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The Effects of Poor Maternal Nutrition during Gestation in Sheep on the Reproductive Efficiency of the Offspring

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Abstract

Poor maternal nutrition during gestation negatively impacts fetal programming and the development of the hypothalamic-pituitary-ovarian axis in the offspring. Reproductive health of ewes can be characterized by concentrations of reproductive hormones including follicle stimulating hormone (FSH), luteinizing hormone (LH), and progesterone, particularly during the estrous cycle. We hypothesized that ewes born to dams that experienced poor maternal nutrition during gestation would have reduced reproductive capability and efficiency. This means that they would take longer to become pregnant and have reduced concentrations of reproductive hormones compared with those born to mothers fed the control diet. Pregnant dams (F0) were fed one of three different diets beginning on day 30 of pregnancy, restricted-fed (RES: 60%), control-fed (CON: 100%), or over-fed (OVER: 140%) based on National Research Council (NRC) requirement for total digestible nutrients. The female offspring (F1) of these dams were fed the same postnatal diet that met the requirements for growing ewe lambs. Thirty-eight multiparous yearling Dorset ewes (F1 generation) were estrous synchronized using the protocol previously described (Jones et al., 2016). Briefly, a controlled intravaginal drug releasing device (CIDR) was placed through vaginal insertion and released progesterone over a 12-day period. After this period, CIDRs were removed and ewes were given an intramuscular injection of prostaglandin (PG) $F_{2\alpha}$ to stimulate luteolysis. After synchronization, ewes were divided evenly and placed with one of two related rams. Blood samples (10 mL) were collected by jugular venipuncture every 7 days from synchronization until one week after confirmation of pregnancy by transabdominal ultrasound. Blood was centrifuged (at 1,200 x g) and sera were collected. Blood samples were analyzed for the concentration of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and progesterone using enzyme-linked immunoassay. Time to

pregnancy was calculated as the number of days between when ewes were first housed with the ram until the first day of gestation. Number of fetuses was determined on between day 30 and day 40 by transabdominal ultrasound, and the number of lambs was counted after parturition. Data were analyzed using RStudio (RStudio, Boston, MA). There was no significant effect of treatment or time on concentration of FSH or LH. Additionally, there was no significant effect of time on concentration of progesterone. However, there was a tendency for the concentration of progesterone in RES to be greater than that of CON ($P = 0.09$). There were no significant effects from treatment on time to pregnancy, number of fetuses, or number of lambs.

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Introduction

The goal of livestock production for food-producing animals is to increase efficiency while decreasing production costs. Production efficiency depends on the ability of the animal to have offspring that are healthy and capable of reproducing (Govoni et al., 2019). In addition to their use as a production model, sheep have also been used as the biomedical research model for humans due to the extensive data available on their reproductive function and similarities to human reproduction. These similarities include relatively long gestation length (150 days), number of fetuses (generally one or two in both species), structure of the placenta (human placental structure is similar in shape to the cotyledon structure in sheep), and development of the fetus, including organ growth rate, metabolic regulation, and neonatal thermoregulation (Vonnahme et al., 2015). Sheep have been used extensively as models for mother and the fetus response to stimuli such as nutrient manipulation during gestation (Vonnahme et al., 2015). Previous studies found that poor maternal nutrition during gestation has adverse effects on the offspring in sheep including altered growth potential and muscle development (Reed et al., 2014; Martin et al., 2019). The outcomes of poor maternal nutrition during gestation on female and male offspring have been studied in several areas, including aspects of the endocrine system. However, the effects on reproductive efficiency in female offspring and the mechanisms of these effects are not well-known (Dupont et al., 2012).

Poor Maternal Nutrition and Fetal Programming

Fetal programming is a process that occurs during embryonic and fetal development that involves the creation of organs and cellular structures and mechanisms to ensure survival of the newborn (Kwon and Kim, 2017). Studies have shown that negative stimuli during this period can lead to alterations that predispose the offspring to permanent endocrine, metabolic, and cardiovascular diseases including type 2 diabetes, coronary heart disease, and hypertension (Kwon and Kim, 2017; Govoni et al., 2019; Martin et al., 2019). Poor maternal nutrition is one of the stimuli that causes negative programming effects on the offspring that persist into adulthood (Kwon and Kim, 2017). Poor maternal nutrition can be characterized as excess or restricted for total nutrient intake or a specific nutrient fed to the mother during gestation (Reed et al., 2014). The duration and period of time in gestation when maternal nutrition is imposed determines the extent of the alterations by fetal programming (Reed et al., 2014). The type of alterations depends on the organ, tissue, or system of the fetus being developed by the fetal programming process at the time of the imposed poor maternal nutrition (Reed et al., 2014). Poor maternal nutrition during gestation including restricted- or over-feeding can have negative effects on fetal programming leading to alteration in birth weight, increased adiposity, decreased muscle, and long-term changes in whole body metabolism and energy efficiency in the offspring (Hales and Barker, 2001; Martin et al., 2019).

Hypothalamic-Pituitary-Ovarian Axis Development and the Onset of Puberty in Sheep

The neuroendocrine components of the hypothalamic-pituitary-gonadal axis are primarily developed and established in the fetus during the gestation period (Da Silva et al., 2003). At day 70 of gestation, a functional hypothalamic-pituitary-gonadal axis is present in the offspring, and the plasma gonadotropins produced by the anterior pituitary gland reach their maximum concentrations in the fetus at day 100 of gestation (Grazul-Bilska et al., 2009). Fetal ovaries are developed by day 75 of gestation and contain a full set of primordial follicles (Grazul-Bilska et al., 2009). During the fetal stage, some ovarian follicles undergo development so that primary, secondary, and tertiary follicles are present in the fetal ovaries at birth (Grazul-Bilska et al., 2009). Prenatal exposure to negative stimuli during the period of gestation in which these critical developments occur could influence the function of the reproductive axis and affect reproductive potential of the offspring (Da Silva et al., 2003).

The onset of puberty is dependent on the amount of body fat and the activation of the components of the hypothalamic-pituitary-ovarian axis (Leka-Emiri et al., 2017; Abou El Ella et al., 2020). Adequate adiposity is needed to achieve appropriate leptin concentrations to stimulate pubertal activation of gonadotropin-releasing hormone (GnRH) neurons (Abou El Ella et al., 2020). The neuroendocrine events that are triggered induce modifications in the neurons of the hypothalamus that cause a surge of GnRH (Dicken et al., 2012). The release of GnRH from the hypothalamus activates the pituitary-ovarian axis in the ewe (Dicken et al., 2012; Merhi et al., 2020). The first onset of estrus marks the onset of puberty in all species (Dicken et al., 2012; Merhi et al., 2020).

The Estrous Cycle in Sheep

According to Bartlewski et al. (2011), before the onset of the first estrus of the breeding season, ewes are in a state of anestrus characterized by decreased concentrations of estrogen and progesterone along with a quiescent reproductive system. Before the start of the first estrous cycle, progesterone concentrations increase to induce estrous behaviors. The average length of the estrous cycle in ewes is 16 to 17 days and it is divided into four stages: proestrus, estrus, metestrus, and diestrus. Sheep are seasonally polyestrous, having ovulatory cycles in the fall when the amount of light per day is decreasing. Reproductive health can be determined by the concentrations of reproductive hormones during the estrous cycle.

The first phase, proestrus, involves the action of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on follicular development for the maturation of primordial follicles (Bartlewski et al., 2011). Luteinizing hormone and FSH are stimulated by the release of GnRH (Dupont et al., 2012). Gonadotropin-releasing hormone is a peptide hormone secreted by the hypothalamus to act on the anterior pituitary gland and stimulates the release of LH and FSH (Dupont et al., 2012). Luteinizing hormone is a glycoprotein hormone that stimulates ovulation and development of the corpus luteum (CL) from an ovulated follicle and ranges from 0.7 ± .2 ng/mL to 253 ± 103 ng/mL during the estrous cycle in sheep (Hauger et al., 1977; Dupont et al., 2012). Follicle-stimulating hormone is a glycoprotein hormone that stimulates follicular growth and development with normal concentrations ranging from a nadir of around 50 to 60 ng/mL and increasing 5-fold to 250 to 300 ng/mL after the onset of estrus (Goodman et al., 1981; Bartlewski et al., 2011). Folliculogenesis involves primordial follicles further maturing into primary, secondary, and finally tertiary follicles before a dominant follicle ovulates (Bartlewski et al.,

2011). Anti-mullerian hormone (AMH*)* is a glycoprotein hormone that is expressed in the granulosa cells of primary, secondary, and tertiary follicles (Campbell et al., 2012).

In rodents, AMH has a role in the control of follicular development by inhibiting selection of primordial follicles for maturation and inhibiting gonadotropin-dependent growth of secondary follicles (Campbell et al., 2012). In sheep, AMH inhibits the response of follicular theca and granulosa cells to LH and FSH to control folliculogenesis during proestrus (Campbell et al., 2012).

Estrus, the subsequent stage, involves a preovulatory surge of LH from 0.5 ng/mL to 15 ng/mL to peak values of 100 ng/mL to 200 ng/mL, along with a peak in estrogen concentrations from 1 pg/mL to average peaks of 10 pg/mL (Wheatley and Radford et al., 1969; Mauer et al., 1972; Karsch et al., 1979; Quirke et al., 1979; Pant et al., 1977, Bartlewski et al., 2011; Husvéth, 2011). Estrogen is a steroid hormone released from the ovaries that causes ovulation of the follicle and stimulation of estrus (Bartlewski et al., 2011; Husvéth, 2011).

Metestrus follows the estrus period and is induced by a decline in estrogen concentrations from peaks of 10 pg/mL to 1 pg/mL (Karsch et al., 1979; Husvéth, 2011). Progesterone concentrations begin to increase from less than 0.4 ng/mL up to 1.6 ng/mL during this period due to the formation of the CL that is created by the action of LH on the ruptured follicle (Stabenfeldt et al., 1969; Thorburn et al., 1969; Hauger et al., 1977; Pant et al., 1977; Husvéth, 2011).

Diestrus is the stage in which progesterone concentrations peak between 1.5 ng/mL and 3.0 ng/mL (Bindon et al., 1979; Quirke et al., 1979; Bartlewski et al., 2011). In the non-pregnant female, diestrus concludes with luteolysis of the CL over 2 to 3 days in sheep (Bartlewski et al., 2011). The CL undergoes luteolysis by secretion of prostaglandin (PG) $F_{2\alpha}$ from the uterus induced by increased concentrations of follicular estrogen (McCracken et al., 1972). If a

pregnancy is not established, there is marked decline in progesterone to less than 0.1 ng/mL (Figure 1; Stabenfeldt et al., 1969; Thorburn et al., 1969; Hauger et al., 1977; Pant et al., 1977; Husvéth; Bartlewski et al., 2011). In the pregnant animal, the CL persists to maintain pregnancy until at least day 60 when the placenta produces adequate progesterone to maintain pregnancy (Al-Gubory et al., 1999; Bartlewski et al., 2011).

Effects and Potential Mechanisms of Poor Maternal Nutrition on Reproduction in Offspring: Rats and Mice

A reduced number of ovarian follicles and CL have been observed in the offspring born to underfed mothers (Rae et al., 2002; Kotsampasi et al., 2009; Khorram et al., 2015; Chan et al., 2018). However, the majority of the research has used rats, not livestock species. Unlike sheep, rats and mice have a relatively short estrous cycle of 4 to 5 days (Hickman et al., 2016). Mice and rats also have a shorter gestation period (19 to 21 days for mice and 21 to 23 days for rats) and a greater number of fetuses (9 to 12 per litter; Hickman et al., 2016).

Chan et al. (2018) investigated the effects from maternal undernutrition in rats on offspring reproductive phenotype on days 4 (neonatal), 27 (pre-pubertal), and 65 (young adult) postnatally. Two groups, each consisting of 25 rats, were fed either an ad libitum control diet throughout pregnancy and lactation or a calorie-restricted diet (50% of the control diet) during the gestation period with an ad libitum control diet during lactation. At days 4 and 27, the number of primordial follicles was not found to differ between maternal treatments. However, the young adult offspring from underfed mothers had a significant loss of growing follicles (decreased tertiary follicles and increased atretic [apoptotic] follicles), a slight decrease in overall follicles and primordial follicles, and fewer CL compared with the controls (Figure 2). At day 4

after birth, rats born to nutrient restricted mothers had reduced AMH receptor type 2 (AMHR2) protein expression (0.09 vs. 0.11, in restricted vs control, respectively) in their ovaries, as well as increased growth and signaling factors involved in primordial follicle recruitment. This included a 200% increase in insulin-like growth factor 1 (*Igf-1*), and an increase in pAKT (0.27 vs 0.22, in restricted vs control, respectively) and pFOXO3 mRNA (0.10 vs 0.06, in restricted vs control, respectively). Reduced AMH concentrations have been associated with increased primordial follicle activation and increased expression of follicular growth factors. Young adult offspring born to nutrient restricted mothers also showed a decrease in AMH concentrations (5 ng/mL) vs controls (9 ng/mL). This corresponds with an increase in tertiary follicles from overactivation of primordial follicles, resulting in an early depletion of the primordial follicle supply in the offspring. Early follicular loss can result in premature reproductive decline. Increased follicular apoptosis in the young adult offspring born to nutrient restricted mothers was associated with an increase in the protein expression of caspase 3 (*Casp3*), a proapoptotic factor, in secondary follicles that causes follicular atresia (12% vs 5%, in restricted vs control, respectively). Reduced expression of *Igf-1* (50% decrease relative to the control) and follicle-stimulating hormone receptor (*Fshr*; 25% decrease relative to the control) were also observed in these offspring associated with an impaired response to FSH in tertiary follicles. The lack of receptivity to FSH impedes the selection of the dominant follicle, thereby causing it to undergo follicular atresia. This increases the number of atretic follicles and decreases the number of CL. Overall, resulting in a loss of reproductive function.

Khorram et al. (2015) investigated the effects of maternal undernutrition using two groups of female rats, a control group and a group fed 50% of the control diet from day 10 until the end of gestation. At ten months of age, the majority of the offspring from underfed mothers

had fewer CL (3 per section) and small follicles (15 per section) compared with the control group at the same age (8 and 20, respectively). Large cystic structures were indicative of anovulation, consistent with the results from Chan et al. (2018). Results of the study indicated that the reproductive ability of the offspring and their premature reproductive senescence was due to programming effects on the hypothalamic-pituitary-ovarian axis from restricted maternal nutrition. However, unlike Chan et al. (2018), no reduction in AMH concentrations were observed. The changes in ovarian status were found to be associated with a 50% increase in ovarian expression of leptin receptor (ObRb) and a 40% decrease in expression of estrogen receptor-α (ER-α) at birth compared with the control. The reverse pattern of the receptor expression reported at birth was observed in 10-month-old offspring born to underfed mothers. The 10-month-old offspring were also found to have increased concentrations of testosterone (0.57 ± 0.08 ng/mL) compared with the control group (0.3 ± 0.07 ng/mL). Decreased estrogen concentrations that are essential to folliculogenesis or an excess amount of leptin can desensitize the follicles to the actions of LH and FSH. Increased testosterone concentrations can also cause follicular apoptosis, which could be the mechanism of increased follicular atresia and reduced folliculogenesis in young adult rats born to underfed mothers.

Changes in concentrations of reproductive hormones have also been observed across studies in sheep and rats with nutrient restricted mothers, contributing to a loss of reproductive function in the offspring (Kotsampasi et al., 2009; Khorram et al., 2015). Khorram et al. (2015) reported that estrogen concentrations were reduced in newborn rats and decreased in 10-month-old rats born to underfed mothers (19.9 ± 4.9 pg/mL) compared with controls (58.3 ± 8.9 pg/mL). However, FSH and LH concentrations were increased at both ages. Follicle-stimulating hormone and LH concentrations in 10-month-old offspring were 7.8 ± 0.9 ng/mL and 0.47 ±

0.08 ng/mL in the group born to underfed mothers, compared with 4.6 ± 0.4 ng/mL and 0.23 ± 0.02 ng/mL in the control group respectively. Increased LH concentrations were consistent with increased testosterone concentrations reported in the offspring born to underfed mothers. However, in newborn offspring, the mechanism of increased LH and FSH concentrations was determined to be independent of GnRH secretion, as hypothalamic protein expression of GnRH at birth was reduced by 50% in the offspring born to underfed mothers compared with controls. Reduced estrogen concentrations were attributed to either the effect of increased leptin concentrations of estrogen synthesis in the ovaries or reduced expression of aromatase (CYP19A1) enzyme.

Another study using rats demonstrated alterations in onset of puberty in offspring as a result of restricted maternal nutrition during lactation rather than gestation (Gúzman et al., 2006). There was no significant difference in the onset of puberty in offspring born to mothers that were underfed solely during the gestation period (Kotsampasi et al., 2009; Gúzman et al., 2006). However, alterations in estrous cyclicity in post-pubertal offspring has been found as a result of restricted maternal nutrition during gestation (Rae et al., 2002; Chan et al., 2018). Chan et al. (2018) reported in rats that 65-day-old female offspring born to dams that were underfed for the duration of gestation were more likely to display irregular estrous cycles compared with the control group. Of the 25 rats born to underfed mothers, 64% exhibited irregular estrous cycles compared with the control group (n = 23) of which 39% displayed irregular estrous cycles (Chan et al., 2018). In the offspring born to underfed mothers, the most common irregularity was prolonged estrus (over 3 consecutive days in estrus) that was displayed by 48% of the total group due to early ovarian aging, follicle loss, and anovulation (Chan et al., 2018).

Merhi et al. (2020) fed female mice increased amounts of advanced glycation end products [diet containing 605 μg/g of N(ε)-(carboxymethyl] lysine compared with 62 μg/g in the control) throughout mating, pregnancy, and lactation and reported effects on the ovaries of the offspring at 16 weeks of age. Young adult female offspring born to mothers fed a greater amount of advanced glycation end products (n = 13) had fewer CL than the control (2 to 3 per section vs 5 to 6 per section, respectively; n = 10). In these offspring, arrested folliculogenesis and fewer ovulatory events with most of the follicles in the secondary stage were also observed compared with controls (5 to 6 vs 3 to 4 secondary follicles per section, respectively). The offspring born to mothers fed increased amounts of advanced glycation end products also exhibited reduced ovarian transcription of *Amh* and *Amhr2* genes involved in folliculogenesis compared with the control group (Figure 3). These findings are similar to those observed in offspring from nutrient restricted mothers observed in Chan et al. (2018) in rats and in Da Silva et al. (2003) in sheep fetuses from overfed mothers. This indicates that decreased AMH concentrations cause a depletion in primordial follicles. However, unlike Chan et al. (2018), the follicular expression of *Fshr* was not found to be reduced, indicating that it may not have a role in the inhibition of folliculogenesis (Merhi et al., 2020).

Merhi et al. (2020) also observed that 16-week-old-mice fed increased amounts of advanced glycation end products had decreased transcription of the ovarian *Cyp19a1* gene for the aromatase enzyme involved in steroidogenesis. This could have a role in the reduction of estrogen concentrations and impact normal vaginal opening and regular estrous cyclicity. Reduction in the *Cyp19a1* gene was also observed in offspring born to nutrient restricted mothers in 10-month-old rats in association with decreased estrogen concentrations (Khorram et al., 2015). There has been no similar genomic study conducted in sheep.

Additionally, Merhi et al. (2020) which found that 66.6% of mice born to mothers fed increased amounts of advanced glycation end products reached puberty by day 7 postnatally. This can be compared with 100% of the control group that reached puberty by day 7, indicative of delayed pubertal onset in the experimental group. Connor et al. (2012), used rats (n = 46), the median age of pubertal onset in offspring born to mothers that were fed high fat diets during gestation and lactation was 31.4 days, whereas offspring born to dams fed high fat diets only during gestation was 32.7 days, and 34.6 days in the control group. These effects were attributed to accelerated maturation due to increased obesity in offspring born to mothers with high fat diets during gestation and lactation. While the results of Connor et al. (2012) were associated with increased adiposity in rat offspring born to mothers with high fat diets during gestation and lactation, the body weight gain that occurred in the offspring was delayed and, in turn, could have delayed pubertal onset. The activation of GnRH neurons needed to initiate puberty could be altered by reduced leptin concentrations in offspring born to dams fed increased advanced glycation end products. This affects the onset of puberty in the offspring. However, the exact mechanism by which maternal nutrition alters pubertal onset in terms of the hypothalamic-pituitary axis in offspring remains largely unknown. Further studies should measure body composition to determine if body fat development becomes delayed resulting in reduced leptin concentrations and, thus, inhibition of pubertal GnRH neurons (Merhi et al., 2020).

Connor et al. (2012) also found that 105-day-old rats born to mothers fed high fat diets during gestation were more likely to have irregular estrous cycles with prolonged periods of 2 or more days of estrus compared with the control group. Thirty percent of the offspring born to mothers with high fat diets during gestation exhibited irregular estrous cycles and over 20% exhibited persistent estrus. Over 20% of offspring born to mothers fed high fat diets during

gestation and lactation displayed irregular estrous cycles, and almost 60% were in persistent estrus compared with less than 20% of control offspring that exhibited irregular estrous cycles and 10% in persistent estrus. Prolonged estrus is a marker of early reproductive and ovarian aging in rats because it is indicative of anovulation and is likely to have a negative impact on fertility. The results of this study are similar to those of Chan et al. (2018), as irregular estrous cycles and persistent estrus were indicative of early ovarian aging observed in 65-day-old rats born to mothers that were underfed during pregnancy and lactation. Merhi et al. (2020) observed that mice born to mothers fed increased advanced glycation end products were more likely to display irregular estrous cycles and were found to spend less time in the proestrus phase (less than 10% of the total cycle) and an increased amount of time in the metestrus phase (45% of the total cycle). In contrast, the control group spent almost 20% of the total time in proestrus and 35% of the total time in metestrus. The findings could be due to reduced concentrations of estradiol required for regular estrous cyclicity and vaginal opening.

Sheep

In sheep, maternal undernutrition during the period of ovarian development in the fetus can lead to a delay in follicular development (Rae et al., 2001). Rae et al. (2001) used sheep and fed groups of 11 to 19 ewes either 100% or 50% of the metabolizable energy (ME) requirement for maintenance from mating to day 50 of gestation, or 50% or 100% of ME from mating to day 30 then 100% or 50% of ME, respectively, until day 50 of gestation. Fetal ovaries were collected and analyzed. At day 110 of gestation, fetal ovaries from mothers that experienced undernutrition for a period during gestation had a reduced number of follicles that had progressed beyond the primordial stage, particularly the tertiary stage (less than 0.2 follicles per

mm^2) compared with controls (0.9 follicles per mm^2), suggesting that folliculogenesis had been inhibited, consistent with the results in young adult rats born to underfed mothers described above (Chan et al., 2018). However, the stage at which they are inhibited differs. At this stage of growth in the fetus, the mechanism is likely independent of the hypothalamic-pituitary axis and mediated instead through germ cells, as no receptors for LH or FSH were detected in fetal ovaries (Rae et al., 2001; Chan et al., 2018). Delayed germ cell maturation was observed in the ovaries of fetuses born to mothers that were underfed at some point during gestation (Rae et al., 2001; Chan et al., 2018).

Another study by Kotsampasi et al. (2009), consisted of three groups of 7 to 8 ewes, a control group fed 100% of the required ME and crude protein, a group fed 50% of the control group diet for the first 30 days of pregnancy, and a group fed 50% of the control for days 31 to 100 of pregnancy. The ovaries of the female offspring were removed and analyzed at 10 months of age. Nutrient restriction for the first 30 days of gestation resulted in offspring with an increased number of small ovarian follicles (2 to 3 mm diameter) relative to the control group; 2.67 follicles vs 2.0 follicles, respectively. These results indicate a delayed follicular development (Rae et al., 2001). However, the number of total follicles did not differ between groups. In the offspring whose mothers were underfed from days 31 to 100 of pregnancy, there was a reduction in the size of the CL (8 to 11 mm average diameter) compared with controls (greater than 12 mm diameter in the control group). This could be associated with permanent infertility if progesterone is not secreted adequately from the CL. The mechanisms for the increase in small follicles and reduction in size of CL were not determined (Kotsampasi et al., 2009). The time and duration of nutrient restriction during gestation appear to be important for the negative impact of the theory.

In contrast with Khorram et al. (2015), Kotsampasi et al. (2009) found that 10-month-old ewes born to dams with a period of undernutrition during gestation had no effect on basal LH and FSH concentrations. However, a GnRH challenge was conducted in ewes born to underfed dams in the first 30 days of pregnancy. The results indicated increased anterior pituitary sensitivity to FSH, which increased the FSH response, and was correlated with a greater number of small follicles and a reduction in mature tertiary follicles.

In more mature ewes, Long et al. (2013) fed 4 sheep a 50% nutrient restricted diet from days 28 to 78 of gestation and reported that progesterone concentrations during the estrous cycles of the offspring at one, two, and six years old were less than the concentrations measured in the control group (n = 4; total progesterone content of luteal tissue of 7.5 μg compared with 10 μg in the treated and control group, respectively). The study also reported a reduction in mRNA expression compared with controls for the steroidogenic enzymes, *stAR* (60% reduction) and *p450scc* (80% reduction), involved in the synthesis of progesterone. This is suggestive of a permanent effect from fetal programming that impairs luteal progesterone secretion (Long et al., 2013).

Kotsampasi et al. (2009) reported that neither maternal undernutrition during the first 30 days or from days 31 to 100 of gestation in sheep affected the onset of puberty in young adult offspring. Additionally, a study using 20-month-old ewes born to dams that were fed 50% of the estimated ME required for the first 95 days of gestation had a decreased mean ovulation rate compared with the control group (1.17 and 1.46, respectively; Rae et al., 2002). Since no changes in basal LH or FSH were observed in response to a GnRH challenge in the study, it was concluded that decreased ovulation rate could be due to gonadotropin-independent mechanisms

in ovarian development potentially mediated through germ cells or the oocyte (Rae et al., 2001; Rae et al., 2002).

Da Silva et al. (2003) showed a reduction in the number of primordial follicles in sheep fetuses (n = 6) at day 103 of gestation born to mothers that were overfed (ad libitum diet) compared with controls; 13 vs 23 follicles per cross-section of ovarian tissue, respectively. Fewer total follicles, per cross-section of ovarian tissue (16 vs 35), were observed in female sheep fetuses from overfed dams compared with fetuses from moderately fed dams. The effect was found to be independent of gonadotropin secretion at this fetal state, since measured LH and FSH concentrations were not found to differ between the overfed and moderately fed groups. Initiation of follicular growth occurs through unknown GnRH-independent mechanisms, but gonadotropins are required beyond the primary follicle stage when there are receptors for FSH present on the follicles. These findings contrast with the results from fetuses from underfed mothers at day 110 of gestation compared with the controls in the study. In fetuses from underfed mothers, there was a reduced number of follicles that progressed beyond the primordial stage, but not an overall decrease in follicle number. However, these effects were also attributed to undetermined GnRH-independent mechanisms potentially linked to interaction of growth factors including stem cell factor (Rae et al., 2001).

Da Silva et al. (2003) also found that sheep fetuses from overfed mothers collected at day 103 of gestation had reduced concentrations of progesterone compared with fetuses from mothers fed the control diet (2.3 ± 0.19 ng/mL vs 3.4 ± 0.23 ng/mL, respectively). The reduced concentrations of progesterone were associated with increased mRNA expression of LH beta subunit (LHβ) in female fetuses (331 ± 22.6 nCi/g vs 213 ± 42 nCi/g in the control group). This could indicate an inhibition in the normal negative feedback of progesterone on LH secretion.

There were no observed differences in the concentrations of LH and FSH between nutritional groups in the study. However, the effects of maternal nutrition on LH and FSH were not conclusive and need investigation further during the lifespan of the offspring. Luteinizing hormone and FSH concentrations were measured solely from fetuses at day 103 of gestation at the time when the majority of ovarian development occurs independently of gonadotropin secretion.

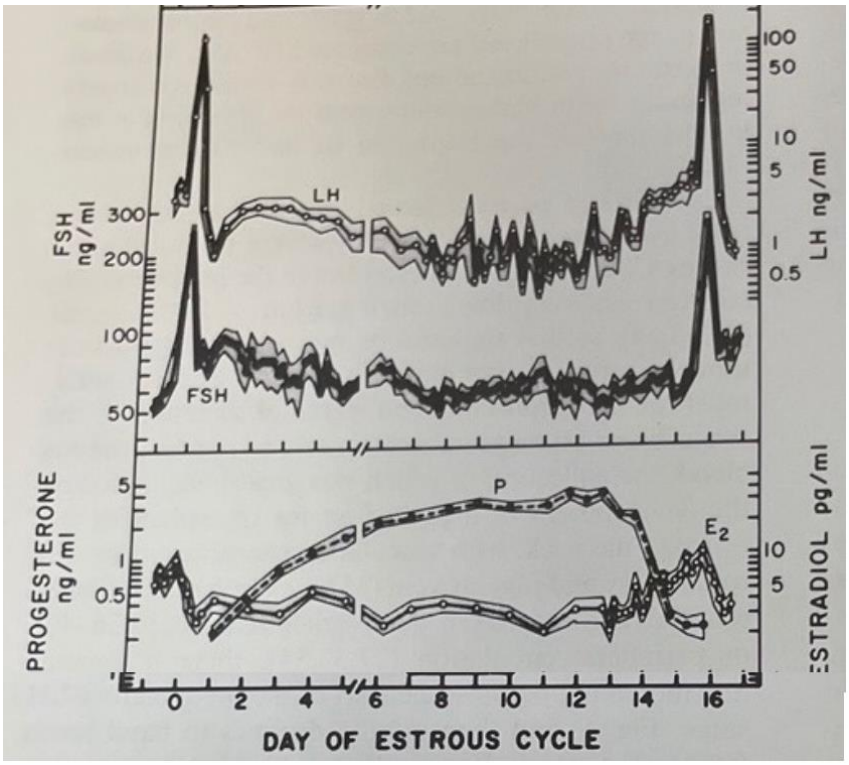
Conclusion

Results from research in sheep and rats show that poor maternal nutrition, either under- or over-nutrition, can lead to adverse fetal programming effects on the hypothalamic-pituitary-ovarian-axis of female offspring. Maternal overnutrition and undernutrition affects the hypothalamic-pituitary-ovarian axis of the offspring in manners dependent on the duration, type, and severity of the nutritional treatment as well as the point of gestation in which it is imposed. These factors determine the component of fetal development that can be affected (Kotsampasi et al., 2009). Further studies may determine the long-term impacts on fertility, reproductive ability, and health of the offspring, as well as the exact mechanisms of fetal programming on the hypothalamic-pituitary-ovarian axis in sheep. The mechanisms for the alterations in hormone concentrations and altered ovarian function, folliculogenesis, and onset of puberty are not well understood, especially in sheep. Long-term effects of this ovarian dysfunction throughout the reproductive lifetime or throughout multiple generations are not well-researched. These effects include the ability of offspring born to mothers that experienced poor maternal nutrition at some point during gestation to conceive, maintain a pregnancy, and produce healthy and viable offspring. Management methods can be identified to mitigate the effects of poor maternal nutrition and

ensure maximum reproductive capability of ewes (Govoni et al., 2019). Additional studies are needed for long-term and multigenerational effects of poor maternal nutrition on reproductive efficiency of the offspring for purposes of production efficiency and alleviation of operational costs (Govoni et al., 2019). While research in this area has important implications in livestock production, it also is significant in biomedical research into human conditions such as polycystic ovarian syndrome, ovarian dysfunction, early menopause, and poor fertility (Connor et al., 2012; Chan et al., 2018).

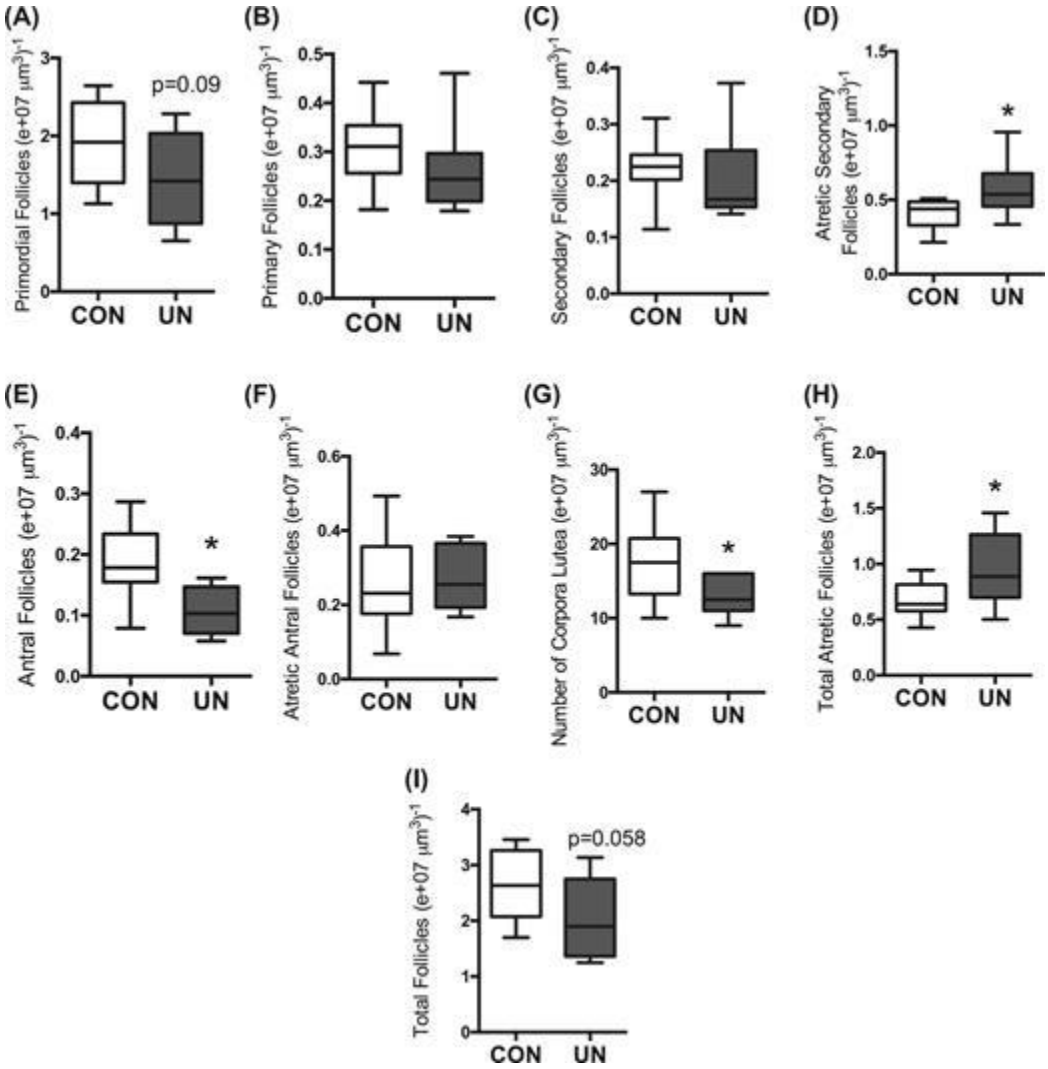
There is little data available describing the effects of poor maternal nutrition on the concentrations of reproductive hormones in offspring using an ovine model. The objective of this experiment was to evaluate the effects of restricted and over-feeding dams on the reproductive efficiency of the offspring. Specifically, concentrations of LH, FSH, and progesterone were evaluated and compared amongst three dietary treatments, over-fed, restricted-fed, and control-fed. We hypothesized that yearling ewes born to dams that experienced either over-feeding or restricted-feeding during gestation would have decreased concentrations of LH and FSH. This means it would take more time to become pregnant and potentially there would be a greater chance of fetal loss.

Figure 1:



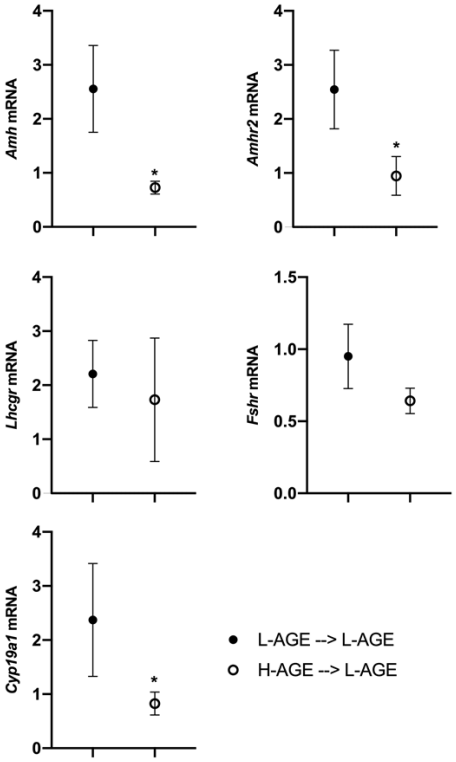
The figure shows the concentrations and patterns in the secretion of FSH, LH, progesterone, and estradiol at each day of the estrous cycle in ewes (Goodman et al., 1981).

Figure 2:



The figure shows the range and mean number of follicle type or CL per total ovarian volume of 65-day-old rats born to underfed mothers (UN) compared with the control group (CON). The number of primordial follicles, antral (tertiary) follicles, and CLs were reduced in the rats born to underfed mothers relative to the control group born to mothers fed ad libitum during gestation. The number of atretic follicles was increased in the group born to underfed mothers compared with the control group indicative of follicular loss (Chan et al., 2018)

Figure 3:



The figure demonstrates the amount of transcription in arbitrary units of genes involved in folliculogenesis and steroidogenesis of mice offspring born to mothers fed high amounts of advanced glycation end products during gestation (H-AGE → L-AGE) and the control fed low amounts of advanced glycation end products (L-AGE → L-AGE). Transcription of *Amh*, *Amhr2* and *Cyp19a1* genes was decreased in offspring born to dams fed increased amounts of advanced glycation end products. Expression of *Fshr* was not found to be significantly reduced (Merhi et al., 2020).

Materials and Methods

Animals and Sample Collection

All procedures done were reviewed and approved by the University of Connecticut's Institutional Animal Care and Use Committee (protocol number: A19-018).

Dams of the F1 Dorset ewes (F0 generation; $n = 48$) used in the study were fed one of three different diets based on National Research Council (NRC) requirements for total digestible nutrients. One group was fed the control diet that met all energy requirements (CON). A second group was overfed (OVER) with a diet consisting of 140% of requirements and the third group was restricted (RES) with a diet consisting of only 60% of the requirements. Ewe lambs were given *ad libitum* access to creep feed and second cut hay until 8 months of age. At 8 months, ewe lambs were fed to meet the NRC requirements for replacement ewes.

Thirty-eight multiparous yearling Dorset ewes (F1 generation) were estrous synchronized using the protocol previously outlined in the literature (12 ewes born to dams in the RES group, 16 OVER, 10 CON; Jones et al., 2016). Briefly, a controlled intravaginal drug releasing device (CIDR) was placed through vaginal insertion and released progesterone over a 12-day period. After this period, CIDRs were removed and ewes were given an intramuscular injection of $PGF_{2\alpha}$ to stimulate luteolysis. After synchronization, ewes were divided evenly and placed with one of two related rams fitted with a crayon. Ewes were checked twice daily to identify marks indicative of breeding (day 0 of gestation) and dates were recorded. Pregnancy and number of fetuses was confirmed by transabdominal ultrasound between days 30 and 40 of gestation (Jones et al., 2016). Ewes with singletons or triplets were rebred to ensure pregnancy with twins. During gestation, ewes were fed diets that meet 100% of the NRC requirements for gestation.

Blood samples (10 mL) were collected by jugular venipuncture and put in serum separator tubes every 7 days from the beginning of estrous cyclicity until day 24 of gestation. Blood samples were initially kept at room temperature for 2 hours before being stored overnight at 4 °C. Samples were then centrifuged for 30 minutes (at 1,200 x g) and sera were harvested. Serum samples taken from two weeks before pregnancy up to 7 days past the date of breeding were utilized for each ewe for enzyme-linked immunoassay (ELISA). Sera were stored at -20 °C until analysis.

Follicle- Stimulating Hormone (FSH) ELISA

Concentrations of FSH present in serum samples for each time point for each individual ewe were determined according to the manufacturer's instructions (Ovine Follicle-Stimulating Hormone, FSH ELISA, Cloud-Clone Corp, Houston, TX). All reagents, kit components, and serum samples were brought to room temperature (18 to 25 °C) before use. Serum samples were diluted 1:2 with 0.01 mol/L phosphate buffered saline (PBS). A triple dilution series was performed using the standard stock solution and standard dilution to create six standards and a blank. Fifty microliters of standards, blank, and samples were added to designated wells in duplicate. The plate was shaken with a microplate shaker and incubated for one hour at 37°C. An autowasher was used to aspirate the solution and wash with 350 µl of 1X wash solution three times. After blotting the plate against absorbent paper, 100 µl of Detection Reagent B was added to each well and the plate was incubated for 30 minutes at 37°C. The wash process was then repeated 5 more times as outlined. Ninety microliters of Substrate Solution were added to each well and the plate was incubated and kept from light for 10 minutes at 37°C. Finally, 50 µl of Stop Solution was added to each well and it was immediately placed in the microplate reader set to 450 nm.

Luteinizing hormone (LH) ELISA

The same process as outlined above for FSH was repeated using the LH ELISA according to the manufacturer's instructions (Ovine Luteinizing Hormone, LH ELISA, Cloud-Clone Corp, Houston, TX). However, the serum samples were not diluted with PBS and concentrations of the standards differed from those used in the FSH or PG ELISA.

Progesterone (P4) ELISA

The same process as outlined above for P4 was repeated using the PG ELISA according to the manufacturer's instructions (Pan-Species Progesterone, PG ELISA, Cloud-Clone Corp, Houston, TX). However, the serum samples were not diluted with PBS and concentrations of the standards differed from those used in the FSH or LH ELISA.

Pregnancy Data

Time to pregnancy was calculated by determining the number of days between when the ewe was placed in a pen with a ram and the date that they received a mark (after which pregnancy was confirmed by ultrasound). The mean was calculated utilizing the values determined for each ewe (RES: n = 12, CON: n = 10, OVER: n = 16). Number of fetuses was determined by ultrasound between days 30 to 40 of gestation. This was determined from the initial pregnancy as estrumate was given to ewes that were not carrying twins so that they could be rebred. Fetus numbers that were determined to be uncertain by ultrasound were omitted. The mean was calculated based on the number of fetuses per each ewe (RES: n = 9, CON: n = 7, OVER: n = 14). The number of lambs was counted after parturition. The mean was determined using the number of lambs counted for each ewe (Number of Ewes utilized: RES: n = 11, CON: n = 9, OVER: n = 13).

Statistical analysis

Data were analyzed using RStudio (Rstudio, Boston, MA). Mean and standard error were calculated using the “emmeans” package and values were analyzed as a factorial repeated measures ANOVA as a linear mixed model using “LME4” package and “NLME” package. Significance was accepted at $P < 0.05$ and $P < 0.10$ was considered a tendency.

Results

Pregnancy Data

There were no observed effects of poor maternal nutrition on the amount of time it took for ewes to get pregnant ($P = 0.16$). Treatment group did not significantly affect the number of fetuses per ewe observed on ultrasound ($P = 0.94$). There was no observed effect of treatment on the number of lambs born per ewe ($P = 0.13$).

FSH ELISA Analysis

There were no observed effects of poor maternal nutrition on the concentrations of FSH in the ewes utilized in the study at the different time points ($P = 0.44$). Treatment group did not significantly affect concentrations of FSH ($P = 0.82$). In addition, time did not significantly affect the concentration of FSH ($P = 0.83$).

LH ELISA Analysis

There were no observed effects of nutritional treatment on the concentrations of LH in the ewes utilized in the study at the different time points ($P = 0.87$). Treatment group did not significantly affect concentrations of LH ($P = 0.18$). Time also did not significantly affect the concentration of LH ($P = 0.43$).

Progesterone ELISA Analysis

There were no significant effects of time on the concentrations of progesterone ($P = 0.47$). In addition, there were no significant effects of treatment x week on the progesterone concentrations ($P = 0.76$). However, there was a tendency for treatment group to affect

progesterone concentration ($P = 0.08$). Specifically, there was a tendency for concentrations of progesterone to be greater in the RES group compared with the CON ($P = 0.09$). The average progesterone concentration in the RES group was 1.09 ng/mL compared with 0.83 ng/mL for the CON group.

Table 1: Pregnancy Data

Item	Treatment[1]				
	RES	**CON**	**OVER**	**SEM[2]**	***P*-value**
Time to Pregnancy, Days	5.25	4.20	2.125	1.75	0.16
Number of Fetuses per Ewe	1.78	1.71	1.71	0.18	0.94
Number of Lambs Born per Ewe	1.82	2.00	2.00	0.12	0.13

Treatment group did not significantly alter time to pregnancy, number of fetuses per ewe, or number of lambs born per ewe ($P > 0.05$).

[1]Ewes from control-fed (CON; 100% TDN requirements from the NRC), restricted-fed (RES; 60% of TDN requirements from the NRC), and over-fed (OVER; 140% TDN requirements from the NRC).

[2]Largest standard error of the mean across treatments for each variable

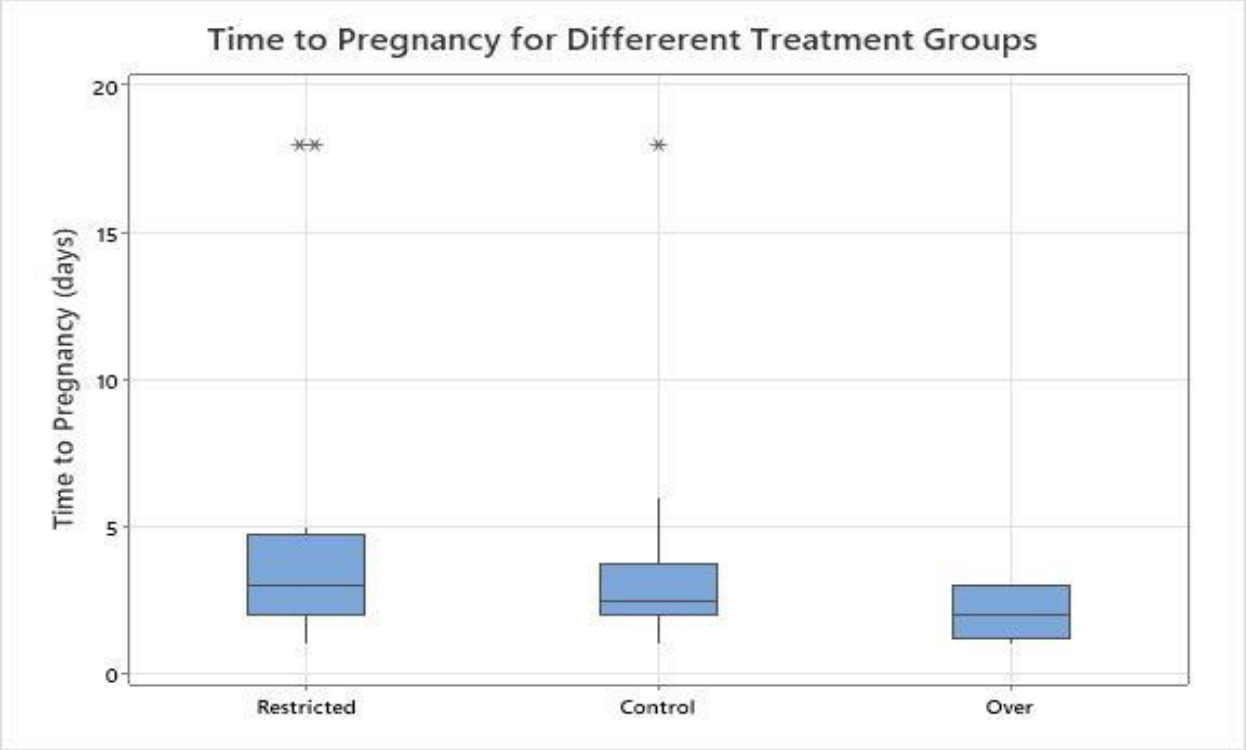


Figure 1: Time to Pregnancy for different treatment groups. There was no difference across the three treatment groups in regards to the amount of time it took to become pregnant (P > 0.05). However, there were significant outliers in the RES and CON groups that could have skewed the data.

Table 2: Effects of maternal diet and week of pregnancy on FSH concentration.

	Treatment[1]						
Item	**RES**	**CON**	**OVER**	**SEM[2]**	**Contrast *P*-values**		
FSH Concentration, ng/mL					Week	Trt[3]	Trt x Week
Week -2	0.82	0.81	0.82	0.26	0.83	0.82	0.44
Week -1	0.75	0.88	1.25	0.19			
Week 0	0.71	1.07	0.81	0.40			
Week 1	0.97	0.70	0.76	0.23			

There were no significant effects or tendencies of treatment or time on FSH concentrations (*P* > 0.10).

[1]Ewes from control-fed (CON; 100% TDN requirements from the NRC), restricted-fed (RES; 60% of TDN requirements from the NRC), and over-fed (OVER; 140% TDN requirements from the NRC).

[2]Largest standard error of the mean across treatments for each variable

[3]Treatment

Table 3: Effects of maternal diet and week of pregnancy on LH concentration.

Item	Treatment[1] RES	CON	OVER	SEM[2]	Contrast *P*-values Week	Trt[3]	Trt x Week
LH Concentration, ng/mL							
Week -2	2.99	2.93	3.12	0.21	0.43	0.18	0.87
Week -1	3.19	2.89	3.27	0.18			
Week 0	3.22	2.89	3.18	0.14			
Week 1	3.21	3.06	3.18	0.19			

There were no significant effects of treatment or time on LH concentrations ($P > 0.10$).

[1]Ewes from control-fed (CON; 100% TDN requirements from the NRC), restricted-fed (RES; 60% of TDN requirements from the NRC), and over-fed (OVER; 140% TDN requirements from the NRC).

[2]Largest standard error of the mean across treatments for each variable

[3]Treatment

Table 4: Effects of maternal diet and week of pregnancy on progesterone concentration.

Item	Treatment[1] RES	CON	OVER	SEM[2]	Contrast *P*-values Week	Trt[3]	Trt x Week
Progesterone Concentration, ng/mL							
Week -2	1.06	0.85	1.12	0.08	0.47	0.08*	0.76
Week -1	1.12	0.82	1.11	0.12			
Week 0	1.08	0.83	1.08	0.08			
Week 1	1.07	0.82	1.03	0.10			

There were no significant effects of time on concentration of progesterone.

*There was a tendency for the concentrations to be greater in the RES group compared with the CON ($P = 0.08$).

[1]Ewes from control-fed (CON; 100% TDN requirements from the NRC), restricted-fed (RES; 60% of TDN requirements from the NRC), and over-fed (OVER; 140% TDN requirements from the NRC).

[2]Largest standard error of the mean across treatments for each variable
[3]Treatment

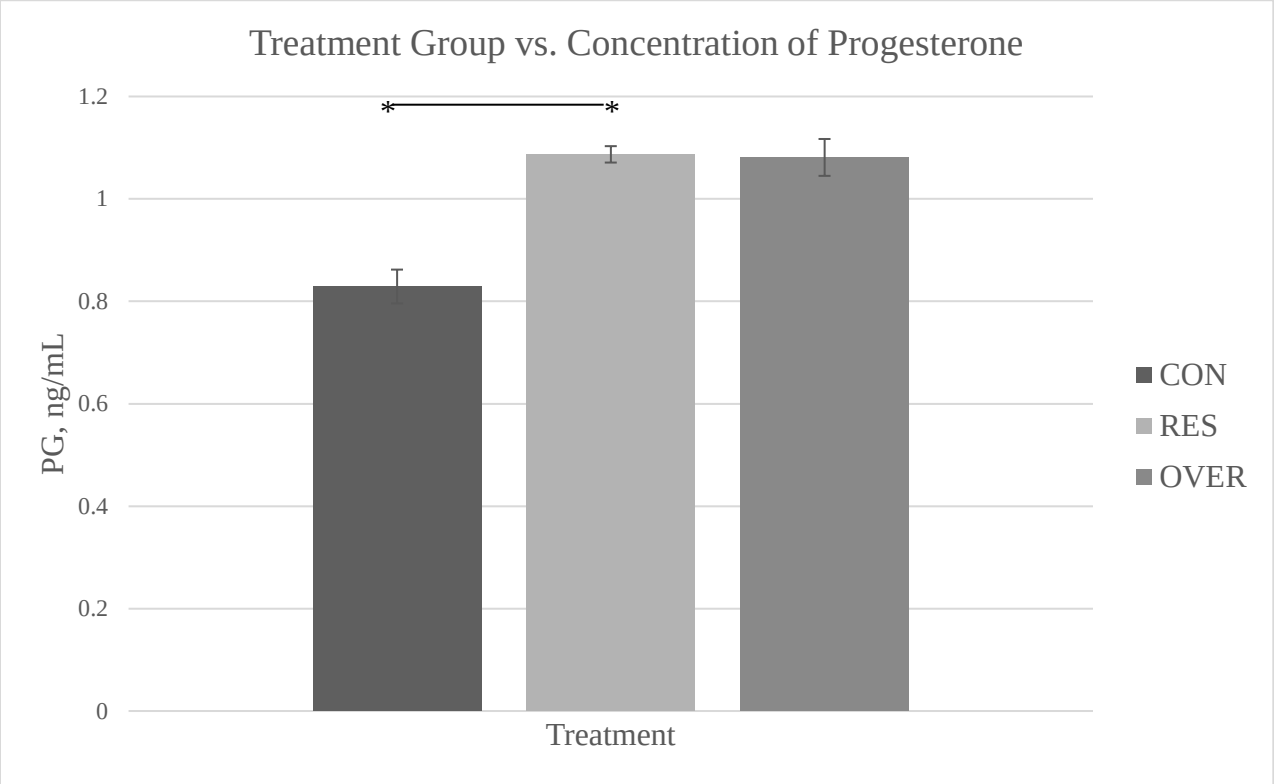


Figure 2: Treatment group vs. concentration of progesterone. *There was a tendency for the concentration of progesterone to be greater in the RES group compared with the CON (P = 0.09).

Discussion

Poor maternal nutrition studies have found that restricted feeding and overfeeding during gestation can cause permanent fetal programming alterations that predispose offspring to certain chronic diseases (Kwon and Kim, 2017; Govoni et al., 2019; Martin et al., 2019). However, the effects of poor maternal nutrition on reproductive efficiency of the offspring are not well-understood. The objective of this study was to analyze the effects of poor maternal nutrition on concentrations of reproductive hormones in the offspring. Blood samples collected from yearling ewes from the beginning of estrous synchronization until one week past the date of conception were analyzed for concentrations of FSH, LH, and progesterone to evaluate differences among those born to over-fed, restricted-fed, and control-fed dams. We found that there were no significant differences in hormone concentrations between the RES, CON, and OVER groups. Treatment group did not significantly affect the amount of time it took for ewes to conceive. However, the results may have been skewed by outliers in the RES and CON groups and should be confirmed by a study with a greater sample size. Treatment group also did not significantly affect the number of fetuses and lambs born per ewe. Overall, the results indicate that treatment did not affect reproductive efficiency of these ewes.

The results indicated that the treatment group did not have a significant difference in concentrations of FSH. This is in agreement with Kotsampasi et al. (2009) that reported that there was no significant difference in basal FSH concentrations of 10-month-old ewes born to dams that experienced restricted maternal nutrition during gestation concentration and ewes born to dams that were adequately fed during gestation. Da Silva et al. (2003) also found that there were no significant differences in basal FSH concentrations in 103-day-old fetuses from ewes born to dams that experienced maternal over-nutrition compared with those from ewes born to

dams that were adequately fed during gestation. However, at 103 days of gestation, organ development occurs mainly independently on gonadotropin secretion. Therefore, this might not be applicable to yearling ewes. Due to the small sample size, further studies are needed to further understand the effects of poor maternal nutrition on the concentration of FSH in the female offspring.

The concentrations of LH were not found to significantly differ between treatment groups. However, Khorram et al. (2015) found that LH concentrations were increased in 10-month-old female rat offspring born to mothers that were underfed during gestation. The increased LH was correlated with greater concentrations of testosterone in these offspring. In accordance with our findings, Kotsampasi et al. (2009) found that 10-month-old ewes born to dams that experienced a period of undernutrition during gestation did not have concentrations of LH that were significantly different from the control group. Da Silva et al. (2003) also found that 103-day old fetuses from dams that experienced maternal overnutrition during gestation did not have significantly different concentrations of LH compared with the control group. However, as previously mentioned, these results may not necessarily apply to yearling ewes.

Time did not significantly affect concentration of progesterone. However, there was a tendency for the treatment group to affect it ($P = 0.08$). The tendency was for the concentration of progesterone in the RES group to be greater than that of the CON ($P = 0.09$). Long et al. (2013) found that ewes born to dams that experienced a period of restricted nutrition during gestation had lesser concentrations of progesterone during the estrous cycle than those born to dams that were adequately fed throughout gestation. Our data may differ from these findings as the period of undernutrition during gestation differed, small sample size, and different type of measurement of progesterone as they measured total luteal content of progesterone.

In addition, Da Silva et al. (2003) found that 103-day-old fetuses from dams that experienced a period of overnutrition during gestation had reduced concentrations of progesterone compared with the control group. However, these results may not necessarily apply to one-year-old ewes as were used in this study that have experienced puberty, and progesterone concentrations for sheep in this study were measured during the estrous cycle and the beginning of pregnancy.

Due to the small sample size of the study, further analysis is warranted to determine the effect of poor maternal nutrition on the concentrations of FSH, LH, and progesterone. In addition, hormone concentrations could be analyzed daily throughout the estrous cycle to assess potential differences between treatment groups to clearly observe the differences between time and concentration. There is previous evidence that poor maternal nutrition in sheep negatively impacts follicular development in female offspring (Rae et al., 2001; Da Silva et al., 2003; Kotsampasi et al., 2009). It would also be beneficial to analyze the effect of treatment on the reproductive capacity of offspring at various ages. Future studies could determine if poor maternal nutrition could lead to early reproductive senescence in ewes as it has in rats (Khorram et al., 2015).

Conclusion

Overall, poor maternal nutrition did not affect the reproductive capability of the sheep in this study. Nutritional group did not have a significant effect on concentration of FSH, LH, or progesterone. In addition, treatment did not have a significant effect on the time it took to become pregnant, number of fetuses, or number of lambs born to each ewe. The findings for concentration of FSH were in agreement with previous research (Da Silva et al., 2003; Kotsampasi et al., 2009). The observations for LH were in agreement with previous studies done in sheep (Da Silva et al., 2003; Kotsampasi et al., 2009). However, the results differed from a study that found that female rat offspring born to mothers that experienced underfeeding during gestation had greater concentrations of LH (Khorram et al., 2015). We found that treatment had no significant effect on progesterone concentration. However, these findings differ from those of Da Silva et al. (2013) that found concentrations of 103-day-old fetuses from underfed dams to have lesser concentration of progesterone compared with the controls. Future studies are needed to further analyze the long-term impacts of poor maternal nutrition on the reproductive efficiency of the offspring.

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