Supplementation of metabolizable protein during late gestation and fetal number impacts ewe organ mass, maternal serum hormone and metabolite concentrations, and conceptus measurements

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This project was supported by National Research Initiative Competitive Grant no. 2009-35206-05276 from the USDA Cooperative State Research, Education, and Extension Service. The authors would like to thank Mr. Jim Kirsch and Ms. Tammi Neville for their assistance in animal work and data collection.

Abstract

To examine the effects of maternal metabolizable protein (MP) supplementation during late gestation on serum hormone and metabolites and organ masses, multiparous ewes (n = 45) carrying singletons or twins were allotted randomly (within pregnancy group) to one of three treatments: 60% (MP60), 80% (MP80), or 100% (MP100) of MP requirements. Blood samples were drawn before initiation of diets (day 100) and prior to slaughter (day 130) for chemistry panel analysis, and weekly for hormone analysis including progesterone (P4) and estradiol-17 β (E2). At day 130 ewe organ masses were recorded. Despite being fed isocaloric diets, MP60 ewes gained less weight throughout pregnancy compared to MP80 and MP100 ewes which were similar. While diet did not impact E2 or P4 concentrations, ewes carrying twins had greater ($P <$

0.05) concentrations of both as gestation advanced. Albumin, aspartate aminotransferase, and total protein were reduced ($P < 0.05$) in MP60 compared to MP100 ewes near term. There was a diet by fetal number interaction ($P = 0.03$) for lactate dehydrogenase. Twin-carrying MP80 ewes had greater lactate dehydrogenase compared to all other groups on day 130 of gestation. Ewes that were fed MP80 had greater body weight on day 130 of gestation compared to MP60 ewes. Kidney and heart weights were lighter in MP60 ewes compared to MP80 ewes. There was a maternal diet by fetal number interaction ($P = 0.05$) on fetal weight per unit empty ewe body weight. In ewes carrying singletons, MP60 ewes supported less fetal weight compared to MP100. In contrast MP60 ewes supported more fetal mass compared to MP100 ewes when carrying twins. The level of protein, and not just total energy, in the diet appears to impact some aspects of the maternal system. Moreover, it appears some measurements of mobilizing maternal body resources are enhanced in ewes carrying twins.

Keywords: metabolizable protein, pregnancy, sheep

1. Introduction

Adequate protein intake is necessary for maternal maintenance, lactation performance, and rebreeding success [1-3]. In beef cows, Sasser et al. [4] reported that diets fed to dams during late gestation and into early lactation that were equal in energy but deficient in crude protein resulted in decreased pre- and post-partum weight gains, decreased first service conception rates, and increased interval from parturition to postpartum estrus compared to dams that had adequate protein. In sows that had a decreased protein content in their gestational diets, piglet weights were decreased and growth was reduced compared to sows receiving greater amounts of protein [5]. Inadequate protein supply in lactating sows has been shown to decrease certain maternal visceral organ weights, such as kidney, heart, and liver, while having less effect on carcass

measurements such as subcutaneous back fat [6]. However, we have recently reported that decreases in metabolizable protein intake during late gestation causes a reduction in body condition score, which is primarily determined by subcutaneous back fat [7]. The protein pool of the body is dynamic with protein turnover rates differing greatly between tissues [8]. Tissues with greater metabolic rate (e.g. liver, small intestine, pancreas, etc.) typically have greater protein turnover rates. Skeletal muscle provides the largest protein pool, and although it can be rapidly mobilized when needed, it typically exhibits decreased protein turnover compared to more metabolically active tissues. When animals are in negative energy and/or protein balance, muscle protein turnover is increased to provide energy and/or amino acids for vital functions [9, 10]. Whole body and tissue specific protein turnover rates increased during gestation and lactation as compared to non-pregnant and non-lactating women and ewes [11].

Recently, our laboratory has demonstrated that maternal dietary nutritional plane during gestation impacts the endocrine and metabolic profile of the ewe [12,13]. It was also noted that placental steroids, estradiol-17 β and progesterone, were increased in ewes experiencing a global nutrient restriction during pregnancy. Thyroxine, but not triiodothyrodine, concentrations were reduced in nutrient restricted ewes compared to adequately fed ewes during mid to late gestation [13]. It is not known what influence protein specifically has on those hormone concentrations in late pregnant ewes, let alone the impact that multiple fetuses may have on those hormones in a protein deficient diet.

In a recent study, we concluded that increased maternal MP intake may positively impact the ewe, without altering offspring performance from birth to weaning [7]. Our hypothesis was that isocaloric diets deficient in metabolizable protein (MP) would not hinder conceptus or mammary development as weaning weights were not affected in a sister study [7]. However, we

hypothesized that ewes deficient in MP would have increased markers of inflammation and experience more fat, organ mass, and skeletal muscle loss (i.e. mobilize their bodily resources) to ensure that their fetuses would have adequate nutrients while in utero. Moreover, hormones associated with maternal metabolism would be decreased with protein deficient diets. Therefore, the objective of this study was to examine the effects of maternal MP supply on ewe weight and condition, visceral organ weights, serum chemistry panel values, hormone concentrations, and fetal and placental weights.

2. Materials and methods

Animal care and use was approved by the Institutional Animal Care and Use Committee (#A0921) at North Dakota State University (NDSU), Fargo.

2.1. Animals and diets

On approximately day 90 of gestation, 45 pregnant multiparous Rambouillet ewes were transported from the Hettinger Research Extension Center (Hettinger, ND, USA) to the Animal Nutrition and Physiology Center at NDSU (Fargo, ND, USA). Upon arrival ewes were individually housed in 0.91 x 1.2-m pens in a temperature controlled (12° C) and ventilated facility for the duration of the study. Lighting within the facility was automatically timed to a 12:12 hour light-dark cycle with lights on at 0700 and off at 1900.

Ewes were acclimated to low-quality hay (Table 1) and a supplement which met 100% of MP requirements (MP100), as determined by National Research Council (NRC, 2007), for 10 d prior to starting dietary treatment [7]. The low-quality hay was used to limit MP intake. Ewes were weighed on two consecutive days (day 99 and 100 of gestation) prior to initiation of treatment.

On day 100 ± 2 of gestation ewes were randomly assigned to one of three dietary treatments (Table 2) designed to be isocaloric and provide: 60% (MP60), 80% (MP80), or 100% (MP100) of metabolizable protein requirements on a DM basis during the last 4 wk of gestation [7,14]. Nutrient requirements were based on NRC [14] recommendations for a 70 kg pregnant mature ewe carrying twins [7]. Thirty-five percent of the total intake was fed as a supplement at 0700, ewes were given one hour to consume the supplement, and then low-quality forage (Table 1) was provided to supply the remaining 65% of total intake. The supplement was always completely consumed. Body weight was measured every 7 d throughout the treatment period and the amount of supplement and forage offered was adjusted for changes in body weight. Throughout the project ewes had free access to water and a trace mineralized salt block [salt (min.) 95.5%, salt (max.) 98.5%, zinc (min.) 3,500 ppm, iron (min.) 2,000 ppm, manganese (min.) 1,800 ppm, copper (min.) 280 ppm, copper (max.) 420 ppm, iodine (min.) 100 ppm, cobalt (min.) 60 ppm; American Stockman, Overland Park, KS].

2.2. Collection of blood samples and ewe performance measurements

Jugular blood samples (10 mL) were collected on day 100 of gestation prior to treatment initiation and weekly thereafter (d 107, 114, 121, and 128). Final blood samples were taken on day 130 ± 1 of gestation prior to necropsy. All blood samples were placed on ice, held a minimum of 45 min, and then centrifuged at $1,500 \times g$ for 30 min. Serum samples were stored at -20°C until further analysis.

Ultrasound measurements (Aloka 500-SSV; Aloka Co. Ltd., Tokyo, Japan; i.e. back fat thickness (BF), body wall thickness (BWT), and loin eye area (LEA) at the 12th rib) were taken prior to treatment initiation (day 89 ± 4 of gestation) and prior to necropsy (day 124 ± 4 of gestation). Back fat thickness was measured at a point three-quarters the length of the

longissimus muscle (LM) from the backbone end, and the LM cross-section was traced to determine LEA.

2.3. Necropsy procedures

Necropsies were performed on day 130 ± 1 of gestation. Immediately prior to slaughter a final BW was taken. Animals were stunned by captive bolt (Supercash Mark 2, Accles and Shelvoke Ltd., Sutton Coldfield, UK), exsanguinated, and blood weighed. The gastrointestinal tract was removed of digesta and weighed. Heart, lungs, perirenal fat, adrenals, kidneys, liver, mammary glands, and thyroid were dissected and weighed. The gravid uterus was removed and weighed. Fetal fluids were collected and measured. The fetus(es) and placentomes were removed and weighed. Placentomes were separated into caruncular and cotyledonary portions and weighed. Intercotyledonary tissue was weighed. Thereafter the empty uterus was weighed. Ewe eviscerated body weight was considered to be the weight of the remaining carcass with head and pelt, but without all thoracic and abdominal internal organs. Empty body weight (EBW) was calculated by subtracting digesta weight from the live weight of the ewe. All organ weights were divided by EBW to normalize organ mass to ewe body weight.

2.4. Hormone analysis

Progesterone (P₄) was analyzed as previously described [15]. Briefly, a 50- μ l sample of maternal serum was analyzed in duplicate. Progesterone concentrations were measured by chemiluminescence immunoassay using the Immulite 1000 (Siemens, Los Angeles, CA), where lesser-, medium-, and greater-P₄ pools were assayed in duplicate (1.72 ± 0.06 , 3.64 ± 0.05 , and 14.23 ± 0.22 ng/mL for lesser-, medium-, and greater-P₄ pools, respectively). The intra- and interassay CV were 3.8% and 7.4%, respectively.

Thyroxine (T4) and triiodothyronine (T3) concentrations were determined by chemiluminescence immunoassay using the Immulite 1000 (Siemens, Los Angeles, CA), utilizing components of commercial kits (Diagnostic Products Corp., Los Angeles, CA) as previously described [16]. Within each assay, lesser-, medium-, and greater-T3 and T4 pools were assayed in duplicate (0.95 ± 0.03 , 1.70 ± 0.29 , and 3.37 ± 0.62 ng/mL and 24.0 ± 0.7 , 78.6 ± 2.1 , and 120.1 ± 2.7 ng/mL, mean $\pm$ SEM for lesser-, medium-, and greater-pools, for T3 and T4 respectively). Twenty-five-microliter and 15- μ L serum samples were assayed in duplicate for T3 and T4, respectively. The intraassay CV was 6.2% and 5.6% for T3 and T4, respectively, and the interassay CV was 4.4% and 6.2% for T3 and T4, respectively.

Ewe serum samples were analyzed for cortisol concentration as previously described [17]. Briefly, serum samples (10- μ L) were assayed in duplicate by chemiluminescence immunoassay using the Immulite 1000 (Siemens, Los Angeles, CA). Within each assay, lesser-, medium-, and greater-cortisol pools were assayed in duplicate (4.29 ± 0.05 , 12.92 ± 0.31 , and 34.35 ± 0.56 ng/mL, mean $\pm$ SEM for lesser-, medium-, and greater-cortisol pools, respectively). The intra- and interassay CV were 9.3% and 4.2%, respectively.

Circulating concentrations of E2 were analyzed in all serum samples by RIA using methodology described by Perry and Perry [18]. Intra- and inter-assay CV values were 5.6% and 13.9% respectively.

2.5. Serum chemistry panel analysis

Serum samples collected prior to treatment initiation and at the completion of the study were analyzed using an automated analyzer (VetTest Chemistry Analyzer; Idexx Laboratories, Inc., Westbrook, ME). Variables measured included: calcium, creatine kinase, creatinine, lactate dehydrogenase (LDH), albumin, globulin, total protein, blood urea nitrogen (BUN), aspartate

aminotransferase (AST), γ -glutamyltransferase (GGT), total bilirubin, glucose, and alkaline phosphatase (ALP).

2.6. Statistical analysis

Ewes carried singletons ($n = 28$; MP60 = 9; MP80 = 12; MP100 = 7) and twins ($n = 16$; MP60 = 6; MP80 = 3; MP100 = 7), and the one ewe (MP100) that carried a triplet was removed from the study. Ultrasound data, organ weights, fetal weights, and chemistry panel data were analyzed using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC) with treatment, fetal number, and their interaction listed in the model statement. Ewe body weight and endocrine data were analyzed with PROC MIXED of SAS with day being a repeated measure. The model included the effects of day, treatment, fetal number, and all interactions. Means were separated with the PDIFF option of the LSMeans statement. The covariance structures used were ante-dependence for P4, cortisol, and E2, unstructured for T3, and autoregressive for T4. In addition, the hormone data were further analyzed by calculating the area under the curve (AUC) with the use of SigmaPlot 8.0 (Systat Software, Inc. San Jose, CA) and were tested with GLM procedure of SAS. The model statement included treatment and fetal number. Means were separated with the PDIFF option of the LSMEANS statement. Least square means and SEM are reported. Data were considered significant when P values were < 0.05 . Tendency for significance were determined if $P \geq 0.06$ but < 0.10 .

3. Results

3.1. Ewe performance

There was no difference ($P \geq 0.23$) in initial BW between treatment groups ($67.1 \text{ kg} \pm 1.1$; Table 3). Treatment did affect final BW with ewes receiving the MP80 diet being heavier ($P = 0.03$) than MP60 ewes, but neither being different from MP100 ewes (Table 3). There was a

treatment by day interaction ($P < 0.01$) for percentage change in BW (Figure 1). Ewes on the MP80 and MP100 treatments had similar weight gain which increased as gestation advanced. The MP60 ewes experienced a decrease in weight by day 107, thereafter returning to weights similar to day 100. Thereafter, MP60 ewes maintained their weight and were lighter than MP80 and MP100 ewes (Figure 1). Neither treatment nor fetal number impacted ($P \geq 0.29$) initial or final maternal LEA ($10.45 \pm 0.23 \text{ cm}^2$, $9.78 \pm 0.26 \text{ cm}^2$), BF ($0.50 \pm 0.015 \text{ cm}$, $0.30 \pm 0.012 \text{ cm}$), or BWT ($1.50 \pm 0.048 \text{ cm}$, $1.17 \pm 0.039 \text{ cm}$), or the percentage change of these variables from day 100 to 130 (Supplementary Table 1).

3.2. Endocrine patterns

Maternal diet did not influence ($P > 0.16$) P4, E2, or cortisol concentrations or their AUC. From day 100 to 130, there was a fetal number by day interaction ($P = 0.01$) in P4 concentrations (Figure 2A) with ewes carrying twins having greater P4 concentrations compared to ewes carrying singletons on all days assessed. Unlike P4, ewes carrying singletons or twins had similar ($P = 0.17$) E2 concentrations on day 100. By day 107 and through day 130, ewes carrying twins had an increased ($P < 0.05$) concentration of E2 compared to ewes carrying singletons (Figure 2B). There was only a main effect of day ($P = 0.03$) on cortisol concentrations which decreased from day 100 to 107 and remained consistent as gestation advanced (Figure 3A).

Neither maternal diet nor fetal number ($P > 0.13$) impacted ewe T3 concentrations. There was a main effect of day ($P < 0.01$) on T3 concentrations which decreased as gestation advanced (Figure 3B). There was a day by fetal number interaction for T4 ($P < 0.01$; Figure 4). On day 100, all ewes had similar T4 concentrations (Figure 4). With advancing gestation, T4 concentrations decreased in both groups with ewes carrying twins exhibiting a greater reduction

in T4 concentrations compared to ewes carrying singletons (Figure 4). While there was no maternal diet effect on T4 concentrations, there was a treatment by fetal number interaction ($P = 0.02$) for T4 AUC (Figure 4 inset). While treatment did not impact T4 AUC in ewes carrying twins, ewes carrying singletons from the MP80 group had more T4 than MP60 and MP100 ewes, with MP100 being least. Moreover, while fetal number did not impact T4 AUC in the MP100 ewes, in both MP60 and MP80, ewes carrying twins had less T4 than ewes carrying singletons.

3.3. Serum chemistry panel

Prior to treatment initiation, there were no differences in panel values within assigned treatment groups ($P \geq 0.11$) or effect of fetal number (Table 4). There were tendencies ($P = 0.07$) for creatinine to be less, and total protein to be greater, in ewes carrying singletons compared to ewes carrying twins (Table 4). There was also a tendency ($P = 0.08$) for a diet by fetal number interaction for globulin measurements (Figure 5) where MP100 ewes carrying singletons had greater globulin concentrations compared to MP100 and MP60 ewes carrying twins.

Lactate dehydrogenase was similar ($P > 0.27$) across ewes on day 100 (Table 4). On day 130, MP60 and MP100 ewes had similar LDH concentrations regardless if they were carrying singletons or twins (Figure 6A). Ewes carrying twins and fed MP80 had increased LDH compared to MP80 ewes carrying singletons and MP60 and MP100 ewes carrying twins (Figure 6A). When the percentage change was calculated, MP80 ewes carrying twins were the only ewes to experience a net increase in LDH (Figure 6B). All ewes carrying singletons fetuses experienced a percentage decrease in LDH, but MP60 ewes carrying singletons had a greater decrease compared to MP100 and MP80 ewes carrying singletons or twins (Figure 6B).

3.3.1. Main effect of maternal diet

There was a main effect of maternal diet for several compounds on day 130 of gestation and the calculated percentage change for their initial measurements (Table 4). Albumin concentrations were similar on day 100, but by day 130, MP60 ewes had decreased ($P = 0.04$) concentrations compared to MP80 and MP100, which did not differ. All ewes experienced a decrease in albumin, but MP60 ewes experienced the greatest percentage loss ($P = 0.02$). While pretreatment and final concentrations of alkaline phosphatase were similar across dietary treatments ($P > 0.15$), there was a tendency ($P = 0.08$) for MP100 to have a greater change, with MP100 ewes experiencing an increase in alkaline phosphatase, and MP60 and MP80 experiencing decreases (Table 4).

Aspartate aminotransferase was not different among treatments on day 100. By day 130, MP60 ewes had decreased concentrations compared to MP100 ewes with MP80 being intermediate. When percentage change was calculated, MP60 ewes experienced a decrease, while MP80 and MP100 ewes had increased concentrations of AST (Table 4).

Blood urea nitrogen and calcium concentrations were not affected by treatment allocation on day 100. By day 130, blood urea nitrogen concentrations increased ($P < 0.01$), while calcium concentrations decreased with increasing amounts of metabolizable protein in the diet. The percentage change of blood urea nitrogen was greater as the amount of MP increased in the diet, and MP60 ewes experienced a urea loss. All ewes experienced a loss of calcium by day 130, with the least reduction being experienced by the MP60 ewes. Lastly, all ewes experienced a loss in total protein when expressed as a percentage change with MP60 losing greater ($P = 0.03$) amounts than MP100 ewes and MP80 being intermediate (Table 4).

3.3.2. Main effect of fetal number

Ewes carrying singletons had greater ($P = 0.03$) circulating protein concentrations on day 130 compared to ewes carrying twins (Table 4). There was a tendency ($P = 0.08$) for ewes carrying singletons to have greater GGT on day 130 than twin-carrying ewes, but when percentage change was calculated, there was a greater ($P < 0.01$) loss in GGT in ewes carrying twins than singletons (Table 4). Moreover, while globulin and glucose concentrations were greater ($P \leq 0.06$) on day 130 of gestation in ewes carrying singletons compared to twins, the percentage change was similar.

3.4. Necropsy measurements

There was no treatment or fetal number effect, or interaction, ($P \geq 0.10$) on eviscerated body weight (kg), blood (g or g/kg EBW), perirenal fat (g or g/kg EBW), thyroid (g or g/kg EBW), spleen, (g or g/kg EBW), gastrointestinal (g), omental fat (g or g/kg EBW), liver (g/kg EBW), pancreas (g), or adrenal (g) masses (Table 3).

There were interactions of maternal diet by fetal number ($P \leq 0.05$) for adrenal weight (g/kg EBW) and fetal weight (g/kg EBW) and a tendency ($P = 0.06$) for empty uterine weight (g/kg EBW). For adrenal glands, (Figure 7) ewes carrying singletons, regardless of maternal diet, had similar adrenal gland mass per unit EBW. However, MP60 ewes carrying twins had greater adrenal gland mass (g/kg EBW) compared to MP100 and MP80 ewes carrying twins. Moreover, while fetal number did not impact adrenal gland mass (g/kg EBW) in MP60 and MP80 ewes, MP100 ewes carrying singletons had greater adrenal gland mass (g/kg EBW) compared to MP100 ewes carrying twins.

Fetal weight, when expressed per unit EBW, decreased as the amount of MP in the diet decreased if the fetuses were singleton, but fetal weight (g/kg EBW) increased as MP decreased, if the ewes were carrying twins (Figure 8A). For empty uterine mass (g/kg EBW), MP80 and

MP60 ewes had reduced empty uterine weights if they carried singletons vs. twins. Moreover, MP100 ewes carrying singletons had greater empty uterine mass (g/kg EBW) compared to MP60 ewes carrying singletons. In twin carrying ewes, MP80 ewes had a greater empty uterine mass (g/kg EBW) compared to the MP100 ewes (Figure 8B).

There was a main effect of maternal diet on heart weight (g) with MP80 ewes possessing heavier ($P < 0.01$) hearts than MP60 and MP100 ewes, which were not different. When expressed per kg of EBW, a tendency ($P = 0.07$) remained where MP80 ewes had heavier hearts compared to MP100 ewes, with MP60 being intermediate. Moreover, kidney weight (g and g/kg EBW) was also affected by treatment with MP60 ewes having lighter ($P < 0.01$) kidneys compared to MP80 and MP100 ewes, which were not different. There was a main effect of maternal diet ($P = 0.04$) for fetal fluids (mL/kg EBW), but upon means separation, there were no differences that reached significance ($P > 0.1$; Table 5).

Fetal number had an effect on several variables (Tables 3 and 5). Ewes carrying twins had increased ($P \leq 0.03$) liver (g), mammary (g and g/kg EBW), and gravid uterine (g and g/kg EBW), fetal weights (g), total placentome (g and g/kg EBW), cotyledonary (g and g/kg EBW), caruncular (g and g/kg EBW), intercotyledonary (g and g/kg EBW), empty uterine (g) weights, fetal fluid (mL and mL/kg EBW) and placentome number compared to ewes carrying singletons (Table 5). There was a tendency ($P = 0.09$) for twins to have a greater fetal to placental weight ratio (Table 6). Ewes with singletons had increased ($P \leq 0.03$) eviscerated body weight (kg/kg EBW), gastrointestinal tract (g/kg EBW), kidney (g/kg EBW), and lung weights (g and g/kg EBW) compared to ewes carrying twins (Table 3). There were tendencies ($P \leq 0.08$) for ewes carrying singletons to have increased heart (g/kg EBW) and pancreas (g/kg EBW) weights (Table 3).

4. Discussion

We hypothesized that ewes deficient in MP would have increased markers of inflammation and experience more fat, organ mass, and skeletal muscle loss (i.e. mobilize their bodily resources) to ensure that their fetuses would have adequate nutrients while in utero, and this would be further exacerbated in ewes carrying twins. We can only partially accept our hypothesis as observed differences in our targeted endpoints were not all inclusive. Firstly, for markers of inflammation it must be noted that according the manufacturer's insert, all markers included in the chemistry panel were considered to be within normal clinical limits, and therefore no animal in the study would have reached diagnostic levels that would cause concern. However, there were statistical difference in 7 of the 13 markers (either final or percentage change or both) with most markers being decreased in MP60 vs. MP100 ewes. Protein concentrations contained in the diet have been shown to affect circulating BUN of various livestock species [2,19,20]. The decreased BUN in MP60 ewes compared to MP100 ewes was expected as protein deficient diets provide fewer precursors for nitrogen supply from breakdown of protein. The kidneys are the most important route for urea excretion. In ruminants, urea entering the rumen is degraded to ammonia, then used to synthesize amino acids and other nitrogen-containing compounds or absorbed into venous drainage [21]. Aspartate is synthesized by a one-step transamination reaction catalyzed by AST using oxaloacetate as the precursor [22]. Aspartate is a precursor of ornithine in the urea cycle [23]. Concentrations of AST have been used as markers of tissue damage with increased serum concentrations indicating increased damage [24]. The decrease in AST in MP60 ewes may predict decreased activity of AST, and therefore the efficiency of the

urea cycle may be compromised leading to a decrease in urea excretion and decreased concentrations of BUN.

One marker associated with skeletal muscle degeneration, creatine kinase [24], was not impacted in the current study. Perhaps this may reflect the lack of ultrasonography differences in LEA and BWT in the current study. Another marker of muscle degeneration is LDH [24], which is found in several tissues including blood cells and cardiac muscle [24]. The concentrations of circulating LDH were greatest in MP80 ewes carrying twins. Similarly, MP80 ewes had the greatest heart weights. Perhaps MP80 ewes had increased cardiac stress resulting in cardiac hypertrophy, thus the greater heart weights we observed. Pregnant rats that were hypothyroidic exhibited increased LDH in skeletal and cardiac muscle [25]. It could be argued that our MP80 ewes were hyperthyroidic if one specifically looks at the T4 AUC, but perhaps abnormalities in overall thyroid function alter LDH and muscle wasting.

Albumin can be used as a marker for protein depleted individuals [26]. Therefore, concentrations observed in this study support those assessments. Deficiencies in albumin are associated with decreased circulating T4 in women [27], which were not observed in the current study as the aforementioned study evaluated samples throughout pregnancy and our study only investigated the last third of gestation.

In domestic animals, GGT is mostly found in the kidneys, pancreas, liver, and intestine. Gamma-glutamyltransferase is highly active in the liver of sheep and serves as an indicator of liver disturbance in ruminants [28,29]. Diets restricted in protein have been associated with decreased liver function [30]. Concentrations of GGT are also increased in mammary glands of pregnant animals [31]. Perhaps a reason why ewes carrying twins in this study had greater serum

GGT concentrations was due to increased oxidative stress and enhanced mammary gland development.

Increased concentrations of calcium in circulation of MP60 ewes could reflect calcium release from bone and skeletal muscle loss as these gestating ewes receive inadequate protein. When dietary protein is increased, there is an enhancement of calcium absorption [32]. While concentrations of calcium were well within normal clinical values, there was a significant alteration in circulating calcium, potentially explaining some of our endpoints as calcium impacts many physiologic, metabolic, and endocrine pathways.

As expected from our previous study [7], we did not observe alterations in placental or mammary gland mass in ewes receiving differing amounts of dietary protein. While there were minimal impacts of protein supplementation on organ mass, the organ masses which were impacted, namely the kidneys, may be important to blood pressure regulation in the ewe. We have reported in a similar experiment that maternal blood pressure (measured during anesthesia) was not different by MP treatments, but interestingly it appears that while not statistically different, there may be a biological increase in MP60 ewes compared to MP100 ewes [33]. Additional studies need to be completed in order to determine if factors that are associated with blood pressure regulation are impacted by maternal protein intake.

In this study, ewes receiving the least amount of MP were lightest near term, which agrees with studies where caloric intake was reduced by 40% [3,34]. While we did not find differences in carcass measurements via ultrasonography, perhaps ultrasonography may not be sensitive enough to measure a difference in these variables. Perhaps carcass measurements do not adequately reflect the ability of the maternal system to mobilize bodily reserves as there were reductions in organ masses from protein restricted ewes. Since the diets were isocaloric, the

protein turnover of visceral organs may have provided the amino acids needed as they have a greater protein turnover than skeletal muscle [11]. Moreover, perhaps 30 d of protein restriction was not long enough to observe differences in carcass measurements. In ewes receiving similar diets, but allowed to lamb (so an additional ~20 d of protein restriction), we reported decreased body condition score of MP60 vs MP100 ewes [7]. The other explanation for not repeating our results compared to the Van Emon study could be that those ewes were fed in a production type setting (i.e. fed outdoors during late gestation) whereas our ewes in the current study were housed in an indoor facility. Therefore, there was a decrease in environmental stressors in the current study compared to the Van Emon et al. study [7].

The observation that adrenal weight (expressed per unit EBW) was altered by protein restriction and fetal number was of interest. In control ewes, carrying an additional fetus decreased adrenal weight. Since there was no impact of carrying an additional fetus on ewe empty body weight, we can interpret this result to mean that adrenal gland weight was reduced when body weight is equalized. In protein deficient ewes, this difference no longer existed. Moreover, it was only in the most protein deficient ewes (MP60) that ewes carrying twins had increased adrenal weight compared to ewes carrying twins. The only hormone that is produced by the adrenal gland that was measured in the current study was cortisol, and it was not impacted by maternal protein level or fetal numbers. Interestingly, there was a reduction in eviscerated body mass per unit empty body weight of ewes that carried twins. Since the majority of mass of the eviscerated ewes is composed of the pelt, skeleton, muscle and subcutaneous fat, perhaps there was greater mobilization of the body reserves to nurture that additional conceptus. Perhaps mineralocorticoids were influenced by maternal protein quantities, fetal number or both, and should be measured in the future.

Another interesting result was that fetal weight per unit maternal body weight decreased as the amount of MP in the diet decreased for singleton-carrying ewes. This pattern was reversed in ewes carrying twins. Moreover, the proportion of the maternal live weight that is composed of fetal mass was increased as the level of MP decreased. This indicates that there is indeed a greater proportion of the maternal body that is being used to support the fetus. The uterus, devoid of any conceptus material, also tended to show as similar pattern as fetal weight. While placental size was not impacted by diet, perhaps overall uterine capacity depends on both placental derived factors and maternal diet to allow for greater fetal mass per unit body weight. In ewes carrying twins, cotyledonary, but not caruncular, tissue had increased mRNA expression of vascular endothelial growth factor and its receptors, indicating the potential for greater vascularity and vascular permeability [35].

We did observe that ewes carrying twins had greater liver, mammary, empty uterus, and gravid uterine weights compared to ewes carrying singletons. Increased placental and mammary gland measurements are logical and would be expected with increased fetal number due to increased demands from the conceptus for size, waste expulsion, and milk output. There have been several studies reporting that placental and mammary gland masses are increased with increased numbers of fetuses [35,36]. Interestingly, there is limited data reporting impacts of litter size on maternal organ weights. Kenyon reported that several gastrointestinal tissues (i.e. rumen, intestinal, etc.), liver and lung weights did not differ between ewes that carried twin vs. triplet lambs [36].

Others have reported that circulating glucose is reduced in ewes carrying twins compared to singletons [37]. As the demand for glucose increases with advancing gestation, decreased

concentrations of glucose in ewes with increased conceptus weight is not surprising, as gluconeogenesis may not be adequate.

While we predicted that restricted ewes would have increased E2 and P4 concentrations, we did not observe a dietary treatment effect. This agrees with Anthony et al. [2] who saw no change in concentration of E2 for beef calves fed differing amounts of crude protein. However, Anthony et al. [2] found P4 concentrations that tended ($P = 0.07$) to be greater for low protein heifers, contrasting our data. Differences may be attributed to the different types of protein restriction, length of restriction, and timing of restriction. We have reported that ewes receiving 40% fewer calories than controls have increased E2 and P4 concentrations [11,12]. We hypothesize that this can be attributed to restricted ewes having greater placental steroid secretion (despite being similar in size), or decreased hepatic catabolism, or both [11,12]. In the current study, ewes carrying twins had greater placental mass, likely allowing for greater steroid secretion. However, liver size was also greater, and assuming steroid catabolism is similar per unit of liver, the greater placental mass and its ability to secrete P4 and E2 outweighed the ability of the liver to clear steroids from circulation. We also observed a tendency for a reduction in circulating globulin in ewes carrying twins. Perhaps with less globulin, there was less bound hormones and more “free” sex steroids in circulation to assay. Interestingly, while P4 is already decreased in singleton vs twin at day 100 of gestation, decreased concentrations of E2 were not observed until day 107. This is of interest as the production of these sex steroids appears to be differentially regulated in the ewe placenta.

Treatment or fetal number independently did not significantly affect T3 or T4 concentrations; however, T4 had a significant treatment by fetal number interaction. In addition, T4 concentrations were significantly affected by the interaction between day and fetal number.

This can be explained by MP80 ewes carrying singleton fetuses had a numerically greater T4 concentrations on day 100 of gestation (data not shown). While this was not statistically different, it surely drove the AUC differences that were observed between the MP60 vs MP80 ewes carrying singletons. Other studies in both sheep and cattle have seen similar results of a linear decrease in T4 with advancing days of gestation [12,13,38,39]. The tendency for singletons to have greater concentrations of T4 may be due to a greater conversion to T3 in twin-carrying ewes allowing for enhanced maternal metabolism to ensure survival of both fetuses in twins; however, more studies are necessary to investigate the differences in how fetal number impacts the thyroid hormone system. The drop in T4 for both ewes carrying singletons and twins late in gestation could have to do with metabolic changes associated with the initiation of lactation. Triiodothyronine concentrations dropped as day of gestation advanced. Again this is likely due to metabolic changes in preparation for parturition and lactation [40].

In conclusion, it appears that while maternal MP dietary level did not affect a large majority of organ masses, there are indications that the maternal system is breaking down bodily reserves and altering its ability to provide nutrients to developing conceptuses during late gestation when experiencing protein restriction. Moreover, alterations in factors included in the serum chemistry panel indicate that specific organ function is compromised. However, while there were some measures of additional maternal compensations in ewes carrying twins in a protein deficient environment, the maternal system seemed quite resilient. More research is needed to determine how the maternal system mobilizes the necessary nutrients to ensure proper conceptus growth, particularly when diets can be protein deficient.

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Table 1. Nutrient composition of fescue straw¹

Item	
Dry matter (DM (%))	96.24
NEm (Mcal/kg)	2.22
CP (% of DM)	2.76
MP (% of DM)	1.95
NDF (% of DM)	80.17
ADF (% of DM)	48.66
Ash (% of DM)	6.00

¹Ewes were fed fescue straw to limit MP intake

Table 2. Ingredient and nutrient composition of dietary supplements fed to ewes

Item	Treatment ^{1,2}		
	MP100	MP80	MP60
Ingredients (% DM³)			
Corn	5.00	15.00	18.50
DDGS ⁴	30.00	20.00	7.00
Soyhulls			9.50
Nutrient composition			
DM (%)	95.90	95.89	95.51
NEm (Mcal/kg)	2.14	2.22	2.00
CP (% of DM)	25.03	20.53	13.45
MP (% of DM)	16.31	13.01	8.41
NDF (% of DM)	40.79	32.11	33.61
ADF (% of DM)	11.61	8.33	15.71
Ash (% of DM)	4.38	3.50	3.17

¹Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements

²Diets formulated based on a 70 kg ewe carrying twins

³Dry matter

⁴Dried distillers grains plus solubles

Table 3. Effects of metabolizable protein and fetal number on ewe organ weights

Item	Nutrition Treatment (Trt) ^{1,2}				Fetal Number (FN)			P-value		
	MP100	MP80	MP60	SEM	Singleton	Twin	SEM	Trt ³	FN	Trt*FN
Initial wt (kg)	66.5	70.1	64.7	2.4	65.9	68.3	2.0	0.23	0.35	0.80
Final wt (kg)	69.1 ^{a,b}	72.6 ^a	64.8 ^b	2.0	67.2	70.4	1.7	0.03	0.20	0.61
Empty BW (EBW ⁴) (kg)	54.69	56.10	51.74	2.06	52.98	55.37	1.71	0.24	0.27	0.78
Evisc BW (kg)	33.61	36.27	32.97	1.40	35.16	33.41	1.19	0.19	0.24	0.82
kg/kg EBW	0.63	0.65	0.63	0.015	0.67	0.60	0.013	0.64	< 0.01	0.70
Blood (g)	3,418	3,558	3,226	209	3,206	3,595	169	0.54	0.12	0.16
g/kg EBW	63.60	63.69	62.59	3.80	61.31	65.28	3.20	0.97	0.33	0.31
Adrenal (g)	4.94	4.68	4.81	0.22	4.91	4.71	0.18	0.64	0.39	0.13
g/kg EBW*	0.092	0.084	0.093	0.0039	0.094	0.086	0.003	0.16	0.05	0.04
Heart (g)	258.31 ^a	308.56 ^b	269.67 ^a	10.82	282.01	275.68	8.78	< 0.01	0.62	0.11
g/kg EBW	4.89 ^a	5.54 ^b	5.23 ^{ab}	0.21	5.42	5.02	0.17	0.07	0.07	0.11
GI tract ⁵ (g)	2,819.1	2,847.1	2,667.9	117.2	2,838.9	2,717.2	97.0	0.41	0.32	0.94
g/kg EBW	51.86	51.00	51.96	1.94	53.9	49.31	1.60	0.92	0.03	0.94
Omental fat (g)	965.2	1,354.2	1,128.7	182.2	1,216.2	1,082.5	150.9	0.27	0.48	0.14
g/kg EBW	18.32	23.97	21.38	2.95	23.08	19.37	2.44	0.35	0.23	0.15
Perirenal fat (g)	249.21	379.23	378.07	70.65	372.78	298.23	60.00	0.25	0.32	0.65
g/kg EBW	4.49	6.67	7.00	1.13	6.83	5.28	0.96	0.17	0.20	0.58
Kidneys (g)	128.95 ^a	134.87 ^a	116.46 ^b	3.79	129.97	123.55	3.16	< 0.01	0.18	0.50
g/kg EBW	2.44 ^a	2.40 ^a	2.25 ^b	0.068	2.51	2.22	0.056	0.06	< 0.01	0.81
Liver (g)	682.05	701.53	628.02	24.58	634.02	707.05	20.51	0.20	0.03	0.59
g/kg EBW	12.66	12.26	12.18	0.41	12.09	12.64	0.34	0.59	0.21	0.73
Lung (g)	574.26	587.62	539.18	23.83	601.51	532.54	19.88	0.35	0.03	0.92
g/kg EBW	10.99	10.54	10.42	0.49	11.65	9.65	0.41	0.61	< 0.01	0.58
Mammary (g)	632.46	783.89	680.92	69.54	507.22	890.97	58.03	0.51	< 0.01	0.89
g/kg EBW	11.50	13.33	13.16	1.46	9.53	15.80	1.21	0.55	< 0.01	0.81
Thyroid (g)	4.38	4.39	4.16	0.47	4.40	4.22	0.40	0.90	0.72	0.25
g/kg EBW	0.084	0.078	0.081	0.0092	0.086	0.076	0.008	0.90	0.34	0.37
Spleen (g)	79.11	95.16	85.21	6.14	86.34	86.65	5.08	0.15	0.96	0.42
g/kg EBW	1.48	1.70	1.63	0.094	1.65	1.56	0.078	0.17	0.38	0.60
Pancreas (g)	77.31	76.41	79.93	5.30	80.88	74.89	4.39	0.86	0.28	0.85
g/kg EBW	1.43	1.37	1.56	0.094	1.54	1.36	0.078	0.26	0.08	0.83

¹Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements

²Diets formulated based on a 70 kg ewe carrying twins

³LSMeans $\pm$ SEM within a row with different superscripts differ; $P < 0.05$

⁴g/kg = organ weight divided by empty body weight (EBW) = live wt – digesta wt.

⁵GI = Gastrointestinal tract includes the sum of stomach, small intestine and large intestine mass that was stripped of digesta.

*Interaction between fetal number and dietary treatment shown in Figure 7.

Table 4. Effects of metabolizable protein and fetal number on selected serum factors associated with protein metabolism and inflammatory markers in pregnant ewes.

Item	Nutrition Treatment (Trt) ^{1,2}				Fetal Number (FN)			P-value		
	MP100	MP80	MP60	SEM	Singleton	Twin	SEM	Trt ³	FN	Trt*FN
Albumin (g/dL)										
Pretreatment ⁴	3.28	3.35	3.32	0.08	3.33	3.30	0.07	0.77	0.72	0.23
Final ⁵	3.16 ^a	3.19 ^a	2.95 ^b	0.08	3.13	3.07	0.07	0.04	0.46	0.55
Δ (%) ⁶	-3.26 ^a	-4.47 ^a	-11.18 ^b	2.49	-5.63	-6.98	2.06	0.02	0.60	0.10
Alkaline phosphatase (U/L)										
Pretreatment	99.90	105.92	112.53	12.27	116.36	96.62	13.52	0.24	0.24	0.82
Final	129.50	84.88	111.39	17.31	113.56	103.62	14.33	0.15	0.58	0.53
Δ (%)	16.67 ^a	-2.88 ^b	-7.95 ^b	9.54	0.94	2.95	7.90	0.08	0.84	0.42
Aspartate aminotransferase (U/L)										
Pretreatment	99.93	105.92	112.53	10.82	102.54	109.71	10.16	0.68	0.57	0.13
Final	109.57 ^a	97.79 ^{ab}	82.25 ^b	9.08	94.44	98.63	7.13	0.02	0.62	0.60
Δ (%)	12.68 ^a	16.21 ^a	-21.20 ^b	8.97	-1.41	6.53	7.04	<0.01	0.35	0.51
Blood urea nitrogen (mg/dL)										
Pretreatment	14.14	14.13	15.86	0.90	14.33	15.09	0.75	0.20	0.42	0.92
Final	24.79 ^a	18.96 ^{ab}	11.03 ^b	2.45	17.52	18.99	2.03	<0.01	0.56	0.77
Δ (%)	75.84 ^a	37.94 ^b	-28.18 ^c	17.70	26.75	30.31	14.66	<0.01	0.85	0.42
Calcium (mg/dL)										
Pretreatment	9.76	10.07	10.12	0.16	9.95	10.02	0.13	0.12	0.65	0.47
Final	8.98 ^a	9.35 ^b	9.78 ^c	0.15	9.43	9.30	0.13	<0.01	0.40	0.32
Δ (%)	-7.95 ^a	-7.13 ^a	-3.21 ^b	1.52	-5.06	-7.13	1.26	0.03	0.19	0.60
Creatine kinase (U/L)										
Pretreatment	225.93	219.42	106.28	65.57	175.53	192.22	54.38	0.24	0.81	0.87
Final	70.21	96.83	40.17	36.46	80.02	58.13	30.20	0.48	0.56	0.94
Δ (%)	-32.33	-40.90	-44.51	22.71	-30.22	-48.28	18.80	0.90	0.44	0.17
Creatinine (mg/dL)										
Pretreatment	1.03	1.02	1.04	0.030	1.00	1.06	0.03	0.90	0.07	0.33
Final	1.22	1.25	1.20	0.05	1.19	1.26	0.04	0.71	0.24	0.20
Δ (%)	19.00	23.23	15.26	4.57	19.41	18.91	3.78	0.41	0.92	0.17
Gamma-glutamyltransferase (U/L)										
Pretreatment	84.14	86.33	89.14	5.68	86.62	86.45	4.70	0.75	0.98	0.90
Final	75.71	79.96	84.25	4.69	84.32	75.63	3.88	0.31	0.08	0.89
Δ (%)	-9.75	-6.97	-3.97	3.14	-2.14	-11.66	2.60	0.30	<0.01	0.77
Globulin (g/dL)										
Pretreatment*	3.84	3.86	3.76	0.09	3.90	3.74	0.07	0.64	0.09	0.08
Final	3.59	3.58	3.50	0.11	3.66	3.45	0.09	0.74	0.06	0.79
Δ (%)	-6.34	-7.01	-6.94	2.45	-5.88	-7.64	2.03	0.97	0.49	0.27
Glucose (mg/dL)										
Pretreatment	51.43	59.96	56.42	3.08	55.65	56.21	2.55	0.11	0.86	0.71
Final	37.14	39.00	40.67	2.88	42.33	35.54	2.38	0.58	0.03	0.97
Δ (%)	-27.99	-31.85	-23.62	6.48	-23.33	-32.30	5.37	0.61	0.18	0.76
Lactate dehydrogenase (U/L)										
Pretreatment	1282.5	1287.7	1287.8	68.5	1325.2	1246.8	56.7	0.99	0.27	0.39
Final*	1152.3	1317.3	1010.6	84.6	1119.3	1200.9	70.1	0.03	0.35	0.03
Δ (%)*	-10.0	-1.0	-20.6	3.8	-15.0	-6.1	3.2	<0.01	0.03	0.05
Total bilirubin (mg/dL)										
Pretreatment	0.22	0.28	0.27	0.05	0.23	0.28	0.04	0.56	0.29	0.75
Final	0.26	0.28	0.23	0.05	0.22	0.30	0.04	0.71	0.17	0.18
Δ (%)	52.38	16.02	10.09	41.07	21.11	34.01	28.20	0.65	0.81	0.52

Total protein (g/dL)

Pretreatment	7.09	7.21	7.08	0.11	7.23	7.02	0.09	0.60	0.07	0.78
Final	6.74	6.75	6.45	0.13	6.79	6.49	0.11	0.11	0.03	0.77
Δ (%)	-5.04 ^a	-6.45 ^{ab}	-9.02 ^b	1.21	-6.05	-7.63	0.99	0.03	0.18	0.93

¹Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements

²Diets formulated based on a 70 kg ewe carrying twins

³LSMeans $\pm$ SEM within a row with different superscripts differ; $P < 0.05$

⁴Pretreatment sample was obtained on day 100 of gestation, prior to treatment initiation.

⁵Final sample was obtained on day 130 of gestation

⁶Percentage change was calculated: % = 100 x ((final-pretreatment)/pretreatment)

*Interaction between fetal number and dietary treatment shown in Figures 5 and 6.

Table 5. Effects of maternal metabolizable protein and fetal number on uterine, placental and fetal characteristics.

Tissue	Nutrition Treatment (Trt) ^{1,2}				Fetal Number (FN)			P-values		
	MP100	MP80	MP60	SEM	Singleton	Twin	SEM	Trt	FN	Trt*FN
Gravid uterus (g)	9,678	10,456	9,523	864	7,039	12,734	691	0.69	< 0.01	0.74
g/kg EBW ³	180.41	186.48	192.05	14.87	138.84	233.78	11.89	0.82	< 0.01	0.46
Fetal wt (g)	4,667	4,947	4,639	418	3,289	6,213	346	0.83	< 0.01	0.21
g/kg EBW*	86.04	87.86	89.44	7.41	63.40	112.16	6.14	0.93	< 0.01	0.05
Placentome wt (g)	694.96	711.44	728.35	37.96	554.87	868.29	31.43	0.75	< 0.01	0.40
g/kg EBW	13.05	12.66	14.18	0.81	10.86	15.73	0.67	0.30	< 0.01	0.20
Fetal wt / placentome wt	6.89	7.03	6.22	0.65	6.13	7.30	0.54	0.56	0.09	0.23
Intercotyledonary wt (g)	412.71	353.00	324.20	39.26	270.57	456.03	32.51	0.16	< 0.01	0.81
g/kg EBW	7.53	6.25	6.24	0.61	5.17	8.17	0.51	0.16	< 0.01	0.43
Cotyledonary wt (g)	522.41	527.63	543.89	31.54	400.62	661.79	26.12	0.84	< 0.01	0.29
g/kg EBW	9.79	9.37	10.59	0.65	7.84	11.99	0.54	0.33	< 0.01	0.14
Caruncular wt (g)	113.64	123.18	121.84	9.36	98.88	140.22	7.75	0.67	< 0.01	0.31
g/kg EBW	2.12	2.21	2.38	0.20	1.93	2.54	0.16	0.54	< 0.01	0.64
Placentome number	76.4	81.4	80.8	5.5	68.18	90.83	4.54	0.72	< 0.01	0.36
Empty uterus (g)	765.59	802.85	700.51	33.16	663.65	849.14	33.48	0.14	< 0.01	0.58
g/kg EBW*	14.11	13.62	14.26	0.53	12.67	15.32	0.43	0.60	< 0.01	0.06
Fetal fluids (mL)	2,683	2,453	2,377	285	1,722	3,286	228	0.65	< 0.01	0.72
mL/kg EBW	46.98	43.57	48.66	5.60	32.24	60.57	4.48	0.04	< 0.01	0.73

¹Treatment: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements

²Diets formulated based on a 70 kg ewe carrying twins

³g/kg = organ weight divided by empty body weight (EBW) = live wt – digesta wt. EBW is reported in Table 3.

*Interaction between fetal number and dietary treatment shown in Figure 8.

Figure legends:

Figure 1. Impacts of maternal nutritional treatment on body weight expressed as percentage change from day 100 of gestation. Nutritional treatments included: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements. * MP60 ewes experienced a decrease in weight compared to day 100; $P = 0.01$. #MP60 < MP80 = MP100 values; $P \leq 0.01$.

Figure 2. Impact of fetal number by day on progesterone (A) and estradiol-17 β (B) concentrations. ^{abcde}LSMeans $\pm$ SEM with different superscripts differ; $P < 0.01$.

Figure 3. Effect of day of gestation on cortisol (A) and triiodothyronine (B) concentrations. ^{abc}LSMeans $\pm$ SEM with different superscripts differ; $P < 0.05$.

Figure 4. Impact of fetal number by day on thyroxine concentrations. Inset: There was a maternal dietary treatment by fetal number interaction ($P = 0.02$) for thyroxine area under the curve (AUC). ^{abcde}LSMeans $\pm$ SEM with different superscripts differ; $P < 0.05$.

Figure 5. Effect of maternal dietary treatment and fetal number on globulin concentrations on day 100 of gestation. Nutritional treatments included: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements. ^{ab}LSMeans $\pm$ SEM with different superscripts differ, $P \leq 0.05$.

Figure 6. Effect of maternal dietary treatment and fetal number on lactate dehydrogenase on day 130 of gestation (A) and percentage change from day 100 (B) of gestation. Nutritional treatments included: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements. ^{ab}LSMeans $\pm$ SEM with different superscripts differ, $P \leq 0.05$.

Figure 7. Effect of maternal dietary treatment and fetal number on adrenal gland weight per empty body weight (g/kg) on day 130 of gestation. Nutritional treatments included: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements. ^{ab}LSMeans $\pm$ SEM with different superscripts differ, $P \leq 0.05$.

Figure 8. Effect of maternal dietary treatment and fetal number on fetal weight per empty body weight (g/kg) (A) and empty uterine weight per empty body weight (g/kg) (B) on day 130 of gestation. Nutritional treatments included: MP100 = diet designed to meet 100% of metabolizable protein requirements; MP80 = diet designed to meet 80% of metabolizable protein

requirements; MP60 = diet designed to meet 60% of metabolizable protein requirements.

^{abcd}LSMeans $\pm$ SEM with different superscripts differ, $P \leq 0.05$.

Figure 1.

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8