (TLR) that promote a pro-inflammatory cytokine cascade that removes a source of harm from the host (Soehnlein and Lindbom, 2010). However, free fatty acids (FFA) serve as ligands for TLR4, demonstrating the nutrient sensing properties of TLR (Hotamisligil, 2006; Shi et al., 2006). Activation of TLR4 by FFA results in the same pro-inflammatory cytokine cascade as observed when TLRs are activated by DAMPs or PAMPs (Shi et al., 2006). The cytokine

cascade is negatively regulated by suppressor of cytokine signaling (SOCS) proteins produced to mediate inflammatory activity (Hotamisligil, 2006). However, cytokines, insulin, and leptin all act through the receptor tyrosine kinase family (Dandona et al., 2004; Hotamisligil, 2006; Shi et al., 2006). Therefore, inhibition of the cytokine cascade via SOCS also disrupts insulin and leptin signaling, demonstrating that chronic nutrient activation of TLRs can contribute to insulin and leptin resistance (Dandona et al., 2004; Hotamisligil, 2006; Shi et al., 2006).

Evidence of metabolic and immune overlap is also demonstrated by the composition of adipose and liver tissues, in which adipocytes and hepatocytes are in close proximity to tissue-resident macrophages (Hotamisligil, 2006). Adipose and liver are both able to produce tissue-specific cytokines, termed adipokines or hepatokines, respectively, and their cross-talk affects tissue-specific and whole-body metabolism (Choi, 2016). Adipose tissue is responsible for regulating fatty acid and glucose storage, while the liver is responsible for maintaining glucose homeostasis (Rosen and Spiegelman, 2006). The presence of TNF disrupts adipocyte function and glucose homeostasis within these tissues. Adipocyte dysfunction is caused when TNF promotes adipocyte hypertrophy rather than hyperplasia by antagonizing peroxisome proliferator-activated receptor gamma (Borst, 2004). This typically occurs during nutrient excess, and results in engorged or necrotic adipocytes that, in turn, shift M2 macrophages toward M1, promoting a pro-inflammatory environment (Borst, 2004). Excessive TNF also causes phosphorylation of the serine residue of the insulin receptor, resulting in progressive insulin resistance and disrupted glucose homeostasis (Borst, 2004). This encourages a futile cycle of pro-inflammatory production that is further aggravated when blood glucose concentrations cannot effectively be reduced, causing glucose to be converted to glycerol and stored as triglycerides, exhausting adipocyte capacity and promoting hyperlipidemia (Borst, 2004;

Hotamisligil, 2006; Rosen and Spiegelman, 2006; Choi, 2016). In turn, TLRs are chronically activated, stimulating ongoing local production of pro-inflammatory cytokines and disrupting whole body metabolism (Shi et al., 2006; Wolfs et al., 2015). Collectively, chronic inflammation is a common denominator underlying whole-body and tissue-specific metabolic disturbances (Hotamisligil, 2006).

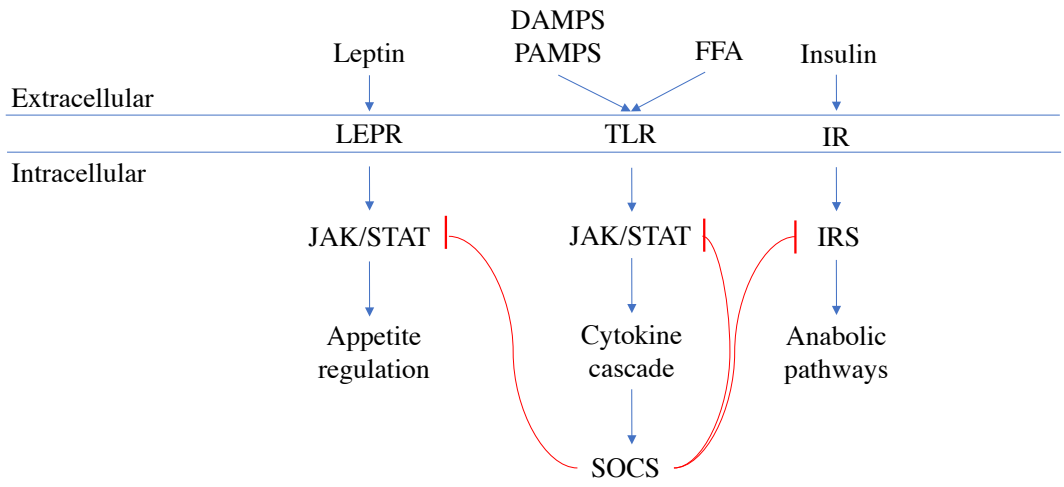

Figure 1. Overlapping signaling systems of the immune and metabolic system. Toll like receptors (TLR) recognize danger or pathogen associated molecular signals (DAMP or PAMP, respectively), which stimulates cytokine production to react to harm in the host (Shi et al., 2006; Rocha et al., 2016). This process is negatively regulated by suppressor of cytokine signaling (SOCS) proteins that inhibit Janus kinase/Signal transducer and activator of transcription (JAK/STAT) proteins via tyrosine kinases (Kazi et al., 2014). Increased concentrations of FFA from the diet can also stimulate TLR with the same downstream cytokine cascade (Shi et al., 2006; Rocha et al., 2016). Excessive signaling of TLR within a host increases SOCS activity, allowing SOCS to inhibit non-target tyrosine kinase signaling pathways, including the leptin receptor (LEPR) and insulin receptor substrate (IRS; Kazi et al., 2014). In this way, excessive free fatty acids (FFA) in the bloodstream contributes to increased inflammation, and leptin and insulin resistance, contributing to further metabolic dysregulation.

Inflammation and pregnancy

Chronic inflammation is also associated with healthy pregnancies and coordinated activation of the cell-mediated immune system. Defense of the mammalian immune system is based on the principle of recognizing antigens that are foreign to the host, which is accomplished by the major histocompatibility complex (MHC). The MHC exhibits a unique pattern of protein expression in each individual, and during pregnancy the fetal MHC expresses paternal antigens that the maternal immune system must tolerate to support fetal development (Rapacz-Leonard et al., 2014). Maternal tolerance of the fetus during pregnancy is mediated by leukocytes through colony stimulating factors (CSF), cytokines, and chemokines (Rapacz-Leonard et al., 2014). Therefore, pregnancy in mammals is associated with mild, chronic activation of the innate immune system and inflammation which is dependent on the stage of gestation (Mor, 2008; Mor et al., 2011).

Early pregnancy involves a Th1-type immune response to facilitate implantation and fetal recognition. The purpose of this Th1 response is to accomplish maternal tolerance and establish a blood supply from the mother to support the fetus (Mor, 2008; Mor et al., 2011; Rapacz-Leonard et al., 2014). Blood flow precedes establishment of the placenta, which will protect the fetus and facilitate nutrient, waste, and oxygen exchange during pregnancy (Reynolds et al., 2005). Ruminants establish a noninvasive placenta where the fetus has no direct contact with maternal circulation (Reynolds et al., 2005; Rapacz-Leonard et al., 2014). Instead, placentome units are comprised of a maternal, caruncular, portion, and a fetal, cotyledonary, portion separated by a microvillar bed that facilitates exchange of nutrients, oxygen, and waste between the maternal and fetal circulation (Reynolds et al., 2005). For comparison, pregnancy establishment in humans is considered invasive because the fetus invades the uterine endometrium to obtain direct contact

with the uterine endoderm to establish a discoid placenta (Rapacz-Leonard et al., 2014). Failure of the initial Th1 response and establishment of the placenta results in early embryonic loss, demonstrating that involvement of the innate immune system is essential for pregnancy success (Mor et al., 2011). A Th2 inflammatory response is observed during mid-gestation, creating a more optimal environment for the fetus to grow and develop (Mor et al., 2011). During late gestation, a shift towards a Th1 inflammatory response is observed and involved in initiating parturition (Mor et al., 2011). This is accomplished through coordinated immune-endocrine actions that increase the abundance of Th1 cytokines and the production of prostaglandins before parturition. Together, these molecules act in a positive feedback manner to promote blood flow to the reproductive tract, cervical dilation, detachment of the placenta, and uterine contractions to facilitate parturition and remove the fetus (Keelan et al., 2003; Gomez-Lopez et al., 2010; Mor et al., 2011). Thus, disruption of the inflammatory response during this time can lead to pregnancy complications such as abortion, pre-eclampsia, or pre-term labor, reiterating the importance of an appropriate innate immune response during pregnancy (Gomez-Lopez et al., 2010).

Potential of maternal inflammation to program fetus

Poor diet and pregnancy are independently associated with chronic inflammation, which may be exacerbated when combined. Inflammation provoked by maternal diet during pregnancy has the potential to alter the maternal environment, intra-uterine environment, and offspring tissue development during gestation, demonstrating a multi-faceted mechanism. Both restricted- and over-nutrition during gestation can alter maternal metabolism and adipose tissue function, resulting in the chronic expression of maternal pro-inflammatory mediators. These pro-inflammatory mediators have the potential to directly affect the fetus by transferring through the

placenta and altering the intrauterine environment (Ingvorsen et al., 2015). It is also possible that maternal inflammatory mediators may indirectly affect the fetus by modifying maternal metabolism and thus altering nutrient composition and availability to the fetus (Ingvorsen et al., 2015). However, limited information is available on the type of maternal inflammatory response that is provoked in response to over- versus restricted-nutrition in livestock, nor how the maternal inflammatory response compares with that of the offspring. Investigation into the inflammatory status of both the dam and offspring may aid in understanding mechanisms by which poor maternal nutrition programs offspring performance.

Maternal diet and offspring inflammation

As described previously, offspring born to nutrient restricted- and over-nourished dams exhibit a postnatal phenotype that is not compliant with efficient growth, proper development, or adequate health. Coincidentally, these offspring often exhibit increased expression of pro-inflammatory mediators indicating that inflammation may be a mechanism by which poor maternal nutrition affects offspring performance. During gestation, inflammation promotes adipogenesis at the expense of myogenesis by disrupting wingless-int (WNT)/β-Catenin signaling (Reviewed in Du et al., 2010a). During normal muscle development, β-Catenin binds to the T-cell factor (TCF) nuclear complex to promote myogenesis (Du et al., 2010a). In the presence of inflammation, β-Catenin is diverted to form a nuclear complex with the forkhead box transcription factor (FOXO), which inhibits myogenesis and promotes adipogenesis (Du et al., 2010a). Male offspring born to over-nourished ewes exhibited increased markers of adipogenesis and insulin resistance in the semitendinosus muscle at d 135 of gestation. This is accompanied by increased inflammatory signaling, demonstrated by phosphorylation of the

inhibitor of nuclear factor κB (NFκB) and increased TLR4 and TNF gene and protein expression (Yan et al., 2010). At 22-mo of age, male offspring born to over-nourished ewes exhibit persistent inflammation in the LM muscle with increased expression of TLR2 and TLR4, and activated NFκB signaling axis. Additionally, maternal obesity increased the gene expression of *IL1B*, *IL6*, *CXCL8,* and *CCL2* and protein expression of TNF in the small intestine of mature lambs at 22 mo of age (Yan et al., 2011b). In a model of maternal nutrient restriction, reduced expression of *SOCS3* was observed in the semitendinosus muscle of newborn lambs, which is indicative of unregulated cytokine activity (Hoffman et al., 2016). Maternal restriction also increased the expression of *TNF* and *TLR4* in the left ventricular tissue of 6 yr old offspring, which was associated with cardiac remodeling (Ge et al., 2013). In response to maternal energy or protein restriction, plasma components C3, C4, IgG, and IgM were reduced in offspring at birth, and accompanied by a muted innate immune response to LPS challenge at 6-wk of age (He et al., 2014). Together, these studies demonstrate that unfavorable inflammation can persist postnatally in multiple tissues of offspring born to both nutrient restricted- and over-nourished dams.

Fetal liver and inflammatory programming

Offspring exposed to maternal nutrient- restriction and over-nutrition exhibit tissue-specific inflammation and dysregulated metabolism, often despite consuming a maintenance diet postnatally (Ford et al., 2007; Hyatt et al., 2011; Yan et al., 2011a; Huang et al., 2012). This suggests that an inflammatory phenotype may be programmed in utero. It has been proposed that inflammation may program the offspring through hepatic priming of the innate immune system

(Heerwagen et al., 2010; Stewart et al., 2013; Wesolowski et al., 2017). Postnatally, the liver is a critical metabolic organ, but prenatally, the fetal liver also has a hematopoietic responsibility.

Fetal liver development begins during early gestation with the hepatic bud generated from the distal end of the foregut of the endoderm (Lemaigre, 2009). Throughout mid-gestation, hepatoblasts will differentiate into hepatocytes, the basic unit of the liver, and cholangiocytes, the basic unit of the bile duct (Lemaigre, 2009; Stewart et al., 2013). Once the basic architecture of the liver is complete, hepatocytes acquire cellular and metabolic capabilities. However, the liver is also the primary location of hematopoietic development during mid- and late-gestation (Kinoshita et al., 1999; Lemaigre, 2009; Stewart et al., 2013; Ginhoux and Jung, 2014). In fact, hematopoietic stem cells account for 60 % of total liver mass during this time (Kinoshita et al., 1999; Stewart et al., 2013; Ginhoux and Jung, 2014). Beginning in mid-gestation, hematopoietic stem cells migrate in from the aorta-gonad-mesonephros area (AGM) to the fetal liver where the hematopoietic stem cells (HSC) continue to proliferate or undergo differentiation (Kinoshita et al., 1999). These cells will either reside in the liver or relocate peripherally to reside in the bone marrow and spleen postnatally (Kinoshita et al., 1999; Ginhoux and Jung, 2014). The liver loses the capability to support hematopoiesis before parturition, at the onset of the glucocorticoid surge (Kinoshita et al., 1999; Ginhoux and Jung, 2014). Due to its prenatal hematopoietic responsibility, it has been proposed that the fetal hepatic microenvironment can prime the postnatal innate immune system through exposure of HSCs to inflammation (Stewart et al., 2013; Wesolowski et al., 2017). That is, T-helper cells and monocytes may be programmed to favor a Th1/M1 or Th2/M2 phenotype postnatally that is dependent upon the prenatal hepatic environment. Thus, the potential of the maternal diet to alter program offspring through the fetal hepatic inflammatory environment warrants further investigation.

Rationale

Optimum livestock production relies on efficient breeding systems and optimal offspring growth to yield high quality products at a volume which meets the demands of the growing population. Proper gestational management of breeding females is a vital component to achieving these production goals. However, multiple factors can stress the maternal or intrauterine environment during gestation, with negative consequences on the subsequent offspring. Integration of ultrasonography during early gestation is a potential management tool to identify intrinsic factors such as litter size that stress fetal development during gestation, while also providing critical information on gestational age to facilitate a pro-active management style. Ultrasonography is a non-invasive technique for monitoring longitudinal fetal growth during gestation, with the advantage of assessing the same fetuses over time. However, previous reports of ultrasonography that monitor fetal growth in livestock rely on time-consuming measurements that are impractical to perform in large production settings. New fetal biometry measurements need to be considered for practical implementation. Pro-active management decisions aid in providing appropriate or interceptive nutrition to gestating females, yet seasonal variability in forage quality and quantity challenge producers to maintain proper gestational nutrition. Poor maternal nutrition negatively impacts fetal and postnatal offspring performance, and inflammation may be an underlying mechanism. Diet-induced inflammation can alter the maternal or intrauterine environment, fetal development, and persist postnatally. Due to importance of gestational management, an applied approach using ultrasonography to diagnose pregnancy and monitor fetal growth, as well as a molecular approach to understanding inflammatory mechanisms contributing to maternal programming, were addressed in the following objectives.

Objectives and hypotheses

Objective 1: Determine the accuracy and sensitivity of transabdominal ultrasound to diagnose pregnancy during early gestation in ewes. We hypothesized that transabdominal ultrasound, using a hand-held ultrasound machine, would accurately detect pregnancy before d 45 of gestation.

Objective 2: Determine the relationship between ultrasound and gross fetal measurements and evaluate differences in fetal ovine growth in utero due to poor maternal nutrition during gestation using transabdominal ultrasound. We hypothesized that paired fetal measurements from ultrasound and gross anatomy would be agreeable when acquired within 24 h of each other and that ultrasound would detect different growth patterns in offspring exposed to poor maternal nutrition compared with offspring of control-fed ewes.

Objective 3: Determine systemic circulatory inflammatory gene expression of pregnant ewes throughout gestation, and systemic circulatory and hepatic inflammatory gene expression of perinatal offspring, when exposed to poor maternal nutrition during gestation. We hypothesized that 1) over- and under-feeding pregnant ewes would disrupt the maternal systemic inflammatory status at specific stages of gestation, 2) offspring exposed to poor maternal nutrition would exhibit increased systemic and hepatic inflammatory gene expression at late gestation and after birth, and 3) the maternal and offspring inflammatory responses would be distinct.

CHAPTER 1: TRANSABDOMINAL ULTRASOUND FOR DETECTION OF PREGNANCY, FETAL AND PLACENTAL LANDMARKS, AND FETAL AGE BEFORE D 40 OF GESTATION IN THE SHEEP

Jones, AK., Gately, RE., McFadden, KK., Zinn, SA., Govoni, KE., Reed, SA. 2015. Transabdominal ultrasound for detection of pregnancy, fetal and placental landmarks, and fetal age before d 45 of gestation in the sheep. *Theriogenology*, 85:939–945. DOI: 10.1016/j.theriogenology.2015.11.002

INTRODUCTION

In 2015, the United States sheep and lamb industry registered its first population increase since 2006, which included an additional 35,000 breeding ewes and replacement lambs (Mathews and Haley, 2015; Sheep and goats, 2015). Such data indicates a current industry focus on increasing flock breeding inventory; thereby generating new interest towards improved management of pregnant ewes. Pregnancy diagnosis is a vital aspect of flock management, and detection of pregnancy during early gestation provides a larger window for sheep producers to make important economic decisions (Bazer et al., 2007). This includes identifying non-pregnant ewes for culling and rebreeding, thereby maintaining reproductive efficiency within the flock and limiting investment into non-pregnant ewes (Ishwar, 1995; Karen et al., 2001; Gonzalez-Bulnes et al., 2010). Additionally, early identification of pregnant ewes ensures adequate nutrition to a gestating ewe and provides an opportunity to estimate litter size. Importantly, the nutritional plane of a ewe can be improved to optimize birth and weaning weights of offspring, prevent pregnancy toxemia, and increase milk production (Gearhart et al., 1988; Karen et al., 2001; Bazer et al., 2007; Scott, 2012).

Real-time ultrasonography is routinely used for pregnancy detection in small ruminants and provides critical information during early gestation, such as estimations of fetal number and gestational age (Buckrell et al., 1986; Scott, 2012; Ginther, 2014). Pregnancy detection via

transrectal ultrasound has been reported to be 77 to 100% accurate as early as d 19 to 29 of gestation in sheep (García et al., 1993; Dinc et al., 2001; Romano and Christians, 2008; Scott, 2012). This level of accuracy using a transabdominal approach has been achieved later in gestation, between d 40 to 80 in sheep (Taverne et al., 1985; Buckrell, 1988; Gearhart et al., 1988; Ganaie et al., 2009) and d 39 to 51 in goats (Karen et al., 2014). Positive indicators of pregnancy, such as placental and fetal landmarks, are detectable by transrectal ultrasound in sheep. This includes identification of a fetal heartbeat as early as d 20, and fetal elongation and placental developments before d 45 of gestation (García et al., 1993; Ali and Hayder, 2007; Romano and Christians, 2008; Godfrey et al., 2010). However, there is limited information on the use of transabdominal ultrasound for identifying such landmarks or fetal elongation (Anwar et al., 2008; Ganaie et al., 2009), and therefore transrectal ultrasound has been favored for early pregnancy detection in sheep (Ali and Hayder, 2007; Ginther, 2014).

Although transrectal ultrasound has been reported to be safe in ewes (Wurst et al., 2007), the application of this approach under field conditions has limitations, requiring a dorsal recumbent position of the ewe for optimal probe contact and ultrasound imaging (Wurst et al., 2007; Romano and Christians, 2008). This positioning is laborious and stressful to both the ewe and technician. Alternatively, the transabdominal approach, applied with ewes in a standing position, may be more adaptable to field conditions and less invasive to the ewe (Gonzalez-Bulnes et al., 2010). Fortunately, advancements in ultrasound technology provide opportunity for more detailed imaging when scanning transabdominally, and therefore potential to use this technique accurately and efficiently for flocks. We hypothesized that transabdominal ultrasound, using a portable ultrasound machine, would accurately detect pregnancy before d 45 of gestation. Therefore, the objectives of this study were to use transabdominal ultrasound during early

gestation to 1) detect pregnancy status and litter sizes, 2) establish a timeline of identifiable early placental and fetal landmarks indicative of pregnancy, and 3) determine the relationship of this information to gestational age.

MATERIALS AND METHODS

The University of Connecticut's Institutional Animal Care and Use Committee approved all animal procedures.

Animals and breeding

Multiparous Western White-faced ewes (n = 99) were estrus synchronized in groups of 24 with progesterone via vaginal insertion of a controlled intravaginal drug releasing device (Easi-Breed CIDR Sheep Insert, Zoetis, Florham, NJ). After 12 d, CIDRs were removed and ewes received 2 mL prostaglandin i.m. (Lutalyse, 5 mg/mL; Zoetis; Florham, NJ; Knights et al., 2001a; Knights et al., 2001b). Ewes were housed with 1 of 4 genetically related Dorset rams for breeding. Each ram was fitted with a marking harness for the entirety of the breeding period. Marking activity was observed and recorded twice daily. The day a ewe received a rump mark was considered d 0 for calculating gestational age. If a ewe was remarked within 20 d, the ewe remained with the ram and calculation of gestational age was restarted. If a ewe was not remarked within 20 d, the ewe was removed from the ram, individually housed and scanned for pregnancy via transabdominal ultrasound. If pregnancy was not confirmed by d 40, the ewe was returned to a ram for rebreeding.

The ewes involved in this study were part of an experiment investigating the effects of poor maternal nutrition on fetal development (Pillai et al., 2015; Raja et al., 2015). At d 20, ewes

in individual housing were transitioned onto a complete pelleted feed over a 10 d period. Beginning at d 30 of gestation, pregnant ewes (n = 82) were randomly fed either a control (100%), over-fed (140%), or restricted-fed (60%) diet based on NRC for TDN requirements for a pregnant ewe carrying twins (National Research Council, 1985). At one of three gestational time points, d 45 (n = 21), d 90 (n = 20), or d 135 (n = 20), animals were euthanized with sodium pentobarbital i.v. (0.22mL/kg; Beuthanasia-D Special; Merek Animal Health; Summit, NJ). A fourth group of ewes was allowed to undergo parturition (n = 21). Ewes remained on diet until euthanasia or parturition. There was no effect of diet ($P \geq 0.27$) on any variable reported herein; therefore data related to diet are not discussed.

Transabdominal ultrasound

Real-time ultrasound was performed transabdominally using a portable EasiScan machine (BCF Technologies, Rochester, MN) with ewes in the standing position. A 5 MHz rectal probe was positioned in the right non-haired abdominal pit with 70% isopropyl alcohol applied topically for optimal contact. A single qualified technician (author R.E.G., DVM) performed all scans (n = 514). Each ultrasound period consisted of scanning each ewe three times/wk beginning at d 26.0 ± 0.3 and continuing through d 40 ± 0.2 of gestation. Due to rebreeding activity, 113 different ultrasound periods were initiated from the 99 ewes.

Ultrasound data collection

During each scan, the ultrasound technician reported a ewe as either pregnant or non-pregnant. Pregnancy was defined by the presence of a fetus(es) with a heartbeat. In pregnant ewes, the first observances of placental or fetal landmarks were recorded (Fig. 2). This included placentome evagination from the uterine wall, leg bud separation from the fetal body, a fetal

genital spot, an umbilical cord, placentome maturation into a hollow donut-shape, and visualization of fetal ribs. Images of enlarged fluid-filled uterine cross-sections, distinct from the bladder, were supportive of pregnancy throughout the entire scanning period. Prediction of litter size was performed for each ewe. Twin and triplet pregnancies were predicted only when more than one fetus could be observed in the same image to prevent recounting the same fetus. Fetal elongation was quantified by measuring fetal length along the longitudinal axis of the fetus using a 1 cm grid super-imposed on the ultrasound screen (Fig. 3). Fetal length measurements were obtained from 16 singleton, 46 twin and 15 triplet pregnancies. Obtaining measurements of fetal length was limited due to improper positioning of the fetus in utero for a longitudinal measurement, or the fetal length extending longer than the ultrasound screen.

Necropsy data collection

At the three gestational time points, a hysterotomy was performed. The uterine horn(s) of fetal implantation were recorded and fetuses were subsequently excised. The number of offspring each ewe carried was recorded at necropsy or following parturition. At the d 45 necropsy, fetal length (n = 36 fetuses) was recorded by measuring craniocaudally along the longitudinal axis of each fetus with a metric digital caliper.

Data analysis

To assess the validity of transabdominal ultrasound to discriminate between pregnancy and non-pregnancy outcomes, each ultrasound result was categorized into a 2 x 2 contingency table as follows (Kaps and Lamberson, 2009; Watson and Petrie, 2010):

True Positive (TP) = pregnant ewe correctly identified as pregnant on ultrasound

False Positive (FP) = non-pregnant ewe incorrectly identified as pregnant on ultrasound

False Negative (FN) = pregnant ewe incorrectly identified as non-pregnant on ultrasound

True Negative (TN) = non-pregnant ewe correctly identified as non-pregnant on ultrasound

Accuracy (Acc) calculated the proportion of pregnant and non-pregnant ultrasound predictions that were correct pregnant or non-pregnant outcomes [$Acc = TP+TN/(TP+FP+FN+TN)$]. Sensitivity (Sen) calculated the proportion of pregnant ewes that were correctly identified as pregnant by ultrasound [$Sen = TP/(TP+FN)$]. Specificity (Sp) calculated the proportion of non-pregnant ewes that were correctly identified as non-pregnant by ultrasound [$Sp = TN/(TN+FP)$]. Positive predictive value (PPV) calculated the portion of ewes with pregnant predictions on ultrasound that were actually pregnant [$PPV = TP/(TP+FP)$]. Negative predictive value (NPV) calculated the portion of ewes with non-pregnant predictions on ultrasound that were actually non-pregnant [$NPV = TN/(TN+FN)$]. Each variable was calculated for every three d of scanning, inclusive of all scans performed during each three-d timeframe.

In pregnant ewes (n = 82), accuracy of fetal counting was calculated as the proportion of litter size predictions made on ultrasound that were correct litter size outcomes at necropsy or parturition. False positive predictions were considered when a larger litter size prediction was made on ultrasound than was present at necropsy or parturition. False negative predictions were considered when a smaller litter size prediction was made on ultrasound than was present at necropsy or parturition.

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). Data were analyzed using the MIXED procedure and included litter size and d of gestation as fixed effects in the class statement. Fetal length was analyzed using repeated measures for the

main effect and interaction of litter size and d of gestation, with d of gestation defined as the repeated unit and ewe defined as the subject. Fetal length measurements obtained from multiple fetuses in a single ewe during a scan were averaged within ewe. Compound symmetry covariance structure was chosen based on the lowest Akaike information criterion (AIC) values. Pairwise incidences were compared using the least square means (LSMEANS) command line and PDIFF option when a main effect or interaction was significant. Data are reported as mean ± SE. Significance was considered at $P \leq 0.05$.

RESULTS

Identification of pregnancy status

Accuracy of detecting pregnancy status increased from 68.8% at d 21 ± 1 to 100% at d 39 ± 1 of gestation (Table 1). Sensitivity of detecting pregnant ewes was 44.4% at d 21 ± 1 and reached 100% at d 39 ± 1 of gestation. Specificity of detecting non-pregnant ewes and the positive predictive value were 100% during the entire scanning period. The negative predictive value increased from 58.3% at d 21 ± 1 to 100% at d 39 ± 1 of gestation. Greater than 90% accuracy, sensitivity and negative predictive value were achieved between d 33 ± 1 and d 42 ± 1 of gestation.

In total, 85 pregnancies were detected at d 28.7 ± 0.4 and 28 non-pregnancies were detected at d 25.5 ± 0.6. Of the 85 pregnancies, three early embryonic losses were identified between d 39 and d 41 (mean: d 39.7 ± 0.7) of gestation, generating a flock embryonic loss rate of 3.5%. The remaining data are presented for the 82 sustained pregnancies, of which pregnancy was detected at d 28.5 ± 0.4 of gestation.

Identification of multiple offspring

Twenty singleton, 46 twin, and 16 triplet litters resulted from the pregnant ewes. Twin fetuses could be observed on ultrasound between d 23 and d 44 (mean: d 34.5 ± 0.5), whereas triplet fetuses were observed between d 25 and d 34 (mean: d 31.0 ± 1.3). Accuracy of predicting fetal number decreased with increasing number of fetuses, with 100, 87 and 31% accuracy for singleton, twin, and triplet pregnancies, respectively. The overall accuracy of predicting fetal number was 78%. No false positive predictions were reported. All inaccuracies were a result of false negative predictions (predicting fewer fetuses than actually present), which included 8 twins and 2 triplets predicted as singletons, and 9 triplets predicted as twins.

Observance of placental and fetal landmarks

In pregnant ewes, placental and fetal landmarks indicative of fetal development were observed throughout early gestation (Table 2). Fluid-filled uterine cross-sections were observed beginning at d 27.9 ± 0.4 and persisted in pregnant ewes throughout the remainder of the scanning period. Immature placentomes emerged from the uterine wall as button-like structures beginning at d 33.8 ± 0.4. Fetal landmarks, such as limb bud separation from the body, a fetal genital spot, and the umbilical cord were first observed at d 35.2 ± 0.7, d 37.9 ± 0.7, and d 38.4 ± 0.7, respectively. Placentome maturation into a hollow donut-shape was observed starting at d 40.6 ± 0.4 and visualization of ribs began at d 42.2 ± 0.7.

Multiple offspring had a significant effect on pregnancy detection and landmarks related to placental development (Table 2). An enlarged uterus ($P = 0.05$) and pregnancy (fetal heartbeat; $P = 0.03$) were detected earlier when multiple offspring were developing compared with singletons (enlarged uterus: d 25.5 ± 1.1, d 26.5 ± 0.8, d 28.6 ± 1.0; pregnancy: d 26.8 ± 1.0,

d 27.3 ± 0.7, 29.7 ± 0.7; triplet, twin, singleton, respectively). Similarly, placentome evagination was observed earlier in triplet than in twin and singleton pregnancies ($P = 0.02$; d 31.8 ± 0.8, d 33.9 ± 0.5, d 34.8 ± 0.7; triplet, twin, singleton, respectively).

Uterine horn distribution

In singletons, fetuses were distributed 56% and 39% in the right and left horns, respectively. One uterine abnormality accounted for the remaining 5% of singletons, where a ewe carrying a singleton had only one uterine horn. A similar uterine distribution was observed in twins, with 53.3% in the right horn and 46.7% in the left horn. For twins, one fetus was implanted in each horn per pregnancy in all but two ewes, which both had two fetuses implanted in the right horn. In triplets, 36.4% of fetuses were implanted in the right horn and 63.6% in the left horn. In all but two ewes, two fetuses were implanted in the left horn and one in the right horn. No cases were observed in which three offspring were implanted in one horn.

Since scanning was performed only in the right abdominal pit, the uterine horn of implantation was analyzed for an effect on pregnancy detection. Only singleton pregnancies were analyzed since those are the only litter size in which the fetus visualized on ultrasound corresponds exactly with fetal information at necropsy. In singletons, uterine horn had no effect on when pregnancy could be detected ($P = 0.29$; right horn d 31.7 ± 1.6; left horn d 29.2 ± 1.3).

Fetal length

Fetal length increased with d of gestation ($P < 0.0001$) but fetal number had no effect on fetal length ($P = 0.72$). A fetal number by day of gestation interaction ($P = 0.01$) was observed for fetal elongation (Fig. 4). At d 29 ± 1, singletons were longer than twins and triplets ($P \leq 0.01$; 20.0 ± 4.9 mm, 12.8 ± 0.5 mm, 13.1 ± 0.8 mm; singleton, twin, triplet, respectively). At d 32 ± 1, singletons were shorter than twins ($P = 0.05$; 13.8 ± 1.8 mm, 18.6 ± 0.7 mm; singleton, twin, respectively). At necropsy at d 45 of gestation, fetuses were 59.7 ± 0.8 mm long, with no effect of fetal number on fetal length ($P = 0.72$).

TABLES

Table 1. Accuracy (Acc), sensitivity (Sen), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of transabdominal ultrasound for pregnancy status prediction.

Gestation, d ± 1	TP[1]	FN	FP	TN	Acc[2], %	Sen, %	Sp, %	PPV, %	NPV, %
21	4	5	0	7	68.8	44.4	100.0	100.0	58.3
24	18	9	0	14	78.0	66.7	100.0	100.0	60.9
27	40	14	0	17	80.3	74.1	100.0	100.0	54.8
30	44	9	0	19	87.5	83.0	100.0	100.0	67.9
33	55	3	0	32	96.7	94.8	100.0	100.0	91.4
36	71	1	0	20	98.9	98.6	100.0	100.0	95.2
39	70	0	0	24	100.0	100.0	100.0	100.0	100.0
42	26	0	0	12	100.0	100.0	100.0	100.0	100.0

[1] TP = correct pregnant prediction; FN = incorrect non-pregnant prediction; FP = incorrect pregnant prediction; TN = correct non-pregnant prediction

[2] *Acc = TP+TN/(TP+FP+FN+TN); Sen = TP/(TP+FN); Sp = TN/(TN+FP); PPV = TP/(TP+FP); NPV = TN/(TN+FN)*

Table 2. Fetal number affects first observance of pregnancy detection and landmarks.

	First Observance, d[1]			
Landmark	Overall	Singleton	Twin	Triplet
Enlarged uterus	27.9 ± 0.4	28.6 ± 1.0[a]	26.5 ± 0.8[b]	25.5 ± 1.1[b]
Pregnancy detection	28.5 ± 0.4	29.7 ± 0.7[a]	27.3 ± 0.7[b]	26.8 ± 1.0[b]
Placentome evagination	33.8 ± 0.4	34.8 ± 0.7[a]	33.9 ± 0.5[a]	31.8 ± 0.8[b]
Limb buds	35.2 ± 0.7	38.0 ± 1.6	34.7 ± 0.8	35.4 ± 1.5
Fetal genital spot	37.9 ± 0.7	39.4 ± 1.2	37.9 ± 0.8	35.8 ± 1.4
Umbilical cord	38.4 ± 0.7	40.3 ± 1.5	38.0 ± 0.9	37.6 ± 1.5
Mature placentome	40.6 ± 0.4	41.5 ± 0.7	40.3 ± 0.5	40.8 ± 0.9
Ribs	42.2 ± 0.7	43.0 ± 1.4	42.4 ± 1.1	38.7 ± 1.7

[1] day of gestation; Mean ± SE

[a-b] Means within a row with different superscripts differ ($P \leq 0.05$)

FIGURES

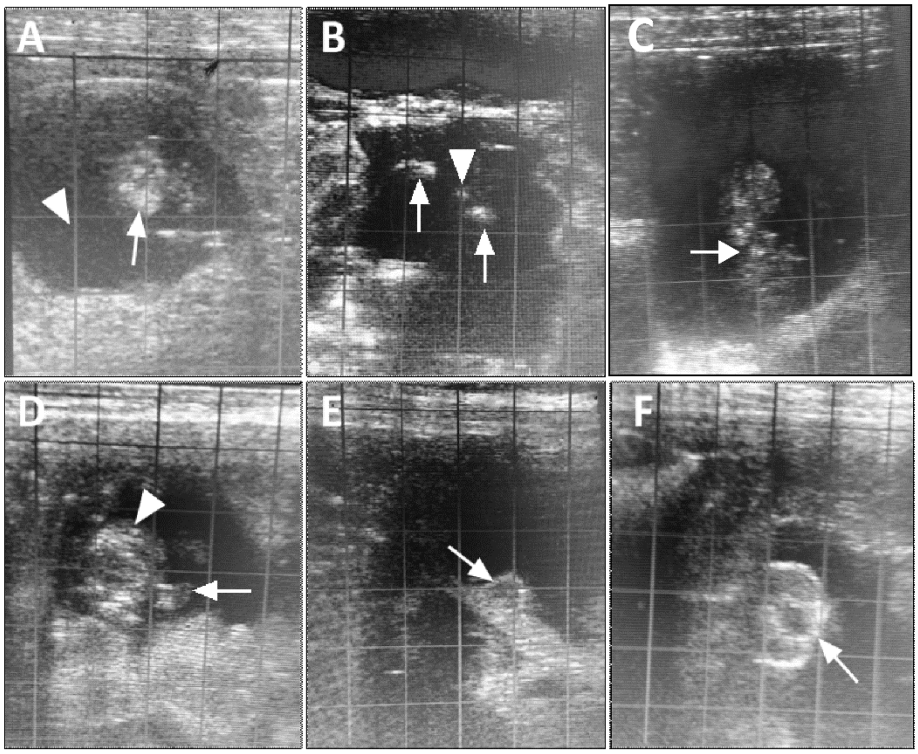

Figure 2. Representative images of ovine placental and fetal landmarks, as viewed transabdominally between d 21 and 45 of gestation. (A) Cross sections of an enlarged, fluid-filled uterus (arrowhead) accompany pregnancy and the presence of a fetus with a heartbeat (arrow). (B) The hind limb buds (arrows) separate from the fetal body and female genitalia (arrowhead) can be identified between the hind legs and tail. (C) The male genitalia (arrow) can be identified in the abdomen of the fetus. (D) The umbilical cord (arrow) extends from the fetal abdomen, with the rib cage (arrowhead) is also visible. (E) An immature placentome (arrow) begins to evaginate from the uterine wall. (F) A hollow, mature placentome unit (arrow) has developed along the uterine wall.

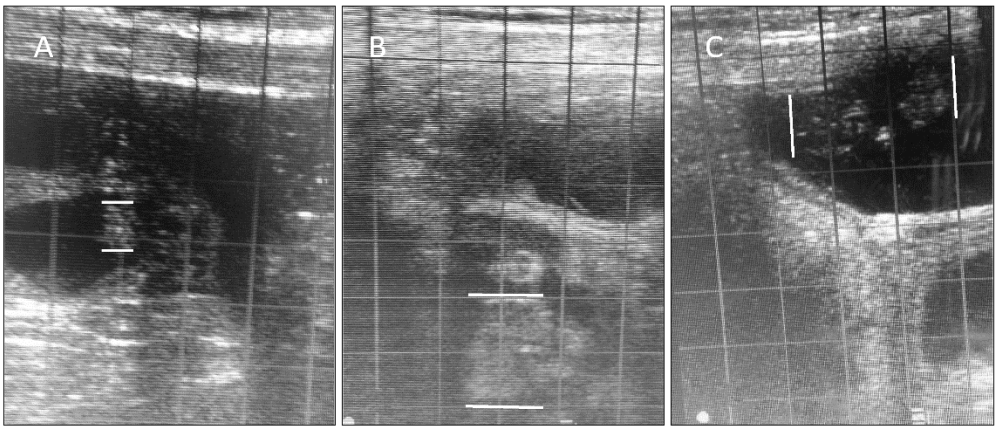

Figure 3. Representative images of fetal length when scanning transabdominally between d 21 and d 45 of gestation in the ovine. Fetal length was measured craniocaudally using a 1-cm grid superimposed on the ultrasound screen. When the longitudinal plane of the fetus could be visualized, the fetus was aligned with the parallel lines of the grid, and the portion of 1-cm squares that a fetus covered was reported in mm. (A) A fetus at d 25. (B) A fetus at d 32. (C) A fetus at d 37.

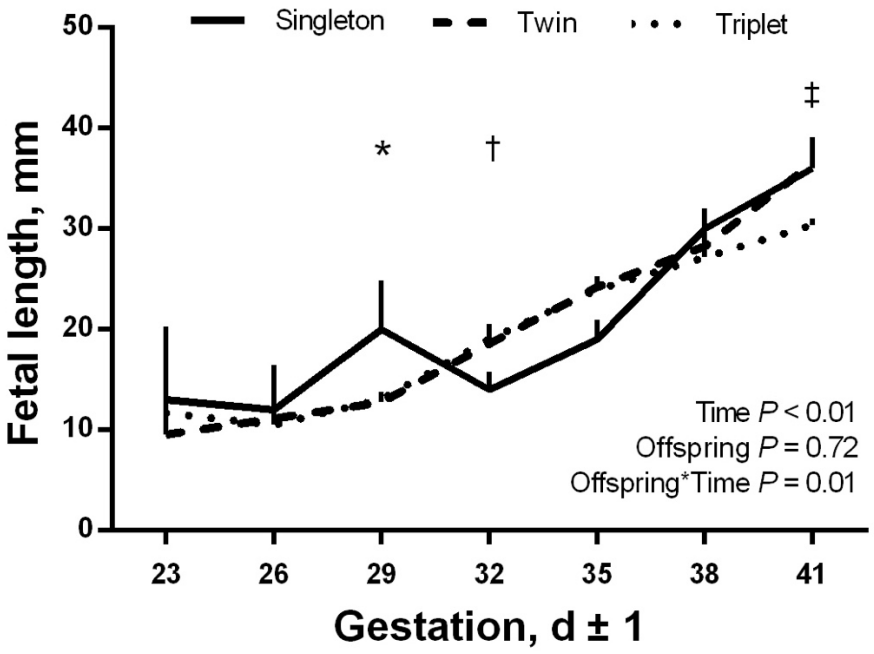

Figure 4. Fetal length increases with gestational age. Fetal length was measured along the longitudinal axis of the fetus using a 1 cm grid superimposed on the ultrasound screen. Fetal length increased with d of gestation ($P < 0.0001$) but not fetal number ($P = 0.72$). A fetal number by d of gestation interaction was observed ($P = 0.01$). * $P \leq 0.01$, singleton vs. twin and triplet; † $P = 0.05$, singleton vs. twin; ‡ $P \leq 0.01$, triplet vs. singleton and twin.

DISCUSSION

In the current study, the overall ability to discriminate pregnancy vs. non-pregnancy using transabdominal ultrasound steadily improved from d 21 to d 33 of gestation, with greater than 90% accuracy and sensitivity achieved from d 33 onward. Increased accuracy and sensitivity were achieved when pregnancy landmarks became identifiable. The low accuracy and sensitivity before d 33 indicate difficulty recognizing positive indicators of pregnancy during this time of gestation. This information is confirmed by the NPV results, which compares the number of non-pregnant predictions on ultrasound with the actual non-pregnant population. Therefore, the low NPV between d 21 and d 30 indicate that a larger number of non-pregnancies were predicted on ultrasound, yet fewer ewes were actually non-pregnant. This information suggests an increased incidence of falsely predicting non-pregnancies during these early days of gestation.

Both specificity and PPV were 100% during the entire scanning period. The high specificity achieved in this study indicates that non-pregnant ewes were adequately detected. However, the aforementioned low NPV between d 21 and d 30 indicates that technicians should use caution when predicting ewes as non-pregnant when using transabdominal ultrasound before d 33. Alternatively, the PPV achieved during this study suggests that once a ewe was detected pregnant by identifying a fetal heartbeat and placental landmarks, there was great confidence that the ewe was, in fact, pregnant.

The increased accuracy, sensitivity and NPV observed from d 30 and d 33 may be due to the uterus expanding from its intra-pelvic location towards the right abdominal wall (Bazer et al., 2012), positioning the uterus and fetus(es) within the depth that the ultrasound probe could penetrate (Bazer et al., 2007; Ganaie et al., 2009). In the current study, identification of placental and fetal landmarks began at approximately d 30 of gestation. This included recognizing

enlarging cross-sections of uterine fluid that were supportive of pregnancy, and placentome evagination and the fetal heartbeat which were positive indicators of pregnancy, between d 28 and d 34. Additionally, the day of gestation that such pregnancy landmarks were identified was indicative of whether multiple or single fetuses were implanted. This is consistent with the biological phenomena of pregnancy, since placental growth is exponential during early gestation, preceding fetal development, and placentome number increases with more fetuses (Vonnahme et al., 2008; Bazer et al., 2012; Vonnahme and Lemley, 2012).

The timeline reported in the current study (Figure 5) for observing early placental and fetal landmarks using the transabdominal approach is consistent with reports using transrectal ultrasound (Ali and Hayder, 2007). Using the transrectal technique, the fetus, limb bud separation and rib structures have been observed at d 25, 38 and 50, respectively using a 6/8 MHz probe (Ali and Hayder, 2007). Transabdominally, using a 3.5 MHz probe, Anwar et al. (2008) observed limb buds and mature placentomes in all ewes between d 51 to 55, when scanning was initiated at d 26 of gestation. The earlier observance in the current transabdominal study (limb buds at d 35.2 and mature placentomes at d 40) may be attributed to improved ultrasound technology and use of a 5 MHz transducer, which lacks the penetration of a 3.5 MHz transducer, but provides a more detailed image (Bazer et al., 2007).

When using ultrasound to estimate litter size in sheep, false positive predictions are reported more commonly than false negative predictions (Fridlund et al., 2013). False positives, or overestimations, are caused by one of two possibilities: unaccounted early fetal loss or recounting of the same fetus (Bazer et al., 2007; Fridlund et al., 2013). False positive predictions were prevented in the present study by 1) accounting for three early fetal losses by scanning ewes repeatedly before d 45, and 2) differentiating fetuses in a single ultrasound image. Reports

of early fetal loss rates in flocks are limited; however, Diskin and Morris, (2008) reported that embryo survival decreased with increasing ovulatory rates in sheep. Mono-ovular sheep had a fetal loss rate of 12%, much less than other mono-ovular species such as bovines (40 to 60%; [Diskin and Morris, 2008]), suggesting that early embryonic loss is a small contributor to overestimations of litter size. This is in agreement with the 3.5% fetal loss rate identified in the current study.

Although false positive predictions were eliminated in this study, 10 false negative predictions of singletons on ultrasound resulting in either twins or triplets at necropsy or parturition did occur. While only a small portion of the flock, such error could result in restricted feeding of a pregnant ewe and compromising fetal development. Importantly, error in predicting fetal number can result in under-feeding (resulting from false negative prediction) or over-feeding (resulting from false positive prediction) pregnant ewes, and therefore these consequences must be considered when using ultrasound to predict litter size.

Measurement of fetal length was dependent on fetal positioning in utero and limited by equipment capacity. With the EasiScan, fetal length was measurable until d 42 of gestation (Figure 5), at which point fetal length reached greater than 40 mm, exceeding the ultrasound image limits. Differences in singleton vs. twin and/or triplet length were observed at d 29 and d 32, but not at other time points. These differences may have resulted from a smaller number of singleton observations obtained at these specific time points, or the limited accuracy of estimating fetal length on a 1 cm grid. Despite these limitations, a significant relationship was observed between fetal length and day of gestation between d 23 and d 41. Similarly, positive correlation between fetal length and age has been established between d 25 and 46 in sheep using the transabdominal technique (Metodiev et al., 2012). Further, Metodiev et al. (2012) reported a

similar fetal length on ultrasound at d 46 (51.8 ± 1.7 mm) to that obtained at necropsy in our study at d 45 (59.7 ± 0.8mm), suggesting agreement between ultrasound estimations and the actual size of the fetus during early gestation.

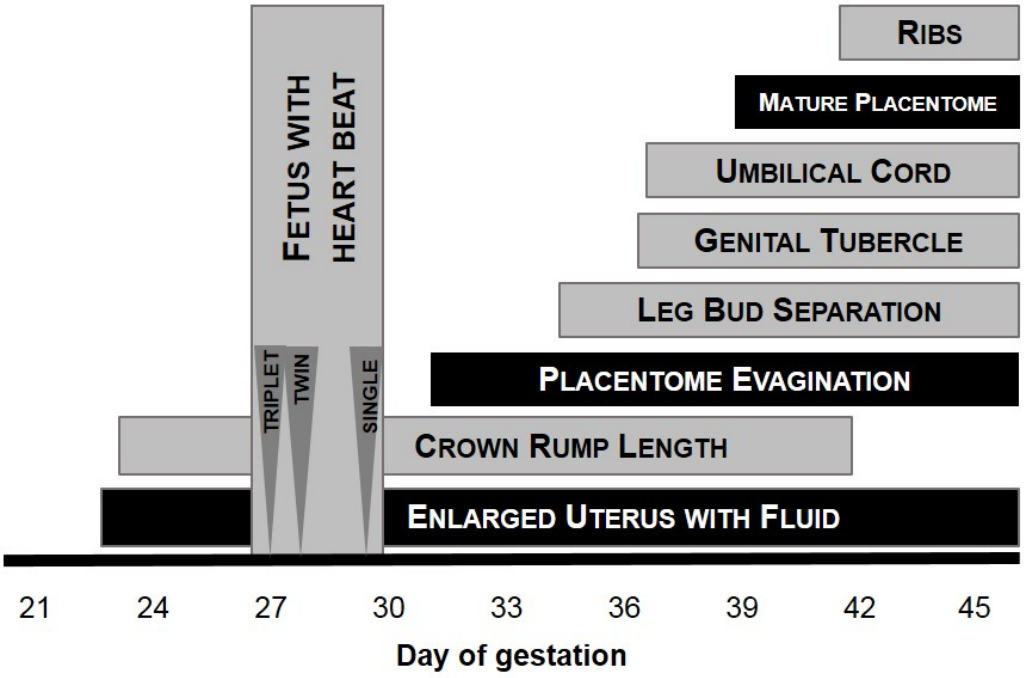

Figure 5. Summary of fetal (grey) and placental (black) landmarks as observed during early gestation via transabdominal ultrasound in sheep as reported by Jones et al. (2016) and published by Jones and Reed (2017).

CONCLUSIONS AND IMPLICATIONS

Transabdominal ultrasound was used to accurately discriminate pregnancy and non-pregnancy before d 45 in the sheep, with greater than 90% accuracy, sensitivity and negative predictive value achieved at d 33 onward. By detecting enlarged uterine cross-sections, technicians can suspect pregnancy even if a fetus is not readily identified. When a fetus and heartbeat are observed, structural developments and fetal length are important indicators of gestational age and breeding date. Furthermore, detection of multiple offspring can be improved by accounting for early placental development and fetal identification. Thus, the use of transabdominal ultrasound before d 45 of gestation in sheep can result in improved flock management by early detection of pregnancy, estimation of fetal number and measurement of fetal length, summarized in Figure 5. Additional research is necessary to determine the accuracy of information acquired on ultrasound to predict parturition information in flocks where breeding records are not maintained. Further, outreach and extension efforts should be conducted by veterinarians and researchers to successfully implement ultrasonography during early gestation into the gestational management of flocks.

CHAPTER II: ULTRASOUND DURING MID-GESTATION: AGREEMENT WITH PHYSICAL FETAL AND PLACENTAL MEASUREMENTS AND USE IN PREDICTING GESTATIONAL AGE IN SHEEP

Jones, AK., Gately, RE., McFadden, KK., Pillai, SM., Hoffman, ML., Zinn, SA., Govoni, KE., Reed, SA. 2017. Ultrasound during mid-gestation: Agreement with physical foetal and placental measurements and use in predicting gestational age in sheep. *Reprod. Dom. Anim.* 00:1-6. DOI:10.1111/rda.12961.

INTRODUCTION

Prenatal growth can be compromised by stressors, including poor maternal diet or litter size (Wu et al., 2006). The gestational age at which fetuses experience stressors can dictate the type and severity of prenatal insult, influencing the postnatal growth and health of offspring (Hoffman et al., 2016a), and impacting economic return of livestock operations. Determining gestational age and monitoring fetal growth are essential components of gestational management that allow producers to provide appropriate nutrition based on the stage of gestation and supervise ewes to improve lamb health and survival.

Ultrasonography is commonly integrated into small ruminant production to improve management of pregnant ewes and monitor the fetus during gestation (Ishwar, 1995). Early pregnancy diagnosis via ultrasound facilitates proactive gestational management decisions and provides litter size estimates (Jones et al., 2016). Mid-gestation is a favorable period to monitor fetal growth via ultrasound because organs and tissues are easily detected. During this time, measurements of fetal or placental structures can be predictive of gestational age, allowing producers to calculate parturition dates and monitor fetal growth (Carr et al., 2011). However, information on the relationship of ultrasonographic measurements to the actual size of the fetus in utero is limited (Carr et al., 2011). Determining the accuracy of ultrasound measurement relative to the actual size of the fetus is important for interpretation of ultrasound data.

The objectives of this study were to 1) employ ultrasound to determine the effects of maternal under- and over-feeding and litter size on fetal growth during mid-gestation, 2) compare ultrasonographic measurements with corresponding postmortem data to determine the precision of ultrasound measurement at d 45 and 90 of gestation, and 3) generate equations predictive of gestational age based on ultrasound data. We hypothesized that poor maternal nutrition and litter size would alter growth of the fetal heart, umbilicus, ribs, and placentomes during mid-gestation and that variables would be predictive of gestational age in the sheep, with ultrasound measures agreeing with postmortem measurement.

MATERIALS AND METHODS

All animal procedures were approved by the University of Connecticut's Institutional Animal Care and Use Committee.

Animals and Breeding

Multiparous Western White-faced ewes were estrus synchronized with progesterone via a CIDR insert (Zoetis, Florham, NJ) for 12 d, followed by i.m. injection of prostaglandin [Lutalyse, 5mg/mL; Zoetis; (Jones et al., 2016)]. Ewes were bred to one of four Dorset rams and confirmed pregnant via transabdominal ultrasound on d 28.5 ± 0.5 of gestation (Jones et al., 2016). Daily marking activity was used to determine gestational age. Pregnant ewes (n = 82) were individually housed and randomly assigned to one of three diets beginning on d 30.2 ± 0.2 of gestation. Diets supplied 60% (RES), 100% (CON) or 140% (OVER) of NRC based on TDN requirements for gestating ewes carrying twins (National Research Council, 1985)**.** Rations were adjusted weekly for BW changes (Reed et al., 2014). A subset of ewes (n = 20 to 21 per time

point) were euthanized at d 45 or 90 of gestation for offspring tissue collection (Pillai et al., 2016).

Sixty-one ewes carrying a singleton (n = 15), twins (n = 35) or triplets (n = 11) were available between d 46.0 ± 0.4 and d 86.0 ± 0.7 of gestation for ultrasonographic evaluation. Transabdominal ultrasound was performed weekly using an Aloka SSD-500 ultrasound (Wallingford, CT) equipped with a 5 MHz linear transducer placed in the right non-haired inguinal pit to visualize the fetus(es). All scans were performed by a single qualified technician (REG). Isopropyl alcohol (70%) was applied topically to enhance probe contact. Fetal and placental biometry were obtained from still-framed images and measured using electronic digital calipers on the ultrasound. Fetal measurements included heart width (HW; transverse axis identified by the septum), umbilical diam. (UMB; obtained from a longitudinal view), and rib width (RW; averaged across three separate ribs). Three placentomes were randomly selected and the outer diam. (OD) and inner diam. (ID) were measured using the longest distance observed. A subset of ewes was euthanized at d 45.1 ± 0.2 and d 89.9 ± 0.2 of gestation (n = 20 or 21 ewes, respectively) for fetal tissue collection (Pillai et al., 2016). Transabdominal ultrasound was performed within 24 h before euthanasia to obtain fetal HW, UMB, RW, placentome OD and placentome ID. Ewes were euthanized with an overdose of sodium pentobarbital administered i.v. (0.22 mL/kg; Beuthanasia-D Special; Merck Animal Health; Summit, NJ) and a hysterotomy was performed to remove the uterus and fetus(es). Postmortem fetal measurements corresponding with ultrasound variables were obtained using handheld digital calipers. These included the transverse axis of the heart, umbilical diam. at three random places, and the width of the 5^{th}, 6^{th} and 7^{th} rib at the ventral aspect of the LM. Three placentome units were detached from

the placenta, the cotyledonary membrane removed, and the longest distance of the caruncle OD and ID measured.

Data analysis

All data were analyzed with ewe as the experimental unit because it was not possible to discriminate between fetuses in ewes carrying multiples during ultrasound scanning. Therefore, sibling data were averaged and reported within ewe. A total of 365 ultrasounds were performed; however, each fetal variable could not be observed on every scan resulting in observance rates of 73% for HW, 81.3% for UMB, 68% for RW, 99% for placentome OD and 98.6% for placentome ID.

Fetal growth

Repeated measures analysis was performed on the weekly HW, UMB, RW, placentome OD and placentome ID using PROC MIXED (SAS Institute Inc., Cary, NC). Fixed effects of gestation, maternal diet, litter size, and all interactions were included with ewe defined as the repeated subject. The Kenward Rogers adjustment was selected to determine the denominator degrees of freedom and account for unequal or missing observations obtained from each subject. The covariate structure for each response variable was chosen based on the lowest Akaike's Information Criteria (Kaps and Lamberson, 2009). Heterogeneous first order autoregressive [ARH(1)] structure was selected for the HW. Heterogeneous toeplitz (TOEPH) structure was selected for the UMB, RW, placentome OD and placentome ID. Significance was considered at $P \leq 0.05$ and data are presented as least square means (lsmeans) ± SE.

Agreement of ultrasonographic and postmortem fetal biometry

The relationship between fetal ultrasound and postmortem measurements was evaluated using correlation and agreement analyses. Pearson's correlation was performed using PROC CORR in SAS. Bland-Altman plots were generated (GraphPad Software Inc., La Jolla, CA) by subtracting the postmortem measurement from the ultrasound measurement. The mean of the differences describes the agreement bias, where a negative bias indicates underestimation, and a positive bias indicates overestimation, of the postmortem measurements by ultrasound. The CI describe the limits of agreement (Bland and Altman, 1986; Watson and Petrie, 2010). Data are presented as mean ± SE.

Generation of equations predictive of gestational age

Regression analyses were performed using PROC REG in SAS (Cary, NC) to generate equations predictive of gestational age. Variables evaluated included HW, UMB, RW, placentome OD and placentome ID. Combinations of these variables were built using the forward and backward command. The goodness of fit of regression equations were assessed using the R^2 and Mallow's C(p) statistic.

RESULTS

Fetal growth

As gestation advanced, fetal HW increased from 6.3 ± 0.14 mm at d 46 ± 3 to 19.4 ± 0.62 mm at d 88 ± 3 ($P < 0.0001$; Table 3); however, no main effects or interactions of maternal diet or litter size with gestation were observed ($P \geq 0.15$). Similarly, a main effect of gestation was observed for UMB ($P < 0.0001$; Table 3), where the UMB increased from 5.6 ± 0.11 mm at d 46

± 3 to 12.5 ± 0.22 mm at d 81 ± 3 ($P < 0.05$), but was similar at d 81 ± 3 and d 88 ± 3 ($P = 0.11$). No main effects or interaction of maternal diet or litter size with gestation were observed for UMB ($P \geq 0.12$).

As gestation advanced, RW increased from 0.85 ± 0.03 mm at d 46 ± 3 to 3.31 ± 0.19 mm at d 88 ± 3 ($P < 0.0001$; Table 3). An interaction of maternal diet by gestation was observed for the fetal RW ($P < 0.0001$; Table 4). Specifically, at d 46 ± 3, RES and OVER were 16.5% and 33% smaller than CON, respectively ($P \leq 0.02$), whereas at d 53 ± 3, RES were 14.5% larger than CON ($P = 0.04$). At d 74 ± 3, RES and OVER were 28.5% and 44.8% larger than CON, respectively ($P \leq 0.04$). At d 88 ± 3, OVER were 18.7% smaller than RES ($P = 0.02$). An interaction of litter size by gestation ($P = 0.0001$; Table 5) was also observed for RW. At d 74 ± 3, twins were 22.6% smaller than triplets (singleton: 2.42 ± 0.27, twin: 2.25 ± 0.13, triplet: 2.90 ± 0.22 mm; $P = 0.02$). At d 88 ± 3 the RW decreased as the number of fetuses increased (singleton: 3.91 ± 0.21, twin: 3.07 ± 0.15, triplet: 2.47 ± 0.26 mm; $P \leq 0.02$).

A main effect of gestation was observed for the placentome OD and ID ($P < 0.0001$; Table 3); however, no main effects or interactions of maternal diet or litter size with gestation were observed ($P \geq 0.15$). Placentome OD increased from 21.0 ± 0.40 mm at d 46 ± 3 to 37.5 ± 0.60 mm at d 74 ± 3 of gestation, but was similar at d 67 ± 3 and 88 ± 3 ($P = 0.82$) and at d 74 ± 3 and 81 ± 3 ($P = 0.48$). Placentome ID increased from 8.2 ± 0.23 mm at d 46 ± 3 to 12.9 ± 0.48 mm at d 67 ± 3 ($P < 0.05$), but was similar at d 53 ± 3, 60 ± 3 and 88 ± 3 ($P \geq 0.07$) and d 67 ± 3, 74 ± 3 and 81 ± 3 ($P \geq 0.15$).

Agreement of ultrasonographic and postmortem fetal biometry

At d 45 of gestation, no significant correlations between ultrasound and postmortem measurements were observed for HW (r = 0.21), UMB (r = -0.03), RW (r = 0.11), placentome OD (r = 0.39) or placentome ID (r = 0.42; $P \geq 0.11$; Table 6). At d 90, correlations were observed between ultrasound and postmortem measurements for the UMB (r = 0.74; $P = 0.0006$) and placentome OD (r = 0.66; $P = 0.0008$), but not for HW (r = -0.08), RW (r = -0.06) or placentome ID (r = 0.28; $P \geq 0.20$; Table 6).

Agreement analysis determined that the mean bias for fetal HW was 0.05 mm (1.3%) at d 45 of gestation; however, at d 90 of gestation, ultrasound underestimated HW by 14.8% (3.17 mm; Table 6). Ultrasound underestimated the actual UMB diam. by 0.29 mm (7.2%) and 1.48 mm (7.3%) at d 45 and 90, respectively. The ultrasound underestimated RW by 0.06 mm (7.7%) at d 45, but overestimated RW by 0.42 mm (23.8%) at d 90 of gestation. Ultrasound measurement underestimated placentome OD at d 45 and 90 of gestation by 0.53 mm (2.7%) and 1.76 mm (4.5%), respectively, and placentome ID by 3.26 mm (27.0%) and 6.48 mm (37.3%). Compared with d 45, larger biases were observed at d 90 for all fetal and placental biometry measurements (Table 6).

Generation of equations predictive of gestational age

All possible combinations of HW, UMB, RW, placentome OD and placentome ID variables were evaluated using regression analysis to determine combination of variables that would explain the most variation in gestational age [greatest R^2 and least Mallow's C(p)]. Seven equations were selected and reported in Table 7. Independent measurement of fetal HW, UMB or RW accounted for 82, 81 or 72% of variability, respectively, when predicting gestational age.

Including two (HW + UMB) or three (HW + UMB + RW) fetal variables accounted for greater variability (88% and 90%, respectively; $P < 0.0001$). By including one placentome measurement (OD or ID), the prediction equation accounted for 91% of variability; however, inclusion of both placental variables did not increase the R^2 beyond 0.91 (data not shown). The combination of HW, UMB, RW and placentome OD decreased the Mallow's C(p) statistic to a value near the number of variables (4) included in the equation, and thus is the strongest multiple-variable equation generated to predict gestational age in the sheep.

TABLES

Table 3. Fetal and placental biometry measured via transabdominal ultrasound in sheep[1].

Gestation, d	HW, mm	UMB, mm	RW, mm	Placentome OD, mm	Placentome ID, mm
46 ± 3	6.3 ± 0.14[a]	5.9 ± 0.11[a]	0.86 ± 0.03[a]	21.0 ± 0.40[a]	8.2 ± 0.23[a]
53 ± 3	8.2 ± 0.21[b]	7.0 ± 0.15[b]	1.31 ± 0.04[b]	27.2 ± 0.58[b]	10.6 ± 0.36[b]
60 ± 3	9.8 ± 0.21[c]	8.6 ± 0.16[c]	1.60 ± 0.06[c]	32.2 ± 0.65[c]	11.4 ± 0.45[b]
67 ± 3	11.8 ± 0.27[d]	9.9 ± 0.22[d]	1.97 ± 0.05[d]	35.8 ± 0.50[d]	12.9 ± 0.48[c]
74 ± 3	14.4 ± 0.30[e]	11.1 ± 0.26[e]	2.49 ± 0.12[e]	37.5 ± 0.60[e]	12.7 ± 0.44[c]
81 ± 3	16.8 ± 0.46[f]	12.5 ± 0.22[f]	2.67 ± 0.09[e]	37.9 ± 0.65[e]	13.5 ± 0.57[c]
88 ± 3	19.4 ± 0.62[g]	15.1 ± 1.68[f]	3.31 ± 0.14[f]	35.6 ± 0.61[d]	11.6 ± 0.43[b]

HW = Heart width; UMB = Umbilical diam.; RW = Rib width; OD = Outer diam.; ID = Inner diam.

[1] Data are presented as lsmean ± SE.

[a-g] Within a column, lsmeans with different superscripts differ ($P \leq 0.05$).

Table 4. Interaction of maternal diet and gestation on fetal rib width, as measured via ultrasound.

	Treatment[1, 2]		
Gestation, d	CON	RES	OVER
46 ± 3	1.03 ± 0.05[a]	0.86 ± 0.04[b]	0.69 ± 0.05[c]
53 ± 3	1.24 ± 0.06[a]	1.42 ± 0.06[b]	1.29 ± 0.06[ab]
60 ± 3	1.62 ± 0.11[a]	1.59 ± 0.11[a]	1.66 ± 0.14[a]
67 ± 3	2.01 ± 0.21[a]	1.90 ± 0.08[a]	1.91 ± 0.10[a]
74 ± 3	2.03 ± 0.15[a]	2.61 ± 0.17[b]	2.94 ± 0.20[b]
81 ± 3	2.71 ± 0.15[a]	2.64 ± 0.15[a]	2.79 ± 0.21[a]
88 ± 3	3.07 ± 0.23[ab]	3.52 ± 0.16[a]	2.86 ± 0.23[b]

[1] Data are presented as lsmean ± SE in millimeters.

[2] Offspring from ewes fed 100%, 60% or 140% of NRC for TDN beginning at d 30.2 ± 0.2 of gestation are referred to as CON, RES and OVER, respectively.

[a-c] Within a day of gestation, lsmeans with different superscripts differ ($P \leq 0.05$).

Table 5. Interaction of litter size and gestation on fetal rib width measured via ultrasound.

	Litter size[1, 2]		
Gestation, d	Singleton	Twin	Triplet
46 ± 3	0.89 ± 0.06[a]	0.89 ± 0.03[a]	0.81 ± 0.05[a]
53 ± 3	1.40 ± 0.07[a]	1.31 ± 0.04[a]	1.23 ± 0.07[a]
60 ± 3	1.61 ± 0.14[a]	1.58 ± 0.08[a]	1.68 ± 0.15[a]
67 ± 3	2.02 ± 0.10[a]	1.98 ± 0.07[a]	1.82 ± 0.11[a]
74 ± 3	2.42 ± 0.27[ab]	2.25 ± 0.13[a]	2.90 ± 0.22[b]
81 ± 3	2.85 ± 0.18[a]	2.59 ± 0.12[a]	2.71 ± 0.23[a]
88 ± 3	3.91 ± 0.21[a]	3.07 ± 0.15[b]	2.47 ± 0.26[c]

[1] Data are presented as lsmean ± SE in millimeters.

[2] Data from siblings are averaged within ewe.

[abc] Within a d of gestation, lsmeans with different superscripts differ ($P \leq 0.05$).

Table 6. Agreement of fetal biometry obtained via transabdominal ultrasound and postmortem measurement.

Gestation, d	Fetal biometry variable	Ultrasound, mm[1]	Necropsy, mm[1]	Correlation (r)	*P* value	Bias[2], mm	Limits of Agreement, mm
45	HW	6.18 ± 0.21 (0.41)	6.10 ± 0.14 (0.27)	0.21	0.45	0.05	(-1.8) (2.0)
45	UMB	5.23 ± 0.23 (0.45)	5.64 ± 0.22 (0.43)	-0.03	0.90	-0.29	(-2.8) (2.2)
45	RW	0.84 ± 0.04 (0.08)	0.91 ± 0.04 (0.08)	0.11	0.75	-0.06	(-0.5) (0.4)
45	Placentome OD	18.98 ± 0.75 (1.47)	19.51 ± 0.72 (1.41)	0.39	0.13	-0.53	(-6.9) (5.8)
45	Placentome ID	7.36 ± 0.44 (0.86)	10.09 ± 0.68 (1.33)	0.42	0.11	-3.26	(-8.5) (2.0)
90	HW	19.25 ± 0.96 (1.88)	22.61 ± 0.46 (1.10)	-0.08	0.75	-3.17	(-12.1) (5.7)
90	UMB	14.30 ± 0.43 (0.84)	15.43 ± 0.55 (1.08)	0.74	0.0006	-1.48	(-4.9) (2.0)
90	RW	2.91 ± 0.19 (0.37)	2.35 ± 0.08 (0.16)	-0.06	0.86	0.42	(-0.8) (2.0)
90	Placentome OD	36.60 ± 1.51 (2.96)	38.36 ± 1.45 (2.84)	0.66	0.0008	-1.76	(-13.0) (9.4)
90	Placentome ID	10.88 ± 0.50 (0.98)	17.35 ± 1.51 (2.96)	0.28	0.20	-6.48	(-19.8) (6.8)

HW = Heart width; UMB = Umbilical diam.; RW = Rib width; OD = Outer diam.; ID = Inner diam.

[1] Ultrasound and postmortem measurements were acquired from the same animal within 24 h. Sibling data are averaged within ewe and presented as mean ± SE (margin of error).

Table 7. Equations predictive of gestational age in sheep.

Ultrasound variable(s)	Regression Equation	SE	R^2	C(p)
HW	GA = 33.91 + 2.65HW	0.59	0.82	166.8
UMB	GA = 26.09 + 4.11UMB	0.77	0.81	194.5
RW	GA = 40.68 + 12.59RW	0.88	0.72	381.4
HW + UMB	GA = 27.42 + 0.50HW + 2.11UMB	0.48	0.88	48.4
HW + UMB + RW	GA = 28.12 + 1.09HW + 1.85UMB + 3.39RW	0.50	0.90	17.4
HW + UMB + RW + PlacentomeOD	GA = 24.45 + 1.02HW + 1.61 UMB + 3.25RW + 0.22PlacentomeOD	0.47	0.91	4.3
HW + UMB + RW + PlacentomeID	GA = 25.95 + 1.09HW + 1.71UMB + 3.47RW + 0.29PlacentomeID	0.42	0.91	9.3

GA = gestational age; HW = Heart width; UMB = Umbilical diam.; RW = Rib width; OD = Outer diam.; ID = Inner diam..

DISCUSSION

The current study employed ultrasound to evaluate growth of the fetal heart, umbilicus, ribs and placentomes during mid-gestation to determine how these organs and structures responded to maternal over- and under-feeding and litter size, and their ability to predict gestational age. The chosen biometry parameters are easily and quickly identified on ultrasound, and thus may be of interest for producers. However, the precision of ultrasound measurement is questionable due to the contractile (heart and umbilicus) and variable (rib and placentome) nature of each organ. Therefore, comparisons of ultrasound measurements with the corresponding organs at slaughter was performed to depict the precision of ultrasonographic data, improving interpretation and implementation of these parameters in pregnant sheep.

Between d 46 and 88 of gestation, growth of the fetal HW and UMB was linear, increasing in size as gestation advanced, and was consistent with previous data reporting that the UMB of fetal sheep increased from 0.35 cm at d 31 to 1.8 cm at d 81 of gestation (Santiago-Moreno et al., 2005). On ultrasound, nutrition-induced changes in fetal HW or UMB growth were not observed at d 45 or 90 of gestation, consistent with postmortem data (Pillai et al., 2016). This is likely due to nutrient prioritization towards the fetal heart and circulation, which are critical for survival (Redmer et al., 2004). Insults to these organs are typically observed later in gestation. For example, the gross weight of the fetal heart from offspring of restricted-fed mothers was less at gestational d 135 and at birth, compared with control (Pillai et al., 2016). Although HW and UMB size were accurate on ultrasound during mid-gestation, the contractile nature and lack of blood fill at necropsy should be considered when interpreting agreement.

The lack of nutrition-induced growth changes during mid-gestation is similar to previous literature investigating the response of fetal organs to maternal diet. In fetuses of over-fed ewes,

the biparietal and abdominal diam.s were not affected by maternal diet between d 40 and 108 of gestation (Lekatz et al., 2013). Likewise, no change in the biparietal or abdominal diam. was observed until d 110 in fetuses of restricted or melatonin supplemented ewes (Lemley et al., 2011) or over-nourished ewes (Carr et al., 2012). Similar observations have been reported for the fetal kidney, femur, and tibia (Lemley et al., 2011; Carr et al., 2012). Thus, this literature and the current study consistently indicate that on ultrasound, changes in fetal growth in response to maternal diet are detected primarily after d 100 of gestation in the sheep.

On ultrasound, fetal RW was inconsistently affected by both maternal diet and litter size during mid-gestation. Contradictory relationships between CON, RES and OVER RW at each d of gestation are likely due to lack of precision and reproducibility, rather than actual asymmetrical growth. This may be explained by discrepancy between ultrasound and postmortem RW measurement, in that a negative bias (7.7%) was observed at d 45 but a large positive bias (23.8%) observed at d 90. These discrepancies may be attributed to intercostal muscle development, which obstructed the delineation of ribs on ultrasound. Previous work in sheep demonstrated a poor relationship of the fetal rib with gestational age when monitored via ultrasound during late gestation in the cervical, thoracic or lumbar region of the spine (Santiago-Moreno et al., 2005). More recently, Kandiel et al. (2015) reported that when the intercostal muscles are measured with six successive ribs, the rib measurement has a stronger relationship with fetal age in goats. Together, this indicates that although the fetal ribs are observable on ultrasound, measurement of a single rib does not accurately detect fetal growth patterns in utero.

Agreement analysis for the placentome variables indicated negative biases for the OD and ID at both d 45 and 90, with limits of agreement that were large relative to the average placentome measurements. The large limits of agreement for the placentome variables were

expected because it was not possible to measure identical placentomes during ultrasound and postmortem. However, the large limits of agreement encompass the variation in placentome size that exists during establishment of the placenta (Doize et al., 1996; Redmer et al., 2004; Vonnahme et al., 2008). In the sheep, the assortment of concave and convex placentomes (Vonnahme et al., 2008) likely contributes to the large biases observed for the placentome ID at both d 45 and 90, indicating that placentomes appear thicker on ultrasound than the gross morphology.

Growth of the placentome OD and ID were consistent with previous ultrasound data in the sheep (Doize et al., 1996; Lekatz et al., 2013) and followed typical placental development (Vonnahme et al., 2013a). In this study, the placentome OD increased until d 67 of gestation, and plateaued between d 67 and 74 (32 to 37 mm). Previously, Doize et al. (1996) reported a plateau in placentome OD between d 67 and 74 (25 to 30 mm). Differences in the placentome OD may be due to the location of the placentomes on the placenta. Doize et al. (1996) performed transrectal ultrasound, measuring caudal placentomes, rather than placentomes deeper in the uterus observed transabdominally. Placentome ID plateaued earlier than the OD. In ewes, the caruncle is hemispherical, and the center becomes filled by the cotyledon, causing the placentome ID to appear more stagnant in diam. (Doize et al., 1996; Vonnahme et al., 2008). Growth patterns of both placental variables were independent of maternal diet and litter size. However, blood flow of the placenta may be altered in the absence of changes to placentome morphology, size or type (Vonnahme et al., 2008; Vonnahme et al., 2013a). Thus, placental hemodynamics may be more informative of placental development during maternal programming studies than placentome size (Lemley et al., 2011).

Seven equations for prediction of gestational age in sheep were derived from a large population of ewes and included measurements from singleton, twin, and triplet pregnancies. The heterogeneity accounted for in these equations is valuable, as differences in lamb birth weight and size are commonly observed in ewes carrying multiple fetuses (Gootwine, 2005). Because no or inconsistent effects of litter size were observed during mid-gestation for the variables evaluated, these equations can be used to determine gestational age, regardless of litter size. Implementing these equations as calculators of gestational age allows producers to improve ewe management according to the stage of gestation, and predict parturition dates to supervise ewes as parturition approaches.

CONCLUSIONS AND IMPLICATIONS

In conclusion, this study demonstrated that during mid-gestation, growth of the fetal HW, UMB and placentomes are predictive of gestational age. However, due to biases of measurement on ultrasound, these biometrics do not necessarily reflect gross fetal growth as gestation advances. Although fetal RW is commonly observed on ultrasound, this measurement is not consistent nor does it accurately portray differences in fetal growth on ultrasound. Importantly, the analysis of ultrasound biases reported in this study improves the interpretation and implementation of information gathered via ultrasound. However, few changes were observed in fetal biometrics in response to poor maternal nutrition, suggesting that monitoring biometrics provides only limited information regarding fetal programming mechanisms. To investigate potential mechanisms by which poor maternal nutrition effects the offspring, a more molecular approach was pursued in Chapter 3.

CHAPTER III. GESTATIONAL RESTRICTED- AND OVER-FEEDING PROMOTE MATERNAL AND OFFSPRING INFLAMMATORY RESPONSES THAT ARE DISTINCT AND DEPENDENT ON GESTATIONAL DIET IN SHEEP

Introduction

Maternal programming is defined as changes to the maternal or intrauterine environment that alter fetal development and can permanently impair tissue function to predispose offspring to chronic diseases postnatally (Nathanielsz et al., 2007). Maternal nutrition is one factor that can cause programming of offspring through altered macro- or micro-nutrient ingestion during gestation. Both maternal nutrient restriction- and over-nutrition result in offspring who exhibit poor postnatal growth, reduced muscle mass, increased adiposity, disrupted metabolism, and altered innate immunity (Wu et al., 2006; He et al., 2014; Hoffman et al., 2017). Further, offspring born to nutrient restricted- and over-nourished mothers are at increased risk to develop chronic metabolic diseases during adulthood, including obesity, type 2 diabetes, atherosclerosis, and hepatic steatosis (Nathanielsz et al., 2007). Chronic inflammation is common to the pathogenesis of these metabolic diseases (Hotamisligil, 2006), and thus may be a persistent consequence of poor maternal nutrition during gestation (Yan et al., 2011a, b;). Although the phenotype of offspring exposed to poor maternal nutrition during gestation is similar in response to maternal restriction and over-nutrition, the mechanisms of each diet are distinct (Ford and Long, 2012; Hoffman et al., 2017). Furthermore, the prevalence of over- and restricted- nutrition is concerning in women of reproductive age (20 to 39 yr). In the United States, 58.5% of women in this age group are overweight (BMI > 24) and 31.8% are obese (BMI > 29; [Ogden et al., 2014]). Likewise, up to 28% of women ages 20 to 39 are underweight (BMI 16 to 18.5) in developing nations (ACC/SCN, 2000). Therefore, it is necessary to understand the effects of both

maternal nutrient excess and restriction on offspring to improve outcomes of fetal programming worldwide.

Inflammation has been proposed as a mechanism contributing to fetal programming because it has the capacity to alter the maternal environment and is known to persist postnatally in metabolically important tissues of offspring exposed to adverse intrauterine environments (Heerwagen et al., 2010; Ingvorsen et al., 2015). In the maternal environment, low-grade, chronic inflammation is present in healthy pregnancies to facilitate maternal tolerance to the fetus, which exhibits paternal antigens (Mor, 2008; Mor et al., 2011). This involves timely coordination of the maternal endocrine and innate immune system throughout gestation and the onset of parturition (Mor, 2008; Mor et al., 2011). Failure of the maternal innate immune system to tolerate the fetus and elicit a chronic but controlled inflammatory response can result in pregnancy complications such as early embryonic loss, abortion, or preterm labor (Mor, 2008; Mor et al., 2011). This is usually provoked by a source of inflammation that is independent of pregnancy, including stress, infection, or diet (Ingvorsen et al., 2015). For example, excessive intake and metabolism of macronutrients is associated with low-grade, chronic inflammation in non-pregnant women (Hotamisligil, 2006), and this inflammation is exaggerated in over-weight and obese pregnant women (Christian and Porter, 2014). When coupled with pregnancy, dietary-induced inflammation has the potential to negatively impact pregnancy success, maternal health, and fetal development.

Diet-associated inflammation in the mother has the potential to impact the fetus through direct transfer of immune modulators across the placenta or by modulating transport of nutrients and oxygen to alter the intrauterine environment (Ingvorsen et al., 2015). Epidemiological evidence demonstrates that offspring born to both over- or under-weight women exhibit an

increased inflammatory score as infants (Broadney et al., 2016). Additionally, animal studies have demonstrated that sheep offspring exposed to maternal restricted- or over-nutrition during gestation exhibit increased tissue-specific inflammation postnatally in muscle, cardiac, intestine, and liver tissues, indicating that inflammation caused by poor maternal nutrition may have persistent underlying effects (Du et al., 2010b; Hyatt et al., 2011; Huang et al., 2012; Ge et al., 2013; Hoffman et al., 2016b).

The fetal liver has been proposed to have an integral role in priming the postnatal innate immune system (Wesolowski et al., 2017). During gestation, the liver is a major hematopoietic organ responsible for differentiating resident and peripheral macrophages, and therefore the fetal hepatic microenvironment has the potential to prime macrophages to favor an M1 (pro-inflammatory) or an M2 (pro-resolving) phenotype postnatally (Stewart et al., 2013; Ginhoux and Jung, 2014; Wesolowski et al., 2017). Exposure to maternal inflammatory factors that would skew the macrophage phenotype towards an M1-type may contribute to pro-inflammatory tissue environments and metabolic disruption after birth. Thus, it is necessary to investigate the relationship of maternal and fetal inflammation resulting from over- and restricted-nutrition during gestation to further understand the long-term consequences of poor maternal nutrition on offspring.

This study investigates changes in maternal systemic gene expression of inflammatory mediators throughout gestation and in response to diet. Contrary to previous studies which have only targeted one stage of gestation (Lekva et al., 2016) or a few specific inflammatory molecules (Christian and Porter, 2014), this study evaluated 5 time-points during gestation and 84 inflammatory genes. Importantly, this information is reported in sheep, a common biomedical model for human pregnancy due to the similar fetal to maternal biomass ratio, delivery of

precocial offspring, and continuity of fetal programming consequences (Barry and Anthony, 2008). Additionally, this study investigates offspring systemic inflammation in response to both maternal restricted- and over-nutrition, allowing comparisons between maternal and fetal inflammation, and between diets, in one study. Finally, we report offspring hepatic changes in inflammatory gene expression to understand how poor maternal nutrition may cause fetal programming through a vital hematopoietic and metabolic organ.

Materials and Methods

Animals and experimental design

All animal procedures were approved by the University of Connecticut's Institutional Animal Care and Use Committee.

Multiparous Western White-faced ewes were estrous synchronized, bred by live cover to one of four related Dorset rams, and confirmed pregnant at d 28.5 ± 0.4 of gestation using transabdominal ultrasound as previously described (Jones et al., 2016). The ewes used for this study were a subset of a larger flock and collaborative experiment detailed by Pillai et al (2017). Briefly, upon individual housing at d 20 of gestation, ewes were transitioned onto a complete pelleted feed to meet 100% of the requirements of a pregnant ewe carrying twins as recommended by the National Research Council (NRC; [National Research Council, 1985]). At d 30.2 ± 0.2 of gestation, pregnant ewes were randomly assigned to one of three experimental diets that met 100% (Control; n = 8), 60% (Restricted; n = 9) or 140% (Over; n = 8) of NRC total digestible nutrient requirements (TDN; [National Research Council, 1985]). Offspring from ewes fed Control, Restricted and Over diets are referred to as CON, RES and OVER, respectively. Body weight and condition score (Russel et al., 1969) of ewes were recorded

weekly, allowing rations to be adjusted for individual changes in BW. Six weeks before parturition, rations were adjusted based on NRC recommendations to meet the TDN requirement for a late gestation ewe expecting twins (28.5% increase of TDN), while maintaining feeding levels of 60%, 100%, and 140% (National Research Council, 1985). Daily requirements were provided in two rations per day and ewes remained on diet until parturition (gestation length 147.4 ± 1.9 d). See Figure 6 for the experimental design and sample collection.

Maternal sample collection

Ewe blood collection was performed in the fasted state, before morning feeding. Whole blood was collected via jugular venipuncture at d 23 ± 1.2, 45 ± 1.4, 90 ± 1.4, and 135 ± 1.6 of gestation for RNA and serum, at d 142 ± 3.3 of gestation for serum only, and within 24 h of parturition (birth) for RNA only. For RNA processing, 3mL of whole blood were transferred into a Tempus Blood RNA Tube (Applied Biosystems, Foster City, CA) containing RNA stabilizer and shaken vigorously to precipitate RNA from whole blood. Tempus tubes were stored on ice until returned to the laboratory and then stored at -20°C until processed. For serum, 5mL of whole blood were transferred into a non-heparinized tube and processed as previously described (Hoffman et al., 2014).

Offspring sample collection

Upon parturition, lambs (n = 6 lambs per diet [3 females and 3 males in CON and OVER, 4 females and 2 males in RES]) from Control, Restricted, or Over ewes (n = 3 to 5 ewes per diet) nursed from their dam for up to 24 h to receive colostrum. Within 24 h of parturition, whole blood was obtained from live lambs via jugular venipuncture and processed for RNA or serum as

described above. Lambs were subsequently euthanized with an i.v. overdose of Beuthanasia-D Special (390 ng/mL Sodium Pentobarbital and 50 mg/mL Phenytoin based on BW; Merck Animal Health, Summit, NJ) and exsanguinated. Liver tissue was collected, snap frozen in liquid nitrogen, and stored at -80°C.

Another group of ewes (n = 3 to 5 ewes per diet) from the same experiment (Pillai et al., 2017) were euthanized at d 135 of gestation to acquire fetus(es) for sampling. Ewes were euthanized with an i.v. overdose of Beuthanasia-D Special (Merck Animal Health) and exsanguinated. Subsequently, a mid-line abdominal incision was performed on the ewe to remove the fetus(es). Following euthanasia, whole blood (3 mL) was collected via cardiac puncture for RNA, and liver was dissected from 6 fetuses per diet (3 females and 3 males in CON and RES, 4 females and 2 males in OVER). Samples were processed and stored as described for lambs.

RNA Isolation

Isolation of RNA from whole blood was performed using the Perfect Pure RNA Blood kit according to the manufacturer's protocol (5 Prime, Inc., Gaithersburg, MD). The GeneJET Cleanup and Concentration Micro Kit (Thermo Scientific, Lafayette, CO) was used to concentrate the eluted RNA into 10 μL of RNase Free water. Hepatic RNA isolation was performed using 100 mg of tissue homogenized using a standard bead beating method in TRIzol Reagent (Invitrogen, Carlsbad, CA; [Reed et al., 2014]). Quantity and quality of RNA was determined using a NanoDrop spectrophotometer (Thermo Scientific) and Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA), respectively.

Real-time reverse transcription (RT)-PCR array

Genomic DNA elimination was performed on each 500 ng sample of RNA and reverse transcribed using the RT^2 First Strand Kit (SABiosciences). Quantitative real-time RT-PCR was performed using the Inflammatory Cytokines & Receptors RT^2 Profiler PCR Array (Catalog # PABT-011Z; SABiosciences, Germantown, MD). This array measures the expression of 84 genes mediating the inflammatory response, and has been previously validated in sheep (Gossner et al., 2013). One cDNA sample was analyzed per PCR array with RT^2 SYBR Green Master Mix (SABiosciences). Cycling conditions were one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min, performed using the ABI 7900 HT Fast Real-time PCR machine (Applied Biosystems). Cycle threshold (C_T) values were obtained and the $^{\Delta\Delta}C_T$ method was used to determine changes in gene expression (Livak and Schmittgen, 2001). Beta-actin (*ACTB*) was chosen as the housekeeping gene for all samples, as the C_T values were not different between diet or stage of gestation in ewes ($P > 0.21$), and were not different between maternal diet or gender in offspring ($P > 0.13$).

C-Reactive Protein (CRP) and NEFA assays

Hepatic protein was isolated by homogenizing 100 mg tissue in phosphate-buffered saline using standard bead beating method and centrifugation to collect the supernatant. The concentration of CRP was determined from undiluted samples using the Sheep C Reactive Protein ELISA kit (assay sensitivity 0.1ug/mL, detection range 0.25 to 8 ug.mL; Catalog # MBS013452; MyBioSource, San Diego, CA). The CRP concentration was measured within a single assay and the intra-assay coefficient of variation were all <10%. Total protein was quantified using the Quick Start Bradford Protein Assay (BioRad, Hercules, CA) to normalize

total CRP concentration. Non-esterified fatty acids were quantified from ewe and lamb serum using the acyl-CoA synthetase-acyl-CoA oxidase method (NEFA-HR, Wako Pure Chemical Industries, Dallas, TX). The NEFA assay detection limit is 0.01 to 4.00 mEq/L and the inter- and intra-assay coefficients of variation were <10%.

Data analyses

The power calculations performed for this study were based on anticipated differences in maternal and offspring BW in response to dietary treatment, with 90% power to detect significance at 5%. We anticipated that a 15% difference in maternal BW (16.5 kg) would be achieved at parturition in response to dietary treatment, with an animal variability of 8 kg, indicating that 7 ewes per diet were needed. Six offspring per diet were selected based on an anticipated 20% difference in BW (996 g) and animal variability of 480 g. Offspring gender could not be controlled for, and therefore the study was not powered to investigate effects of gender within a diet.

All data analyses were performed using the mixed procedure of SAS version 9.4 (SAS Institute, Cary, NC). Ewe data were analyzed as a completely randomized design. Percent change in BW from d 30 of gestation, percent change in BCS from d 30 of gestation, and NEFA concentrations were analyzed with repeated measures. The model included fixed effects of diet, day of gestation, and their interaction, with the subject defined as ewe. Covariate structures were chosen based on the lowest Akaike Iteration Criterion (AIC) value for each dependent variable (Kaps and Lamberson, 2009). Compound symmetry was chosen for percent change BCS, autoregressive was chosen for percent change in BW, and unstructured was chosen for NEFA concentration. Data are presented as least squares mean (lsmean) ± SE. Due to insufficient RNA

quantity from ewe blood (< 500 ng from 25% of samples), PCR arrays could not be performed for all days of gestation within each ewe. Thus, ewe array data were analyzed as a cross-sectional study using the MIXED procedure with fixed effects of diet, day of gestation, and their interaction. Heat maps of ewe inflammatory gene expression were generated in the gplots package in R version 3.3.1. Ewe gene expression data are expressed relative to Control at d 23 in the presence of an interaction, relative to Control in the presence of a main effect of diet, or relative to d 23 in the presence of a main effect of gestation.

Offspring data were analyzed as a completely randomized design using the MIXED procedure with fixed effects of maternal diet, gender and stage of gestation, and the interaction of maternal diet by stage of gestation for all variables except gene expression. For the blood and liver PCR Arrays, the housekeeping gene *ACTB* cycled differently at 135 d of gestation and birth ($P < 0.0001$). Thus, offspring array data from each time point were analyzed separately. Offspring gene data are expressed relative to CON or females. For the NEFA assay, two observations are missing from OVER. All data are expressed as lsmean ± SE. Significance is discussed when $P \leq 0.05$ and main effects are discussed only in the absence of an interaction.

Results

Maternal BW, BCS and circulating NEFA concentration

There was a significant interaction of diet by day of gestation (Figure 2) on ewe BW ($P < 0.0001$), BCS ($P = 0.001$) and circulating NEFA concentrations ($P < 0.0001$). Upon the start of the dietary treatment at d 30 of gestation, the BW of pregnant ewes did not differ between diet groups (78.2 ± 2.4, 81.2 ± 2.2, 75.7 ± 2.4 kg for Control, Restricted and Over, respectively; $P \geq 0.09$). Between d 79 and 135 of gestation, the percent change in BW from d 30 of gestation was

different between Control, Restricted and Over ewes ($P \leq 0.05$; Figure 2A). That is, the BW of Control and Over ewes increased by 19.4% and 22.7%, respectively, between d 79 and 135, whereas the BW of Restricted ewes was reduced by 3.5% at d 79, and increased by 5.8% at d 135 of gestation (Figure 7A). Five d before parturition (d 142) the percent change in BW from d 30 was greater in Over (31.4 ± 5.3%) and Control (26.6 ± 2.1%) ewes than in Restricted ewes (5.9 ± 2.4%; $P < 0.0001$; Figure 2A). Similar to ewe BW, ewe BCS did not differ between diets at d 30 of gestation (3.07 ± 0.04, 3.00 ± 0.04, 2.99 ± 0.04 for Control, Restricted and Over, respectively; $P \geq 0.85$). However, by d 142 of gestation, the percent change in BCS from d 30 was greater in Over (5.7 ± 1.4%) and less in Restricted (-5.7 ± 0.83%) ewes, compared with Control (0.6 ± 2.6%; $P \leq 0.02$; Figure 7B). Circulating NEFA concentrations were not different in ewes before beginning of experimental diets ($P \geq 0.71$; Figure 7C). Throughout dietary treatment, Restricted ewes had greater ($P < 0.0001$) circulating NEFA concentrations compared with Control and Over, which did not differ ($P \leq 0.55$; Figure 7C).

Maternal systemic inflammatory gene expression

Analysis of inflammatory related gene expression in pregnant ewes indicated that there was an interaction of diet and day of gestation on the expression of interleukin 6 receptor (*IL6R*) and platelet factor 4 (*PF4*) in the circulation of pregnant ewes ($P \leq 0.04$; Figure 8; Supplementary Table 1). Compared with d 23 of gestation, the expression of *IL6R* did not change as gestation advanced in Control ewes ($P > 0.05$). Expression of *IL6R* increased at d 135 of gestation in Restricted compared with Control and Over ewes and within 24 h of birth in Over compared with Restricted ewes (Figure 8A; $P \leq 0.05$). As gestation advanced, expression of *PF4* did not change in Control ewes, compared with d 23. (Figure 8B: $P \leq 0.05$). At d 45 of gestation,

PF4 gene expression in Over ewes was greater than Control, whereas Restricted was greater than Control at d 90 of gestation ($P \leq 0.05$).

Thirteen genes exhibited a main effect of diet and are reported as a heat map in Figure 9 and as relative expression in Supplementary Table 2. Regardless of the stage of gestation, the expression of C-X-C motif chemokine ligand 9 (*CXCL9*), *IL4, IL7*, lymphotoxin beta (*LTB*), TNF super family member 10 (*TNFSF10*), and interleukin 10 receptor subunit alpha (*IL10RA*) increased in Over ewes compared with Control ($P \leq 0.05$). In Restricted ewes, expression of bone morphogenetic protein 2 (*BMP2*), C-X-C motif chemokine receptor 1 (CXCR1), *IL1B* and *TNFSF13B* increased compared with Control ($P \leq 0.05$). Expression of C-C motif chemokine ligand 4 (*CCL4*), lymphotoxin alpha (*LTA*) and *TNFSF11* was greater in Over ewes than Restricted ($P = 0.001$), but neither differed from Control ($P \geq 0.09$).

Forty-one genes exhibited a main effect of day of gestation, reported as a heat map in Figure 10 and as relative expression in Supplementary Table 3. Genes with a main effect of gestation exhibited different expression patterns throughout gestation. Compared with d 23 of gestation, expression of *CCL2* and *IL2RB* increased at d 45, 90 and 135 of gestation ($P \leq 0.04$). Expression of secreted phosphoprotein 1 (*SPP1*) peaked at d 45 of gestation ($P \leq 0.04$), whereas *IL17B* was greatest at d 135 of gestation ($P \leq 0.05$). Expression of C-C motif chemokine receptor 6 (*CCR6*) increased at d 45 and 90 of gestation ($P \leq 0.03$) whereas colony stimulating factor 3 (*CSF3*) increased at d 90 and 135 of gestation ($P \leq 0.05$). Compared with d 23 of gestation, *CXCR1* expression decreased at d 45 and increased at d 135 of gestation and birth ($P \leq 0.05$). At d 90 of gestation and after parturition, *IL5* expression was reduced ($P \leq 0.02$). Between d 23, 45 and 90 of gestation, expression of *CCL4, CXCL9, C-X3-C motif* chemokine receptor 1 (*CX3CR1*), CD40 ligand (*CD40LG*), Fas ligand (*FASLG*), and *TNFSF13B* did not differ, but all

were reduced at d 135 and 24 h after parturition ($P \leq 0.05$). Expression of aminoacyl tRNA synthase complex interacting multifunctional protein 1 (*AIMP1*)*, CCL3, CCL5, CCL22,* macrophage migration inhibitory factor (*MIF*)*, CCR1, CCR3, CCR5, CCR8, CXCR3, CSF1,* interferon gamma (*IFNG*)*, IL7, IL13, IL16,* interleukin 6 signal transducer (*IL6ST*)*, IL9R, LTA, LTB, TNF* and *TNFSF11* did not change during gestation ($P > 0.06$)*,* but exhibited reduced expression 24 h after parturition ($P \leq 0.05$). Expression of *CXCL8* decreased only at d 45 of gestation ($P \leq 0.03$), whereas *CXCL10, IL4, IL15,* nicotinamide phosphoribosyltransferase (*NAMPT*) and *TNFSF10* continued to decline from d 45 of gestation through parturition ($P \leq 0.05$).

Offspring BW, hepatic CRP and serum NEFA concentrations

A main effect of maternal diet was observed on offspring BW in that regardless of age, RES offspring weighed 15.4% and 7.7% less than CON and OVER, respectively (CON: 5,007.8 ± 187.6, RES: 4,236.5 ± 188.5, OVER: 4,591.8 ± 188.6 g; $P = 0.02$). Offspring BW did not differ with stage of gestation (d 135: 4,667.3 ± 604.0 g; Birth: 4,556.3 ± 822.6 g; $P = 0.611$) or gender (Males: 4,802 ± 835.4 g; Females: 4,421.4 ± 557.3 g; $P = 0.09$).

No interactions ($P \geq 0.07$) or main effect differences were observed in the concentration of CRP in offspring liver between d 135 of gestation and birth (5.02 ± 0.81 and 5.67 ± 0.88, μg, respectively; $P = 0.59$), males and females (4.41 ± 0.91 and 6.28 ± 0.77, μg, respectively; $P = 0.13$) or maternal diets (CON: 4.65 ± 1.03, RES: 6.18 ± 1.03, OVER: 5.20 ± 1.06, μg; $P = 0.57$).

No differences were observed in the lamb serum NEFA concentration at birth between maternal diets (CON: 872.3 ± 109.7, RES: 803.9 ± 112.0, OVER: 1,138.3 ± 134.3, μmol/L; $P = 0.18$) or males and females (924.2 ± 91.6 and 952.1 ± 102.4, μmol/L, respectively; $P = 0.84$).

Offspring systemic inflammatory gene expression

In offspring circulation at d 135 of gestation, there were main effects of maternal diet for five genes ($P \leq 0.05$; Figure 11A) and gender for six genes ($P \leq 0.05$; Table 8). Systemic *CCL22* expression was reduced in RES compared with CON ($P \leq 0.05$). Systemic expression of *CXCL12, CXCR1,* and *IL1A* were reduced in OVER and RES compared with CON ($P \leq 0.05$). Systemic expression of *MIF* was reduced in OVER compared with CON ($P \leq 0.05$). At d 135 of gestation, systemic *CCL22, CXCL1, CXCL9, CXCL12, IL4,* and *IL15* expression was reduced in male fetuses compared with females, regardless of maternal diet ($P \leq 0.05$).

In offspring circulation at birth, there were main effects of maternal diet for five genes ($P \leq 0.05$; Figure 11B) and gender for two genes ($P \leq 0.05$; Table 8). Systemic *CXCL8* increased in RES and OVER compared with CON ($P \leq 0.05$). Systemic *LTB* was reduced in RES compared with CON and OVER ($P \leq 0.05$). Systemic *PF4, TNFSF13,* and *VEGFA* were increased in OVER compared with CON and RES ($P \leq 0.05$). Compared with females, systemic *CXCR1* expression was reduced, whereas *TNFSF13* expression increased in male lambs at birth ($P \leq 0.05$).

Offspring hepatic inflammatory gene expression

In offspring liver at d 135 of gestation, there were main effects of maternal diet for three genes ($P \leq 0.05$; Figure 12A) and gender for seven genes ($P \leq 0.05$; Table 9). Hepatic *CCL16* expression increased in RES compared with CON ($P \leq 0.05$). Hepatic LTB was reduced in OVER compared with CON and RES ($P \leq 0.05$). Hepatic TNFSF11 was increased in OVER compared with RES, with CON intermediate ($P \leq 0.05$). Compared with females, hepatic

expression of Complement C5 (*C5*)*, CCL3, CCR1, CCR6, IL2RG,* and *LTA* was reduced in male fetuses ($P \leq 0.05$). Oppositely, hepatic *TNFSF14* was increased in male fetuses compared with females ($P \leq 0.05$).

In offspring liver at birth, there were main effects of maternal diet for four genes ($P \leq 0.05$; Figure 12B) and gender for eight genes ($P \leq 0.05$; Table 9). Hepatic *BMP2* and *CXCL12* expression was reduced in OVER compared with CON and RES, whereas *CXCL10* was reduced in OVER compared with RES with CON intermediate ($P \leq 0.05$). Hepatic *TNF* expression increased in RES compared with CON and OVER ($P \leq 0.05$). Compared with females, hepatic expression of *CCL16, CXCL9, CXCL10, IL1R1, IL5, IL7, IL15,* and *TNFSF10* was reduced in males, but *MIF* expression was greater ($P \leq 0.05$).

TABLES

Table 8. Main effect of gender on gene expression of offspring systemic inflammatory mediators.

Gene[1]	Gender		SEM	*P* value	
	Female (n = 10)	Male (n = 8)		Diet[2]	Gender
D 135					
CCL22	1.52	0.53	0.30	0.02	0.03
CXCL1	1.37	0.40	0.20	0.32	0.02
CXCL9	2.07	0.43	0.56	0.25	0.02
CXCL12	2.22	0.59	0.68	0.04	0.04
IL4	1.10	0.49	0.14	0.32	0.002
Birth					
CXCR1	1.11	0.63	0.11	0.17	0.02
TNFSF13	1.23	2.55	0.36	0.02	0.002

[1] Expression relative to females.

[2] Six offspring per diet at each time point.

Abbreviations: C-C- motif chemokine ligand (CCL)-22; C-X-C motif chemokine ligand (CXCL)-1, 9, 12; C-X-C motif chemokine receptor (CXCR)-1; Interleukin (IL)-4, 15; TNF super family member (TNFSF)-13.

Table 9. Main effect of gender on hepatic gene expression of inflammatory mediators in offspring.

Gene[1]	Gender		SEM	*P* value	
	Female (n = 10)	Male (n = 8)		Diet[2]	Gender
D 135					
C5	1.84	0.42	0.35	0.52	0.03
CCL3	1.12	0.50	0.18	0.58	0.01
CCR1	3.04	0.27	0.72	0.28	0.01
CCR6	6.36	0.19	1.54	0.56	0.04
IL2RG	1.04	0.68	0.12	0.41	0.03
TNFSF14	1.23	2.94	0.43	0.61	0.02
Birth					
CCL1	1.52	6.80	0.96	0.41	0.0005
CXCL9	1.35	0.33	0.22	0.96	0.004
CXCL10	1.50	0.46	0.26	0.02	0.04
IL1R1	1.43	0.48	0.27	0.31	0.03
IL5	1.12	0.61	0.16	0.55	0.02
IL7	1.14	0.52	0.14	0.77	0.01
IL15	1.08	0.61	0.11	0.95	0.01
MIF	1.11	3.59	0.70	0.76	0.05

[1] Expression relative to females.

[2] Six offspring per diet at each time point.

Abbreviations: Complement C5 (C5); C-C motif chemokine ligand (CCL)-1, 3; C-C motif chemokine receptor (CCR)-1, 6; C-X-C motif chemokine ligand (CXCL)-9, 10; Interleukin (IL)-2G, 5, 7, 15; Interleukin 1 Receptor 1 (IL1R1); Lymphotoxin alpha (LTA); Macrophage migration inhibitory factor (MIF); TNF super family member (TNFSF)-14.

FIGURES

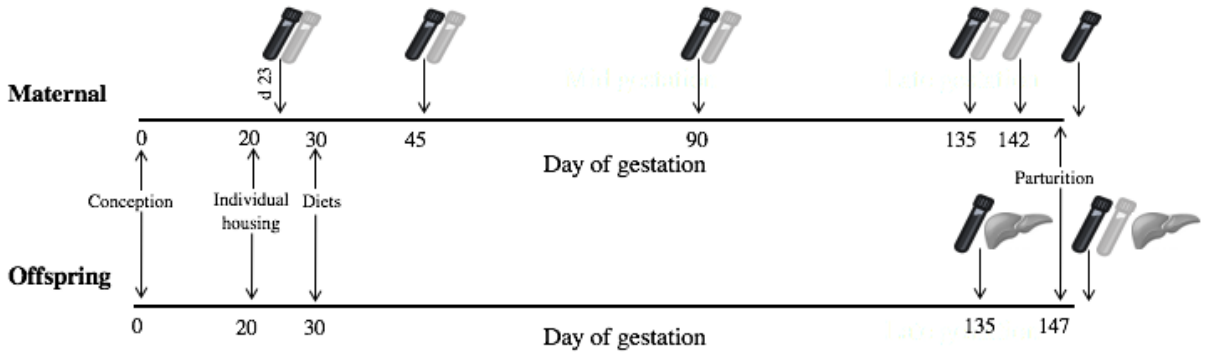

Figure 6. Schematic of experimental design and sample collection. Pregnant ewes were individually housed beginning at d 20 of gestation and transitioned onto a diet providing 100%, 60% or 140% of NRC beginning at d 30 of gestation. Whole blood was collected for RT-PCR arrays (black tube) or serum (grey tube) from pregnant ewes at d 23, 45, 90, 135, and 142 of gestation, or within 24 h of birth, as well as offspring at d 135 of gestation and within 24 h of birth. Liver was collected from offspring for RT-PCR array and CRP assays at d 135 of gestation and within 24 h of birth.

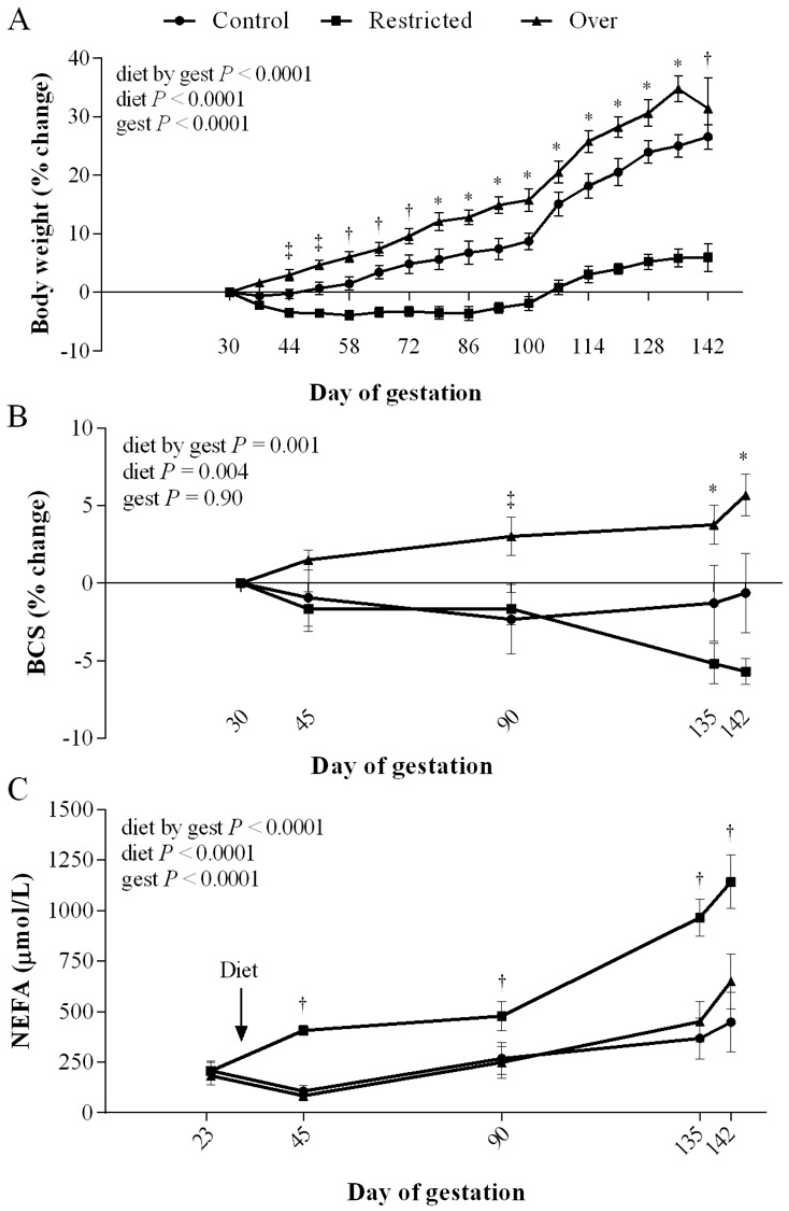

Figure 7. Change in maternal BW (A), BCS (B) and circulating NEFA concentration (C). Pregnant ewes were individually fed 100% (Control; n = 8), 60% (Restricted; n = 9), or 140% (Over; n = 8) of NRC total digestible nutrient requirements from d 30.2 ± 0.2 of gestation through parturition (National Research Council, 1985). Significant interactions of diet by d of gestation (gest) were observed for ewe BW ($P < 0.0001$), BCS ($P = 0.001$) and circulating NEFA

($P < 0.0001$). Data are presented as lsmean ± SE. Within a day of gestation, pairwise comparisons between diets are denoted when $P \leq 0.05$ by ‡ (Restricted vs. Over, with Control intermediate), † (Restricted vs. Control and Over) or * (Restricted vs. Control vs. Over).

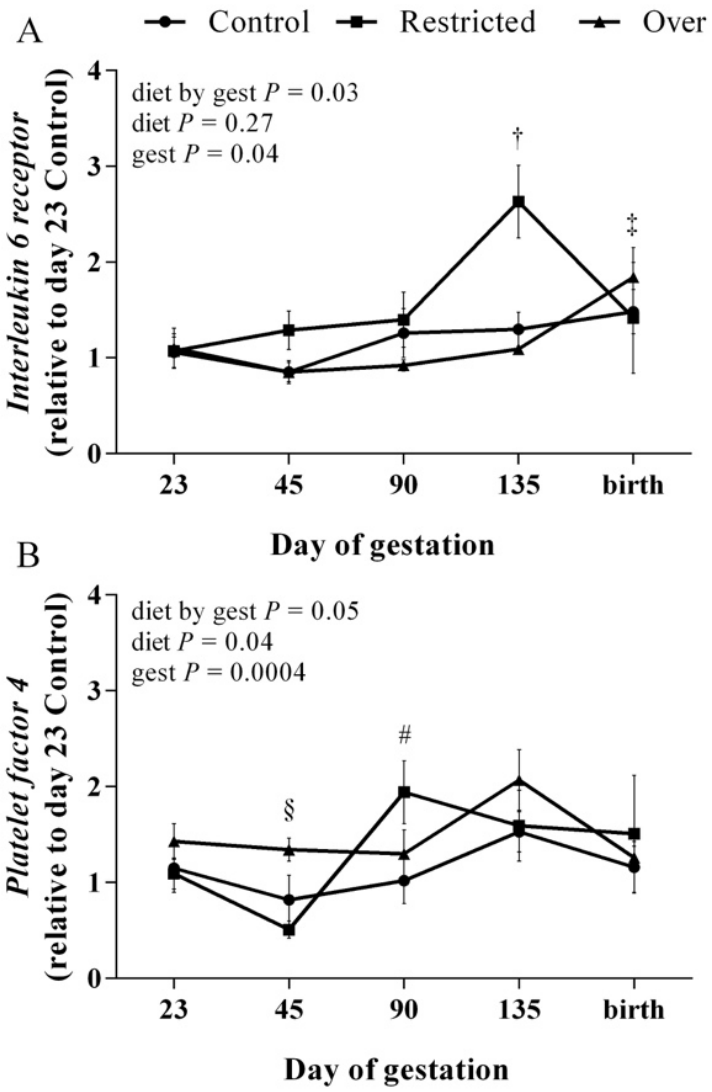

Figure 8. Maternal systemic inflammatory gene expression is altered by gestational diet and stage of gestation (gest). Pregnant ewes were individually fed 100% (Control; n = 8), 60% (Restricted; n = 9), or 140% (Over; n = 8) of NRC total digestible nutrient requirements from d 30.2 ± 0.2 of gestation through parturition (National Research Council, 1985). An interaction of diet by day of gestation was observed for the expression of Interleukin 6 receptor (*IL6R; P* = 0.03) and Platelet factor 4 (*PF4*; *P* = 0.05). Messenger RNA is expressed relative to d 23

Control. Within a day of gestation, pairwise comparisons between diets are denoted when $P \leq 0.05$ by † (Restricted vs. Control and Over), ‡ (Restricted vs. Over, with Control intermediate), § (Over vs. Control and Restricted) or # (Restricted vs. Control, with Over intermediate). Relative expression values are available in Supplementary Table 1.

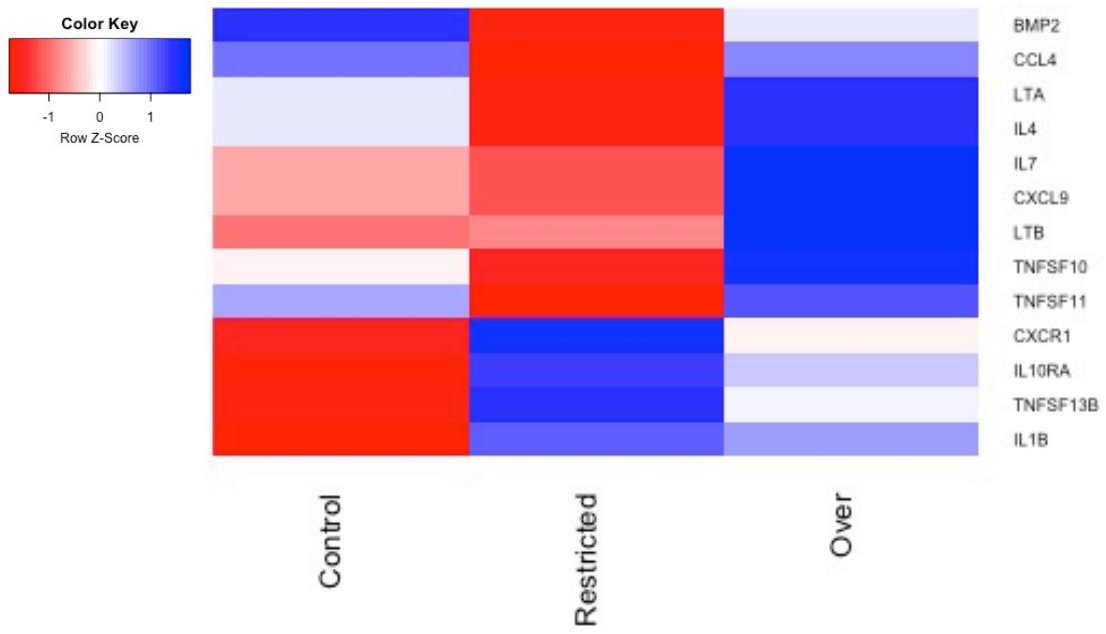

Figure 9. Maternal systemic inflammatory gene expression is altered by gestational diet. Pregnant ewes were assigned to 100% (Control; n = 8), 60% (Restricted; n = 9), or 140% (Over; n = 8) of NRC requirement for total digestible nutrients from d 30.2 ± 0.2 of gestation through parturition (National Research Council, 1985). A main effect of diet ($P \leq 0.05$) was observed for the expression of thirteen inflammatory genes. Within a gene, red indicates the least expression and blue indicates the greatest expression. Relative expression values are available in Supplementary Table 2. Abbreviations: Bone morphogenetic protein 2 (BMP2); C-C motif chemokine ligand 4 (CCL4); C-X-C motif chemokine ligand 9 (CXCL); C-X-C motif chemokine receptor 1 (CXCR1); Interleukin 1 beta (IL1B), Interleukin (IL)-4, 7; Interleukin 10 receptor subunit alpha (IL10RA); lymphotoxin alpha (LTA); lymphotoxin beta (LTB); TNF super family member 10 (TNFSF10), TNFSF11, TNFSF13B.

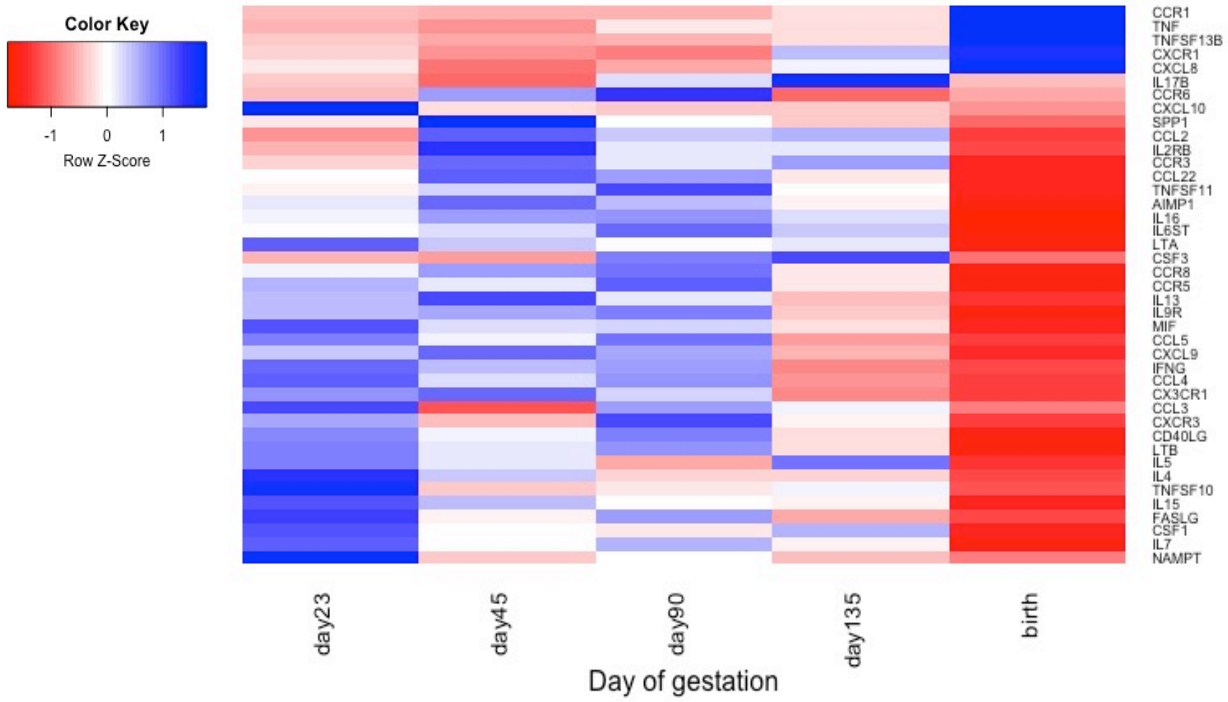

Figure 10. Maternal systemic inflammatory gene expression is altered by stage of gestation. Pregnant ewes were assigned to 100% (Control; n = 8), 60% (Restricted; n = 9), or 140% (Over; n = 8) of NRC total digestible nutrient requirements from d 30.2 ± 0.2 of gestation through parturition (National Research Council, 1985). A main effect of gestation ($P \leq 0.05$) was observed for the expression of 41 inflammatory genes. Within a gene, red indicates the least expression and blue indicates the greatest expression. Relative expression values are available in Supplementary Table 3. Abbreviations: Aminoacyl tRNA synthetase complex interacting multifunctional protein 1 (AIMP1); CD40 ligand (CD40LG); C-C motif chemokine ligand (CCL)-2, 3, 4, 5, 22; C-X-C motif chemokine ligand (CXCL)-9, 10; C-C motif chemokine receptor (CCR)-1, 3, 5, 6, 8; C-X-C motif chemokine receptor (CXCR)- 1, 3; C-X3-C motif chemokine receptor 1 (CX3CR1); Colony stimulating factor (CSF)-1, 3; Fas ligand (FASLG);

Interferon gamma (IFNG); Interleukin (IL)-4, 5, 7, 8, 13, 15, 16, 17B; Interleukin receptors (IL6R; IL9R); Interleukin 6 signaling transducer (IL6ST); Lymphotoxin alpha (LTA); Lymphotoxin beta (LTB); Macrophage migration inhibitory factor (MIF); Nicotinamide phosphoribosyltransferase (NAMPT); Secreted phosphoprotein 1 (SPP1); Tumor necrosis factor (TNF); TNF factor super family member (TNFSF)-10, 11, 13B.

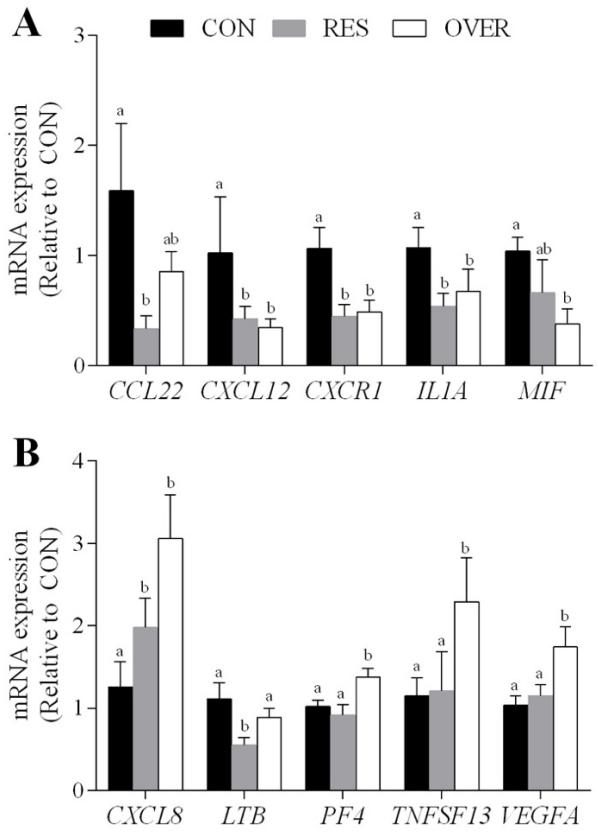

Figure 11. Systemic mRNA expression of offspring at d 135 of gestation (A) or within 24 h of birth (B). Offspring (n = 6 per diet at each stage) from ewes fed 100%, 60% or 140% of NRC total digestible nutrient requirements beginning on d 30.2 ± 0.2 through gestation are referred to as CON, RES, and OVER, respectively. At each stage of gestation there are 10 female and 8 male offspring. Within a stage of gestation, mRNA expression is expressed relative to CON. At d 135 of gestation (A), significant effects of maternal diet ($P \leq 0.05$) were observed for the mRNA expression of C-C motif chemokine ligand 22 (*CCL22*), C-X-C motif chemokine ligand 12 (*CXCL12*), C-X-C motif chemokine receptor 1 (*CXCR1*), interleukin 1 alpha (*IL1A*) and macrophage migration inhibitory factor (*MIF*). At birth (B), significant effects of maternal diet ($P \leq 0.05$) were observed for the mRNA expression of *CXCL8*, lymphotoxin beta (*LTB*), platelet

factor 4 (*PF4*), TNF super family member 13 (*TNFSF13*) and vascular endothelial growth factor A (*VEGFA*). Within genes, bars with differing superscripts are significantly differ ($P \leq 0.05$).

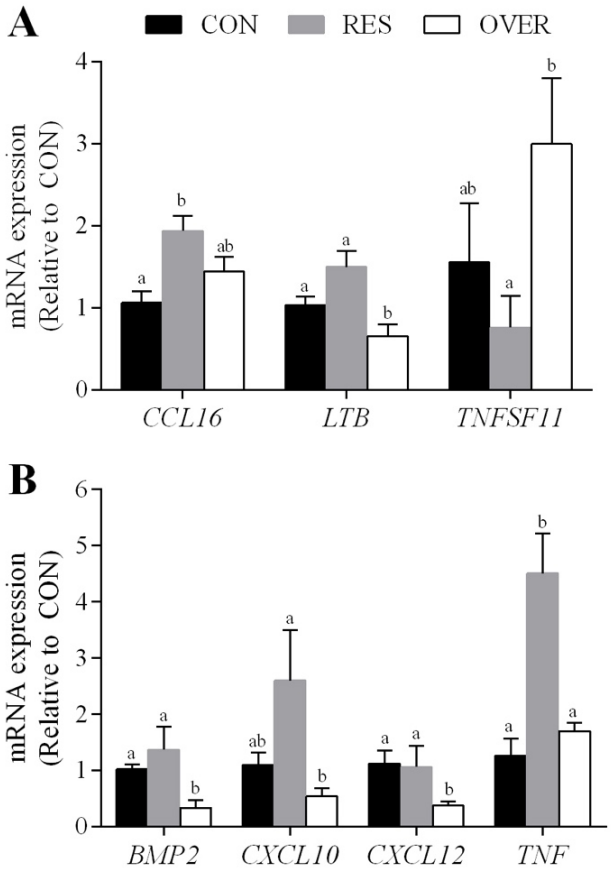

Figure 12. Hepatic mRNA expression of offspring at d 135 of gestation (A) or within 24 h of birth (B). Offspring (n = 6 per diet at each stage) from ewes fed 100%, 60% or 140% of NRC total digestible nutrient requirements beginning on d 30.2 ± 0.2 through gestation are referred to as CON, RES, and OVER, respectively. At each stage of gestation there are 10 female and 8 male offspring. Within a stage of gestation, mRNA expression is expressed relative to CON. At d 135 of gestation (A), significant effects of maternal diet ($P \leq 0.05$) were observed for the mRNA expression of C-C motif chemokine ligand 16 (*CCL16*), lymphotoxin beta (*LTB*) and TNF super family member 11 (*TNFSF11*). At birth (B), significant effects of maternal diet ($P \leq 0.05$) were observed for the mRNA expression of bone morphogenetic protein 2 (*BMP2*), C-X-C motif

chemokine ligand 10 (*CXCL10*), *CXCL12*, and tumor necrosis factor (*TNF*). Within genes, bars with differing superscripts are significantly differ ($P \leq 0.05$).

SUPPLEMENTARY TABLES

Supplementary Table 1. Interaction of diet and stage of gestation on the gene expression of systemic inflammatory mediators of pregnant ewes.

	Diet				*P* value		
Gene[1]	Control	Restricted	Over	SEM	Diet*Gest	Diet	Gest
IL6R					0.03	0.27	0.04
d 23	1.06^{ab}	1.07^{ab}	1.10^{ab}	0.18			
d 45	0.85^{a}	1.29^{ab}	1.21^{ab}	0.14			
d 90	1.26^{ab}	1.40^{ab}	0.92^{ab}	0.21			
d 135	1.30^{ab}	2.63^{d}	1.09^{ab}	0.21			
Birth	1.48^{bc}	1.42^{ab}	1.84^{cd}	0.92			
PF4					0.05	0.04	0.0004
d 23	1.15^{bcd}	1.09^{bcd}	1.44^{cde}	0.21			
d 45	0.82^{ab}	0.51^{a}	1.34^{cde}	0.16			
d 90	1.02^{bc}	1.94^{de}	1.30^{cde}	0.28			
d 135	1.53^{cde}	1.59^{cde}	2.08^{e}	0.31			
Birth	1.16^{bcd}	1.51^{cde}	1.26^{cde}	0.34			

[a-e] Within a gene, relative expression with different superscripts differ ($P \leq 0.05$).

[1]Expression relative to d 23 Control.

Abbreviations: Interleukin 6 receptor (IL6R); Platelet factor 4 (PF4).

Supplementary Table 2. Main effect of diet on gene expression of systemic inflammatory mediators of pregnant ewes.

	Diet				*P* value		
Gene[1]	Control	Restricted	Over	SEM	Diet*Gest	Diet	Gest
Chemokine ligand							
CCL4	1.57[ab]	0.91[b]	1.53[a]	0.21	0.60	0.05	<0.0001
CXCL9	1.92[a]	1.76[a]	2.60[b]	0.47	0.66	0.03	<0.0001
Chemokine receptor							
CXCR1	1.35[a]	2.36[b]	1.82[a]	0.26	0.34	0.002	<0.0001
Interleukin ligand							
IL1B	1.55[a]	2.49[b]	2.34[ab]	0.37	0.55	0.03	0.23
IL4	1.52[a]	1.06[a]	1.84[b]	0.22	0.85	0.005	<0.0001
IL7	1.20[a]	1.13[a]	1.47[b]	0.12	0.16	0.04	<0.0001
Interleukin receptor							
IL10RA	1.12[a]	1.68[ab]	1.51[b]	0.14	0.37	0.05	0.39
Transforming growth factor superfamily							
BMP2	2.88[a]	0.43[b]	1.83[a]	0.47	0.90	0.04	0.69
Tumor necrosis factor super family							
LTA	1.28[ab]	0.97[a]	1.51[b]	0.16	0.94	0.006	0.05
LTB	1.22[a]	1.23[a]	1.45[b]	0.13	0.76	0.05	<0.0001
TNFSF10	1.27[a]	1.01[a]	1.57[b]	0.14	0.60	0.009	<0.0001
TNFSF11	1.84[ab]	1.09[a]	2.01[b]	0.29	0.86	0.007	<0.0001
TNFSF13B	1.51[a]	2.06[b]	1.81[ab]	0.68	0.07	0.007	<0.0001

[ab] Within a gene, relative expression with different superscripts differ ($P \leq 0.05$).

[1]Expression relative to Control.

Abbreviations: Bone morphogenetic protein 2 (BMP2); C-C motif chemokine ligand 4 (CCL4); C-X-C motif chemokine ligand 9 (CXCL9); C-X-C chemokine receptor 1 (CXCR1); Interleukin 1 beta (IL1B); Interleukin 4 (IL4), IL7 7; Interleukin 10 receptor subunit alpha (IL10RA); lymphotoxin alpha (LTA); lymphotoxin beta (LTB); TNF super family member 10 (TNFSF10), TNFSF11, TNFSF13B.

Supplementary Table 3. Main effect of stage of gestation on gene expression of systemic inflammatory mediators of pregnant ewes.

	Stage of gestation						*P* value		
Gene[1]	d 23	d 45	d 90	d 135	Birth	SEM	Diet*Gest	Diet	Gest
Cytokine ligand									
AIMP1	1.09^{a}	1.38^{a}	1.20^{a}	1.01^{a}	0.49^{b}	0.17	0.80	0.44	<0.0001
CCL2	1.76^{a}	4.74^{b}	3.51^{b}	3.82^{b}	0.76^{c}	0.22	0.77	0.52	<0.0001
CCL3	1.21^{a}	0.65^{bc}	1.08^{ab}	0.95^{ab}	0.72^{c}	0.14	0.53	0.06	0.01
CCL4	1.40^{a}	1.06^{a}	1.25^{a}	0.63^{b}	0.40^{c}	0.18	0.60	0.05	<0.0001
CCL5	1.60^{a}	1.23^{a}	1.66^{a}	0.82^{a}	0.49^{b}	0.19	0.66	0.22	0.01
CCL22	1.16^{a}	1.66^{a}	1.48^{a}	1.10^{a}	0.52^{b}	0.20	0.50	0.82	<0.0001
CXCL9	1.19^{a}	1.51^{ab}	1.28^{ab}	0.72^{b}	0.29^{c}	0.24	0.66	0.03	<0.0001
CXCL10	1.65^{a}	0.36^{b}	0.30^{b}	0.27^{b}	0.07^{c}	0.13	0.36	0.08	<0.0001
MIF	1.49^{a}	1.09^{a}	1.12^{a}	0.85^{a}	0.30^{b}	0.96	0.17	0.06	<0.0001
SPP1	2.20^{bc}	6.15^{c}	2.48^{bc}	1.72^{b}	0.42^{a}	0.13	0.24	0.30	0.0002
Chemokine receptor									
CCR1	1.12^{a}	1.04^{a}	1.07^{a}	1.38^{a}	3.83^{b}	0.22	0.66	0.13	<0.0001
CCR3	1.20^{a}	1.70^{a}	1.40^{a}	1.57^{a}	0.76^{b}	0.24	0.55	0.33	0.002
CCR5	1.15^{a}	1.06^{a}	1.32^{a}	0.95^{a}	0.53^{b}	0.13	0.85	0.12	<0.0001
CCR6	1.18^{ab}	1.86^{b}	2.26^{b}	0.87^{a}	1.11^{a}	0.32	0.22	0.14	0.04
CCR8	1.10^{a}	1.30^{a}	1.40^{a}	0.99^{a}	0.47^{b}	0.16	0.72	0.23	<0.0001
CXCR1	1.20^{a}	0.78^{b}	0.58^{a}	2.07^{c}	3.14^{d}	0.23	0.35	0.002	<0.0001
CXCR3	1.07^{a}	0.76^{a}	1.27^{a}	0.87^{a}	0.50^{b}	0.10	0.40	0.46	<0.0001
CX3CR1	1.24^{a}	1.35^{a}	1.06^{a}	0.54^{b}	0.33^{c}	0.18	0.92	0.69	<0.0001
Colony stimulating factor									
CSF1	1.14^{a}	0.84^{a}	0.78^{a}	0.97^{a}	0.44^{b}	0.12	0.71	0.31	0.0002
CSF3	1.09^{a}	1.05^{a}	1.55^{b}	1.65^{b}	0.94^{a}	0.14	0.15	0.59	0.0003
Interferon type II									
IFNG	1.31^{a}	1.13^{ab}	1.20^{a}	0.71^{bc}	0.57^{c}	0.20	0.52	0.69	0.0007

Interleukin ligand									
IL4	1.16[a]	0.79[b]	0.52[b]	0.53[b]	0.20[c]	0.09	0.85	0.005	<0.0001
IL5	1.19[ab]	0.97[ab]	0.71[b]	1.21[a]	0.47[c]	0.14	0.78	0.81	<0.0001
IL7	1.10[a]	0.87[a]	0.97[a]	0.84[a]	0.53[b]	0.10	0.88	0.04	<0.0001
IL8	1.50[a]	0.66[b]	1.00[a]	1.85[a]	3.56[a]	0.10	0.50	0.06	0.02
IL13	1.16[a]	1.45[a]	1.06[a]	0.86[a]	0.52[b]	0.14	0.83	0.27	<0.0001
IL15	1.15[a]	0.92[ab]	0.80[b]	0.75[b]	0.31[c]	0.15	0.85	0.25	<0.0001
IL16	1.07[a]	1.19[a]	1.21[a]	1.11[a]	0.69[b]	0.11	0.75	0.61	0.002
IL17B	1.34[a]	0.84[a]	1.85[a]	2.88[b]	1.29[a]	0.33	0.25	0.54	0.05
Interleukin receptor									
IL2RB	1.18[ab]	2.30[b]	1.57[b]	1.57[b]	0.80[a]	0.33	0.67	0.65	0.04
IL6ST	1.08[a]	1.12[a]	1.28[a]	1.16[a]	0.73[b]	0.12	0.96	0.98	0.005
IL9R	1.09[a]	1.12[a]	1.21[a]	0.85[a]	0.50[b]	0.28	0.80	0.21	<0.0001
Nicotinic acid phosphoribosyltransferase family									
NAMPT	1.12[a]	0.63[b]	0.74[b]	0.62[b]	0.53[c]	0.11	0.97	0.41	<0.0001
Tumor necrosis factor super family									
CD40LG	1.07[a]	0.85[ab]	1.08[ab]	0.75[b]	0.35[c]	0.08	0.87	0.16	<0.0001
FASLG	1.16[a]	0.75[bc]	0.97[ab]	0.61[c]	0.41[d]	0.10	0.76	0.36	<0.0001
LTA	1.24[a]	1.08[a]	0.98[a]	1.03[a]	0.61[b]	0.13	0.94	0.006	0.05
LTB	1.09[a]	0.89[a]	1.06[a]	0.76[a]	0.37[b]	0.22	0.76	0.05	<0.0001
TNF	1.14[a]	1.07[a]	1.29[a]	1.24[a]	1.98[b]	0.13	0.15	0.06	<0.0001
TNFSF10	1.10[a]	0.56[b]	0.61[b]	0.70[b]	0.34[c]	0.10	0.60	0.009	<0.0001
TNFSF11	1.17[a]	1.42[a]	1.92[a]	1.23[a]	0.36[b]	0.25	0.86	0.007	<0.0001
TNFSF13B	1.12[ab]	0.87[a]	0.92[a]	1.28[b]	3.71[c]	0.19	0.07	0.007	<0.0001

[a-d] Within a gene, relative expression with different superscripts differ ($P \leq 0.05$).

[1] Expression relative to d 23 of gestation.

Abbreviations: Aminoacyl tRNA sunthetase complex interacting multifunctional protein 1 (AIMP1); CD40 ligand (CD40LG); C-C motif chemokine ligand (CCL)-2, 3, 4, 5, 22; C-X-C motif chemokine ligand (CXCL)-9, 10; C-C motif chemokine receptor (CCR)-1, 3, 5, 6, 8; C-X-C motif chemokine receptor (CXCR)- 1, 3; C-X3-C motif chemokine receptor 1 (CX3CR1); Colony stimulating factor (CSF)-1, 3; Fas ligand (FASLG); Interferon gamma (IFNG); Interleukin (IL)-4, 5, 7, 8, 13, 15, 16, 17B; Interleukin receptor (IL6R; IL9R); Interleukin 6 signaling transducer (IL6ST); Lymphotoxin alpha (LTA); Lymphotoxin beta (LTB); Macrophage migration inhibitory factor (MIF); Nicotinamide phosphoribosyltransferase (NAMPT); Secreted phosphoprotein 1 (SPP1); Tumor necrosis factor (TNF); TNF super family member (TNFSF)-10, 11, 13.

Discussion

This study evaluated inflammatory gene expression in both the mother and fetus to begin to understand inflammation as a potential fetal programming mechanism. Our findings indicate that maternal inflammatory gene expression is altered by poor nutrition during gestation, but the expression of inflammatory molecules differs during restricted- or over-nutrition (Figure13). Further, our data indicate that the offspring inflammatory response to maternal nutrient restriction- or over-nutrition is distinct from the mother, indicating that maternal inflammation may program fetal development through indirect mechanisms (Figure13). Finally, we report alterations in pro-inflammatory cytokine and chemokine expression in the liver of offspring, which may be responsible for predisposition to hepatic dysfunction later in life (Figure13).

In addition to evaluating the maternal and fetal inflammatory response to gestational diet, this study also reports changes to maternal inflammation at multiple stages of gestation, in the absence of dietary effects. Previous literature evaluating inflammation throughout gestation has only evaluated one stage of gestation, or performed longitudinal analyses of few inflammatory molecules (Madan et al., 2009; Christian and Porter, 2014; Lekva et al., 2016). Our study is unique in that inflammatory changes are reported at multiple stages of gestation in the same cohort of females, with the mRNA abundance of 84 inflammatory genes evaluated. These data have implications in understanding how the maternal innate immune system responds during gestation using a common biomedical model of pregnancy (Barry and Anthony, 2008). Healthy pregnancies are associated with a chronic, low-grade inflammatory response which is pertinent to pregnancy recognition, maintenance, and parturition (Mor, 2008; Mor et al., 2011). It has been proposed that *CCL2*, *CCL3*, *CCL4* and *CCL5* are relevant chemokines during pregnancy due to their role in controlling leukocyte chemotaxis between the systemic and local intrauterine

environment (Gomez-Lopez et al., 2010). This is supported by our data, in which *CCL2* increased 3- to 4-fold between d 23 of gestation and parturition. Further, the expression of *CCL3*, *CCL4,* and *CCL5* was maintained during early- and mid-gestation and decreased during late gestation or postpartum, indicating a greater role in pregnancy maintenance than parturition. In our study, the mRNA expression of *TNF* and *CXCL8* was greatest after parturition, consistent with longitudinal reports in women in which the serum concentrations of TNF and CXCL8 were greatest postpartum (Christian and Porter, 2014; Lekva et al., 2016). During labor, TNF is considered a primary signal for the secretion of secondary inflammatory molecules and induces the production of prostaglandins to coordinate uterine contractions (Gomez-Lopez et al., 2010). C-C- motif chemokine ligand 8 is a secondary inflammatory molecule produced by multiple reproductive tissues, such as the cervix, to induce blood flow and dilation (Gomez-Lopez et al., 2010). Typically, CXCL8 expression is induced by TNF to support parturition events and tissue repair postpartum (Gomez-Lopez et al., 2010). In addition to these hallmark inflammatory molecules, our data demonstrate that the maternal immune response involves the expression of multiple cytokine, chemokine, and TNFSF ligands that previously may have not been considered during gestation. Although additional research and protein expression is necessary to investigate the roles and relationships of each specific inflammatory molecule reported, our data support the integral role of the innate immune system during pregnancy.

While chronic, low-grade inflammation is associated with healthy pregnancies (Mor, 2008; Mor et al., 2011), improper activation of the innate immune system due to maternal stressors may have negative consequences on the success of the pregnancy and development of the fetus during gestation (Mor, 2008; Mor et al., 2011). In our study, pro-inflammatory gene expression was observed in both restricted- and over-nourished mothers, yet there was no

evidence of disrupted pregnancy success as no early fetal losses occurred after dietary treatment began (Jones et al., 2016), and the gestation length of ewes was not different based on diet (Pillai et al., 2017). However, the gene expression of pro-inflammatory molecules may indirectly affect fetal development by modulating fetal nutrient availability through altered placental transport (Ingvorsen et al., 2015). Although our study did not investigate the placental tissues, we did observe increased systemic *PF4* during early- and mid-gestation of both Restricted and Over pregnancies. In sheep, placental development is rapid during early to mid-gestation, and complete by d 90 (Redmer et al., 2004). Increased *PF4* may play a role in altered angiogenesis and vascularization during this timeframe, potentially having negative consequences on the formation and transport capacity of blood to and across the placenta to affect fetal development (Wallace et al., 2002; Cetin and Alvino, 2009). Previous models of maternal over-nutrition have associated reduced uterine and umbilical blood flow in ewes with reduced fetal nutrient delivery (Wallace et al., 2002; Reynolds et al., 2005; Cetin and Alvino, 2009). Additionally, in ewes who were nutrient restricted during the first 40% of gestation, placental vasoconstriction contributed to fetal intrauterine growth restriction (Reynolds et al., 2005). In our model, OVER lambs at birth exhibited increased systemic gene expression of *PF4* and *VEGFA*, which promote cell survival in response to hypoxic conditions. Thus, future research may investigate the role of PF4 on placental structure and transport during over-nutrition, and the presence of a hypoxic intrauterine environment.

Nutrient restriction- and over-nutrition resulted in differing maternal metabolic and inflammatory phenotypes (Figure13), indicating that inflammation may affect maternal health and fetal development differently based on nutrient status. Nutrient restricted ewes demonstrated depletion of nutrient reserves by the gradual loss of body condition (Russel et al., 1969) and

increased circulating NEFA concentrations as gestation advanced. Additionally, RES offspring were smaller at both d 135 of gestation and birth. This maternal-fetal phenotype is consistent with previous pregnant sheep models of limited maternal nutrient intake that are associated with slowed fetal growth in late gestation (Vonnahme et al., 2013a; He et al., 2015; Wallace et al., 2015). Mobilization of NEFA may have contributed to inflammation in Restricted mothers because sustained increases of NEFA are known to activate the innate immune system through toll like receptors (Hussey et al., 2014), a network of transmembrane receptors that typically respond to pathogen or danger associated molecular patterns. Specifically, stimulation of TLR4 by NEFA causes a pro-inflammatory signaling cascade through the activation of master inflammatory axes, nuclear factor kappa B (NFκB) and mitogen activated kinase (Shi et al., 2006; Hussey et al., 2014). In our model, Restricted ewes exhibited a pro-inflammatory response during gestation via increased mRNA expression of cytokines *IL1B* and *TNFSF13B*, and decreased mRNA expression of anti-inflammatory cytokine *IL4* and chemokine *CCL4*. The cytokines IL1B and TNFSF13B activate both canonical and non-canonical inflammatory signaling pathways, and act in a positive feedback loop to mediate a chronic inflammatory environment and depress anti-inflammatory molecules (Lawrence, 2009).

During gestation, the fetal liver receives the first exposure of nutrients and oxygen from the maternal circulation for secondary distribution to the brain, heart, and peripheral tissues (Godfrey et al., 2012). Thus, the fetal liver is also susceptible to sensing altered nutrients delivered from the maternal circulation. Although we did not investigate transport of nutrients to the fetus, we did observe increased *CCL16* gene expression in RES fetuses at d 135 of gestation. C-C motif chemokine ligand 16 is primarily expressed in the liver in response to TLR stimulation (Nomiyama et al., 2001). It is possible that transport of maternal NEFA to RES

fetuses was increased, increasing hepatic TLR signaling to create a pro-inflammatory environment, which would include increased *CCL16* expression.

At the onset of parturition and the glucocorticoid surge, the fetal liver partakes in glucose metabolism rather than hematopoiesis, and is responsible for maintaining glucose homeostasis postnatally (Wesolowski et al., 2017). Offspring born to restricted mothers often exhibit liver dysfunction postnatally (Hyatt et al., 2011; George et al., 2012; Thorn et al., 2013). Intrauterine growth restricted offspring exhibit premature hepatic glucose production at late gestation, which is not suppressed by insulin, indicating predisposition to insulin insensitivity (Thorn et al., 2013). One-yr old lambs born to restricted mothers exhibited hepatic steatosis and reduced oxidative metabolism when challenged with nutrient excess at weaning (Hyatt et al., 2011). At 6-yr of age, offspring born to restricted mothers had increased hepatic glycogen and lipid content and reduced insulin sensitivity (George et al., 2012). In our model of maternal restriction, newborn lambs exhibited a 4-fold increase in hepatic *TNF* gene expression. Tumor necrosis factor causes insulin insensitivity by inducing serine phosphorylation of the insulin receptor subunit and is associated with the pathogenesis of insulin resistance and hepatic steatosis (Borst, 2004). Although immediate effects of this cytokine on neonatal metabolism are unknown, hepatic *TNF* may have long-term negative consequences on the offspring if persistent by disrupting insulin signaling and promoting lipid deposition in the liver, contributing to hepatic metabolic disease at maturity.

In our study, Over ewes did not demonstrate increased circulating NEFA concentrations. However, the BCS of Over ewes increased as gestation advanced, indicating increased accumulation of maternal adipose tissue (Russel et al., 1969). Therefore, the maternal inflammation observed in response to over-nutrition was not related to increased circulating

NEFA. Rather, increased adiposity and adipocyte dysfunction may have contributed to inflammation in Over ewes. During over-nutrition, adipocyte hypertrophy is promoted to store excess nutrients instead of hyperplasia because local TNF production antagonizes peroxisome proliferator-activated receptor gamma, inhibiting adipocyte differentiation (Guilherme et al., 2008). Consequently, engorged adipocytes drive local and systemic inflammation in two ways; first, enlarged adipocytes induce local hypoxia by constricting the adipose capillary network, and second, engorged adipocytes undergo necrosis and apoptosis, resulting in macrophage infiltration and the production of adipokines that travel peripherally to induce systemic inflammation (Guilherme et al., 2008). Although this study did not directly measure hypoxic or inflammatory gene expression in maternal adipose tissue, adipocyte hypertrophy and hypoxia are hallmark events linking over-nutrition, adipose accumulation, and inflammation in both non-pregnant and pregnant women (Guilherme et al., 2008). The systemic mRNA expression of TNFSF ligands and lymphotoxins in Over ewes supports an environment that would promote adipocyte hypertrophy and hypoxia (Guilherme et al., 2008), and thus, may be a mechanism promoting maternal inflammation during over-nutrition.

To investigate the offspring inflammatory response to maternal restricted- and over-nutrition, our study focused on the perinatal period as T and B cells of the innate immune system are mature by late gestation, and differentiation of monocytes into macrophages is ongoing (Yan et al., 2011a; Ginhoux and Jung, 2014). Postnatally, the systemic inflammatory response is a critical component of the innate immune system because it is responsible for eliciting the first-line of defense in response to an immune challenge. At d 135 of gestation, both RES and OVER lambs exhibited reduced chemokine expression, which may be characteristic of a depressed or immature innate immune system that may impair health and survival postnatally. Previous

studies have demonstrated that the ability of neonate offspring from restricted mothers to elicit an immune response to lipopolysaccharide challenge was diminished compared with control offspring (He et al., 2014). This dampened immune response was observed in the absence of changes to CRP expression at birth (He et al., 2014), similar to the current study.

Our study identified increased systemic *CXCL8* expression in RES and OVER at birth which is indicative of pro-inflammation and neutrophil recruitment. This has the potential to antagonize growth and alter metabolism of multiple tissues if persistent postnatally (Elsasser et al., 2007). Previous studies have demonstrated that inflammation does, in fact, persist postnatally in response to maternal diet, and is associated with adulthood disease. Maternal obesity induced the gene expression of *IL1B*, *IL6*, *CXCL8* and *CCL2* and protein expression of TNF in the intestine of mature lambs at 22 mo of age (Yan et al., 2011a). Maternal obesity also enhanced *TLR2* and *TLR4* gene expression and protein phosphorylation of the p65 subunit of NFκB in the longissimus muscle of mature lambs at 22 mo of age, which was associated with attenuated insulin signaling and collagen formation (Huang et al., 2010; Yan et al., 2011b). Maternal nutrient restriction reduced the expression of suppressor of cytokine signal 3 (*SOCS3*) in the semitendinosus muscle of newborn lambs (Hoffman et al., 2016b), indicative of unregulated cytokine activity. Maternal restriction also increased the gene expression of *TNF* and *TLR4* in the left ventricular tissue of 6- yr old offspring, which was associated with cardiac remodeling (Ge et al., 2013). Thus, persistence of tissue specific inflammation is associated with predisposition to developmental and metabolic disorders, and systemic *CXCL8* gene expression may be a common mechanism programming offspring exposed to poor maternal nutrition during gestation.

Our study also identified gender differences in offspring inflammatory gene expression, with an overall observation of increased inflammatory mRNA expression in females. Although

this study was not powered to investigate gender differences within a diet, gender is an important consideration for future research in the maternal programming field, considering that baseline gender differences exist.

Gestational diet	Maternal	Late gestation fetus	Neonate
Nutrient Restriction	↓ BW, BCS ↑ NEFA ↑ Pro-inf. cytokines	↓ BW, size ↓ Systemic chemokine exp. ↑ Hepatic *CCL16*	↓ BW, size ↑ Systemic *CXCL8* ↑ Hepatic *TNF*
Over-nutrition	↑ BW, BCS ↔ NEFA ↑ TNFSF	↔ BW, size ↓ Systemic chemokine exp. ↓ Hepatic TNFSF exp.	↔ BW, size ↑ Systemic *CXCL8, PF4, VEGFA* ↓ Hepatic *BMP2*
Restricted ≠ Over	Maternal ≠ Offspring		Inflammation = hepatic dysfunction

Figure 13. Summary table depicting the effect of gestational diet on maternal and offspring BW, metabolism and inflammatory gene expression. The expression of maternal inflammatory molecules differs during restricted- or over-nutrition and may be due to differing body composition and circulating non-esterified fatty acid (NEFA) concentrations in maternal circulation. The offspring inflammatory response to maternal nutrient restriction- or over-nutrition is distinct from that of the mother, indicating that maternal inflammation may program fetal development through indirect mechanisms. Alterations in pro-inflammatory cytokine and chemokine expression in the liver of neonate offspring may be responsible for predisposing offspring to hepatic dysfunction later in life.

CONCLUSIONS AND IMPLICATIONS

In conclusion, the current data demonstrate that nutrient restriction- and over-nutrition to the ewe during gestation promote a pro-inflammatory environment in both the mother and offspring, yet the inflammatory responses of each diet are distinct. During nutrient restriction, maternal inflammation is linked with mobilization of NEFA, whereas over-nutrition is not. We propose that maternal inflammation provoked by diet indirectly promotes systemic and hepatic inflammation in the perinatal offspring, which may have long-lasting effects on innate immune system function and hepatic metabolism postnatally. Identifying inflammatory molecules in both the mother and offspring in response to poor maternal nutrition is important to further investigate the mechanisms contributing to fetal programming, and create strategies to mitigate the long-term consequences. Future research regarding the effect of maternal inflammation on placental transport and integrity is necessary to further understand how maternal inflammation contributes to fetal programming.

GENERAL DISCUSSION

The livestock sector is an essential component of the food supply because it contributes wholesome protein products to the human diet. Current demands for animal-based protein are increasing in parallel with the growing global population (Reynolds et al., 2015; Global Harvest Initiative, 2016). To meet these demands, animals must grow efficiently and yield a nutritional, palatable product for consumption. However, many animals fail to reach their genetic potential postnatally, and increasing evidence indicates that this may be due to the intrauterine environment encountered during gestation (Wu et al., 2006). Maternal programming refers to alterations in the maternal or intrauterine environment which can program postnatal growth, development, and metabolism of offspring (Wu et al., 2006; Hoffman et al., 2017). Many factors can result in maternal programming, and understanding the mechanisms by which these factors affect offspring is necessary to improve livestock production . Understanding these mechanisms aids producers in optimizing gestational management and mitigating the negative consequences that maternal programming can have on livestock production.

Gestational management is an important component of livestock production because it contributes to the health and performance of both the mother and subsequent offspring. During gestation, maternal physiological adaptations occur to support maternal maintenance and the developing fetus. This includes immune adaptations that tolerate the fetal immune system, circulatory adaptations that support placental vascularization and fetal blood flow, and metabolic adaptations that partition nutrients to the fetus (Redmer et al., 2004; Reynolds et al., 2005). When it is not possible for the maternal system to accomplish these adaptations, prenatal fetal development is impaired and the consequences may persist, impairing postnatal performance. Gestational nutrition is a common factor that impacts pregnancy because of seasonal variability

in forage quality and quantity. Our research group previously demonstrated that both nutrient restriction- and over-nutrition during gestation (d 30 through parturition) reduces muscle fiber cross-sectional area in 3 mo old lambs, irrespective of muscle fiber type (Reed et al., 2014). These lambs also exhibit metabolic disturbances including increased insulin concentrations and glucose intolerance (Hoffman et al., 2016). Further, when gestational nutrition is restricted- or over-fed during only late gestation, 3 mo old lambs exhibit reduced IGF-1 and IGFBP-3 in circulation despite the short period of nutrient insult (Hoffman et al., 2014). Collectively, this demonstrates the importance of maintaining optimal nutrition throughout gestation, as deviations above or below nutrient recommendations may impair offspring postnatal performance.

The research in this dissertation addressed the importance of gestational management, with an emphasis on gestational nutrition, using both applied and molecular approaches. Gestational management can only be performed once females are diagnosed as pregnant. Thus, diagnosis of pregnancy as close to conception as possible provides the longest timeframe during gestation during which producers can monitor the needs of gestating females. Numerous tools are available to producers to diagnose pregnancy in livestock, including observational heat detection, blood progesterone assays, amplitude-mode ultrasound, real-time ultrasound, and Doppler ultrasound (Ganaie et al., 2009). Real-time ultrasound is favored for pregnancy diagnosis because it provides immediate results, can verify fetal viability and number, and is user-friendly to non-veterinarians (Ishwar, 1995; Ginther, 2014; Jones and Reed, 2017). Chapter 1 utilized real-time ultrasound to diagnose pregnancy transabdominally, and demonstrated that this technique can be accurately used as early as d 33 of gestation in ewes. This early diagnosis has been reported when scanning transrectally (García et al., 1993; Doize et al., 1996; Gonzaled de Bulnes et al., 1998), but transabdominal reports had been inconsistent before d 45 of gestation

(Gearhart et al., 1988; Suguna et al., 2008). The advantage of our research is that we reported a detailed fetal and placental developmental timeline that aids technicians in detecting signs of pregnancy to make accurate diagnoses, as well as estimate breeding dates. This research may significantly improve gestational management of ewes if producers integrate information gained during early gestation ultrasound to calculate gestational age and parturition dates, as well as determine litter size. This information further provides producers a proactive management strategy for monitoring flock feeding regimens during gestation, and managing ewes according to gestational age, to minimize the incidence of over- or restricted- nutrition during gestation.

Proactive gestational management also involves detecting maternal or fetal stress, and adjusting management protocols to mitigate the stress. Ultrasound is a non-invasive approach commonly used to monitor fetal growth during gestation, which can potentially indicate stress if deviations in fetal growth are detected. Doppler ultrasound is commonly utilized to monitor indices of placental and fetal blood flow (Carr et al., 2012; Vonnahme and Lemley, 2012), but real-time ultrasound is more practically applied in production settings. Real-time ultrasound has been successfully utilized in sheep to detect changes in fetal growth due to maternal nutrition; however, previous research has focused on late-gestation because this is the timeframe of rapid fetal growth (Carr et al., 2011; Lemley et al., 2011; Lekatz et al., 2013). The research in this dissertation applied real-time ultrasound during the first half of gestation because detection of abnormal fetal growth in mid-gestation rather than late- gestation provides more time-sensitive information for producers to make effective adjustments to their gestational management. The results from Chapter 2 indicated that alterations in fetal growth during mid-gestation were not evident due to maternal nutrition. These results were consistent with previously reported gross necropsy data from the same fetuses (Pillai et al., 2017), and thus, we can conclude that

ultrasound could detect accurate fetal growth trends during this time. The data in Chapter 2 are also unique in that ultrasound measurements were compared with gross measurements at necropsy, providing insight into the precision of ultrasound measurement during this timeframe of gestation.

When gestational management is not optimal, pregnant females cannot adequately adapt to sufficiently provide for the developing fetus. Maternal nutrient restriction- and over-nutrition are two common examples of factors that compromise gestation and the intrauterine environment; however, the contributing mechanisms are poorly understood. Inflammation is a candidate mechanism of maternal programming because it has the potential to alter the maternal and intrauterine environment, and fetal development (Heerwagen et al., 2010; Ingvorsen et al., 2015). Inflammation is also commonly observed in the tissues of neonate and mature offspring born to nutrient restricted- and over-nourished mothers (Du et al., 2010a; Hyatt et al., 2011; Yan et al., 2011a, b; Huang et al., 2012; Dong et al., 2013; He et al., 2014; Hoffman et al., 2016b). Chapter 3 investigated inflammation in mothers and perinatal offspring to gain more knowledge of inflammation as a potential mechanism of programming. Our research approach was unique by utilizing RT-PCR arrays to more comprehensively evaluate inflammation. We identified that in both the mothers and offspring, the inflammatory response provoked by gestational nutrition is distinct and based on diet. This is consistent with previous research by our group and others (Ford and Long, 2012; Hoffman et al., 2016b), and reiterates that despite similar observational changes to body mass and composition, strategies to mitigate the effects of gestational nutrient restriction versus over-nutrition must be diet specific. The data in this dissertation also demonstrated that the inflammatory response of the mothers compared with the offspring are not similar. It is likely that the physical barrier of placenta prevents direct transfer of maternal

inflammatory molecules to the fetus, and rather placental transport or integrity should be researched in the future to completely understand how maternal dietary inflammation affects the fetus. By understanding inflammatory mechanisms of maternal programming, anti-inflammatory interventions provided during gestation or postnatally should be further investigated.

In conclusion, managing gestation is a vital component of livestock production because inadequacies or stressors during gestation impact both the mother and offspring. Resources such as ultrasonography are available to producers to proactively manage gestating females, while also aiding researchers in understanding fetal growth under normal and suboptimal gestational situations. Nutrition is an important aspect of gestational management, and information gained from ultrasound regarding gestational age and litter size aids producers in providing appropriate nutrition at differing stages of gestation. In the event that adequate nutrition cannot be provided during gestation, nutritional interventions may be implemented. Our research identified inflammation as a potential mechanism by which poor nutrition during gestation affects the mother and offspring, and therefore anti-inflammatory diets should be investigated in the future with the caveat that these dietary interventions will differ during situations of restricted- or over-nutrition. These considerations will improve the overall production livestock and overall contribute to a more secure and nutritious global food source in the future.

APPENDICES
APPENDIX I. Descriptive Statistics

Descriptive information should be evaluated to determine patterns and relationships of the dependent variable (DV) to the independent variable (IV) within the fixed effects of the experimental design. This also allows identification of missing data.

Scatter Plot: Create a scatter plot to assess distribution of values in the dataset, observe potential patterns or relationships within the data set that may be explained by fixed effects, and determine which modeling of the DV may be most appropriate.

```
Proc gplot;
Plot variable1*variable2;
run;
```

Combinations of IVs and DVs may be plotted. A class statement can be added to the univariate procedure if evaluating normality within fixed effect comparisons is desired.

Means: Obtain descriptive values from the dataset. Using the means procedure, the raw values will be used to calculate the mean, standard deviation, standard error, minimum value and maximum value. In addition, the n of observations compared to the total n will be reported, allowing patterns of missing values to be identified.

```
Proc means n mean stddev stderr min max; ways 1, 2, 3;
class fixed effects;
var DV;
run;
```

Means vs. LSMeans: If the dataset contains missing values, or if the number of observations is not exactly balanced in within the fixed effects of the experimental design, then the least square means (lsmeans) reported will be different from the actual means when the dataset is analyzed further. The lsmeans will estimate values for missing data or unbalanced effects, and the pairwise comparisons will be based on lsmeans, NOT means. Because of this, in cases of unbalanced fixed effects, the pairwise statistics reported may be discrepant for the actual means. In any manuscript, it should be noted if values are means or lsmeans are reported.

Example 1: In a dataset evaluating the effect of two treatment groups, if n = 10 for both treatments, and no data are missing, means = lsmeans. If this is not true, the data was input into SAS incorrectly or an error exists in the original data file.

Example 2: In a dataset evaluating the effect of two treatment groups and gender, if n = 10 for both treatments, but treatment A contains 4 females and 6 males, whereas treatment B contains 7 females and 3 males, the means and lsmeans will be different for the fixed effect of gender and interaction of gender by treatment. However, the lsmeans of treatment will be the same as the actual means. SAS will impute gender data because this fixed effect is not balanced. Any result evaluating gender will reflect the imputed data.

APPENDIX II. Missing Data

In datasets that contain missing data, R software can be used to further assess patterns of missing values, impute missing data, and compare imputed values with actual values in the dataset.

```
#Evaluate variables in the spreadsheet called 'ewe'
summary(ewe)
str(ewe)

#Impute missing data into a new dataset 'impewe' by using the original dataset 'ewe'. Allow 5
#imputation (default)
library(mice)
impewe<-mice(ewes, m = 5)

#Evaluate the imputed values to compare with original values.
complete(impewe)

#Inspect pattern of imputed (red) and observed (blue) values.
#This code will build a plot of all dependent variables in the spreadsheet.
library(lattice)
stripplot(impewe, pch = 20, cex = 1.2)

#This code allows you to specify a dependent variable of interest (i.e. TNF), and observe the
#imputed data pattern based on subject (i.e. EweID) or independent variable (i.e. Treatment or
#Timepoint)
library(lattice)
xyplot(impewe, TNF ~ EweID, pch=18, cex=1)
xyplot(impewe, TNF ~ Treatment, pch=18, cex=1)
xyplot(impewe, TNF ~ Timepoint, pch=18, cex=1)
```

APPENDIX III: Normality

Normality of a dataset should be evaluated to determine what type of analysis will most appropriately model the data.

To determine normality:

```
proc univariate NORMAL PLOT;
var DV;
histogram;
qqplot DV/normal (mu=est sigma=est color=red l=1);
run;
```

A class statement can be added to the univariate procedure if evaluating normality within fixed effect comparisons is desired.

Points of interest on SAS Output:

Shapiro Wilk's Statistic: A statistic of 1 indicates normality. If $P > 0.05$, then the distribution of the data set is not different than normal and the Shapiro Wilk statistic will also be close to 1. If $P < 0.05$, the distribution of the data set is significantly different than normal. Subsequent analyses should be chosen with this consideration and interpreted with caution.

Histogram: The histogram visualizes the distribution of the values of the DV. Observe histogram to determine if data is distributed as a normal bell curve, or if data are skewed to the right or left.

Box and Whiskers plot: This plot visualizes statistical outliers. The numeric values of the DV is plotted on the y-axis. If an outlier is present, it can be found by identifying the point in the dataset that corresponds to the outlier value. Prior to removing any outlier from a dataset, it should be considered WHY the value is an outlier (poor assay CV, sampling error, biological variation, etc.). If an outlier is removed, this should be reported in the manuscript methodology.

QQPlot: If normal, data will have been plotted very close to the line.

APPENDIX IV. Intraclass Correlation

When deciding if a multilevel analysis is necessary for a dataset, the Intraclass correlation (ICC) can be calculated. The ICC describes how strongly units within a Level 2 variable resemble each other, or how much variability is accounted for by the Level 2 variable. Level 1 variables are the independent observations (ex: offspring, cow, student). Level 2 variables are a higher-order grouping (ex: mother, farm, school, etc.)

To calculate the ICC, an empty model must be performed. The empty model will provide the amount of error accounted for by the Level 2 variable (random error) and the residual error. The ratio of random variability to the total variability is the ICC (random/random+residual).

An ICC of 1 indicates high similarity of values within a group. An ICC of 0 indicates that values from the same group are no more similar than values of the entire population. If the ICC is >0.15, a level 2 variable should be included in the random statement of the statistical model.

Empty model in SAS

```
proc mixed COVTEST;
class subjectID;
Model DV = /SOLUTION;
Random intercept / subject = subjectID;
run;
```

Empty model in R

```
Library(lme4)
Model1 <-lmer(responsevariable ~ 1 Time + (1 | ID), data=dataset, REML=0)
summary(Model1)
```

APPENDIX V: Mixed Models

Mixed models: In cases where a Level 2 variable needs to be accounted for, both fixed and random effects need to be evaluated. This is possible using a mixed model, which includes a class statement to define the fixed and random effects and a random statement to define the Level 2 variable.

Example: The effect of two treatments are being evaluated on the BW of twin offspring. The fixed effect is treatment and the level 2 variable, or random effect, is mother of the offspring.

```
Proc mixed;
Class mother treatment;
Model BW = treatment;
Random mother;
run;
```

APPENDIX VI: Repeated Measures

Repeated measures analysis is conducted to model longitudinal data, or multiple values obtained within the same subject. The code for repeated measures accounts for the response of a DV that has been measured at multiple intervals, specified by the 'repeated' command. By specifying the subject, the code accounts for similarity of responses within the same subject, and variability of responses between different subjects.

For example, the effect of two dietary treatments on the BW of ewes is being evaluated. Ewe BW is repeatedly measured from the subject, EweID at weekly intervals.

```
Proc mixed;
Class EweID treatment week;
Model BW = treatment|week;
Repeated week/ subject = EweID type = cs;
run;
```

Covariate structure: The appropriate covariate structure for modeling each DV must be chosen by coding different covariate structures in the "type = ;" command. In the output, the AIC, AICC and BIC values should be assessed for all covariate structures. The lower the AIC, AICC and BIC values, the better fit the structure is for the responses of the DV.

Covariate structures, code
Compound symmetry, cs
Variance component, vc
Unstructured, un
Hyunh Feldt, hf
Autoregressive, ar(1)
Heterogeneous autoregressive, arh(1)
Toeplitz, toep
Heterogeneous Toeplitz, toeph

Once the appropriate covariate structure has been chosen further analyses can be performed to obtain the pairwise comparisons. The p-value reported should always be from the appropriate covariate structure, and the covariate structure modeling each DV should be reported in the results of a manuscript.

```
Proc mixed;
Class EweID treatment week;
Model BW = treatment|week;
Repeated week/ subject = EweID type = cs;
Lsmeans treatment|week/ pdiff;
Run;
```

Degrees freedom adjustment: The default degrees of freedom in SAS is containment**.** In cases of an unbalanced experimental design or unequal observations obtained per experimental unit, a Kenward Rogers degrees of freedom adjustment may be used to correct the denominator degrees of freedom in the model statement.

```
Model BW = treatment|week/ ddfm = kr;
```

Longitudinal model in R: The appropriate covariate structure is automatically used in R software. Therefore, the longitudinal model can be performed. The output includes the intercept (starting value) and slope (change over time) to model the DV response over time.

```
#slopes and random intercepts, explained by the predictor variable of diet.
require(lme4)
data.frame(ewes)
DV<- lmer(DV ~ Diet*Time + (Time|EweID), REML = FALSE, data = ewes))
summary(DV)

#same code using imputed data set 'impewe'
require(lme4)
library(mice)
data.frame(ewes)
DV<- with(impewe, lmer (DV ~ Diet*Time + (Time|EweID), REML = FALSE, data = ewes))
summary(DV)
```

APPENDIX VII: Regression Analysis

Regression Analysis: To explain how changes of values of a DV are influenced by changes of value for the IVs, regression analysis can be performed. In cases of multiple IVs, the regression can be built by adding IVs or condensed by removing IVs from the analysis to determine a final regression model with the greatest R^2. The R^2 can acquire a value between 0 and 1, and indicates the percentage of the DV output accounted for by the values of the IVs. An R^2 of 1 indicates that 100% of the DV value can be explained by values of the IVs.

Example: It is desired to predict the day of gestation (daysg) based on measurements of the heartwidth, umbilicus, and ribs. Build and remove IVs to determine the model with the greatest R^2:

```
Proc reg;
Model daysg = heartwidth umbilicus ribs / selection = forward selection = summary;
Model daysg = heartwidth umbilicus ribs / selection = backward selection = summary;
Run;
```

APPENDIX VIII: Heat maps

Heat maps can be created in R to visually represent data results. The data to be represented as a heatmap should be in a .csv spreadsheet format and set up in columns and rows. The format of the heatmap will exactly replicate the columns and rows setup in the spreadsheet.
The color gradient in R does not reflect statistical analyses. Rather, the color gradient will reflect the numeric values in the spreadsheet.

#Create matrix from the dataset "Gest"
```
Gest_matrix <- data.matrix(Gest)
```

#Create heatmap titled "Maternal inflammation".
#Color choice and color gradient scale are specified by "col=" and "scale=", respectively
#Heatmap legend is specified by "key="
#Font size of the column and row is specified by "cexCol=" or "cedRow=", respectively
#Clustering pattern within a column or row can be specified by "Colv=" or "Rowv=", respectively
```
heatmap(Gest.matrix, main = "Maternal inflammation", col=redblue(50), scale="none", key=TRUE, keysize=1.5, density.info = "none", trace="none", cexCol=0.9, cexRow = 0.6, Rowv = NA, Colv = NA)
```

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