Effects of maternal supplementation with rumen-protected methionine and niacin on gene expression: the role of fetal programming on growing beef cattle

by

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Abstract

The role of supplementation with dietary rumen-protected methionine (RPM), and niacin (RPN) on beef cattle was assessed in two different, 2-year studies.

In the first study, Angus × Simmental dams (n = 44) were divided based on parities, either primiparous (PRIM) or multiparous (second calf; MULT). Simultaneously, parity groups were split based on two nutritional treatments: control (CTRL) and RPM groups. Control group received bermudagrass hay, and corn gluten and soybean hulls pellets supplementation (base diet); whereas RPM group received the base diet in addition to 8 g/hd/day of RPM at a fixed rate the last trimester of gestation and the first ~85 days of lactation, in which calves were early weaned. Only male calves were included in this study. After weaning, calves born to RPM dams also received RPM from weaning (Day 1) to Day 100. Blood sampling and skeletal muscle biopsies for subsequent quantitative PCR analysis were conducted at Day 1, 25, 50, and 100 on calves. There was no difference in maternal body weight (BW), body condition score, and milk production by the inclusion of RPM. Similarly, offspring BW and average daily gain were similar within each parity category. Glucose and blood metabolites that served as biomarkers for liver health (e.g., aspartate transaminase, albumin, alkaline phosphatase, and alanine transaminase) were in the normal levels for all calves. Calves in the PRIM-RPM group had a greater expression of adipogenic genes (e.g., *PPARg*, *LPL*, and *CEBPD*) at Day 100 compared with PRIM-CTRL. In addition, DNA methylation (*DNMT1*) and oxidative stress-related genes (*SOD2* and *NOS3*) in PRIM-RPM group were upregulated at Day 100 compared with PRIM-CTRL. These results may suggest that calves born to PRIM dams exposed to RPM supplementation are more prone to develop greater adipose tissue than CTRL calves.

Furthermore, RPM supplementation may improve methylation processes in addition to a possible hypertrophy acceleration due to greater free radicals in skeletal muscle cells. However, the mechanisms in which methionine executes these nutrigenetic changes only in calves born to PRIM dams remain to be elucidated.

In the second study, a total of 28 Angus × Simmental pregnant dams (11 cows and 17 heifers) were selected based on genetic resistance to fescue toxicosis based on a commercial genetic test (T-snip™, Ag. Botanica, Columbia, MO). Subsequently, dams were divided based on genetic resistance to fescue toxicosis and randomly assigned to dietary treatments: 1) Susceptible Control (SC; n = 7); 2) Susceptible Niacin (SN; n = 7); 3) Tolerant Control (TC; n = 7); and 4) Tolerant Niacin (TN; n = 7). All animals received 1.16 kg of endophyte-infected (E+) tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort.) seeds for a period of 30 days. Ergovaline concentration in fescue seeds was ~5,000 ppb on a DM basis. Dams in SN and TN groups received 6 g/hd/day as-fed of top-dressed rumen-protected niacin (RPN), following manufacturer's maximum dose recommendation. Dams in the TN group experienced an accelerated reduction in BW as compared to the rest of the treatments. However, TN offspring did not show BW differences at birth or weaning as compared to those born to dams in other treatments. Milk production was estimated by the weigh-suckle-weigh method, and no difference was observed among treatments. All dams had a reduction in circulating prolactin concentration, indicating that all animals were experiencing fescue toxicosis. In addition, blood metabolites that indicate liver health were markedly decreased at the end of the study. Overall, these results suggest that dams receiving E+ tall fescue seeds effectively are exposed to fescue toxicosis symptoms. Therefore, neither RPN supplementation nor genetic test for fescue toxicosis tolerance could be used as an effective mechanism of dampening these symptoms. In addition,

steers and heifers born to dams receiving E+ tall fescue seeds were also exposed to 30 days feeding trial period following maternal dietary treatments. A total of 20 μg/kg BW/day was the daily dietary dose of ergovaline offered to the animals under study to produce characteristic signs of fescue toxicosis. Complete blood count analysis was performed in the offspring to identify hematological changes of ergovaline consumption. Animals in the SC group presented low mean corpuscular hemoglobin and mean corpuscular volume at the end of the study, which is indicative of anemia; whereas animals in the TN group presented signs of inflammation or infection due to a high level of white blood cells and basophils, and low neutrophil to lymphocytes ratio. Furthermore, there was a reduction in rectal temperature in SC animals. The utilization of RPN supplementation or genetic test did not improve performance parameters (i.e., BW and average daily gain). Lastly, we performed RNA-sequencing on liver samples of steers (n = 4) and heifers (n = 2) receiving E+ tall fescue seeds and born to cows exposed to E+ tall fescue during mid-gestation; and a total of 3 animals (2 steers and 1 heifer) receiving endophyte-free (E-) tall fescue seeds. All animals received tall fescue seeds (E+ or E-) for a period of 30 days. Results showed an overall downregulation in KEGG pathways in E+ group compared with E- group. More specifically, a downregulation in ‘Cellular processes’ showed that liver cells may experience less senescence due to ergot alkaloids consumption. There was a lower expression of ‘Environmental information processes’, which is related to several endocrinal and immune pathways. In addition, ‘Organismal system’ KEGG category was also downregulated in E+ group, in which the most impacted pathway was ‘B cell receptor signaling pathway’. Thus, a possible immunosuppression may exist in cattle exposed to E+. Based on our results, future research could be aimed at developing pharmacological strategies to dampen fescue toxicosis.

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List of abbreviations

ADG	Average Daily Gain
ALT	Alanine Transaminase
ALP	Alkaline Phosphatase
AST	Aspartate Transaminase
BW	Body Weight
CK	Creatine Kinase
CTRL	Control Treatment
DM	Dry Matter
DMI	Dry Matter Intake
E-	Endophyte-free Tall Fescue
E+	Endophyte-infected Tall fescue
IUGR	Intrauterine Growth Restriction
LDH	Lactate Dehydrogenase
MULT	Multiparous
PRIM	Primiparous
PRL	Prolactin
RNA-seq	RNA sequencing
RPN	Rumen-protected Niacin / Rumen-protected Niacin Treatment
RPM	Rumen-protected Methionine / Rumen-protected Methionine Treatment
ROS	Reactive Oxygen Species

Introduction

Pregnancy is an event in which, preferably, most of the available cows and replacement heifers in the herd experience once per year. The main objective of producing one calf per cow per year in a cow-calf operation system depends on one priority: gestation. The study of the consequences of changes on growth and development during fetal life in ruminants is not a novel branch of science. The first evidence was reported in the 1950s in sheep when Short (1955) showed the effect of adverse maternal nutrition on the developmental modification of the fleece structure of the offspring (Short, 1955). The concept of fetal programming, also called developmental programming, is widely defined as the changes in the utero environment during fetal growth which affect the offspring in a positive or negative manner, throughout its entire lifetime in a direct or indirect fashion (Broadhead et al., 2019). Different factors affect fetal growth, such as maternal age and plane of nutrition, environmental conditions, and stress. Based on this concept, from a production standpoint, it is convenient to expose animals to beneficial conditions for both the dam and the offspring, avoiding nutritional and environmental negative disturbances. The programmed changes in offspring are frequently aimed at essential characteristics for the cattle industry production, such as growth, efficiency, carcass quality, immune function, among others (Robinson et al., 2013). Fetal adipogenesis and skeletal muscle hypertrophy occur during mid to late gestation; therefore, maternal nutritional disturbances during this period impact the development of both tissues (Du et al., 2010). Consequently, the targeted periods for impacting skeletal muscle and adipose tissue development are mid-gestation and late gestation.

Beef production systems, especially cow-calf operations, experience variations in feed supply during the year. Most of the cow-calf operations in the US are pasture-based systems,

where the forage availability depends on the environmental conditions and seasonality of rains (Short, 2001). Gestating dams present high nutritional requirements, and the calving period must be timely matched with the best forage quality. Generally, during mid to late gestation, energy and protein demands increase, and supplementation is required for achieving optimum performance in cows on pasture (LeMaster et al., 2017; Maresca et al., 2018).

The present dissertation has two objectives: a) to understand the effects of maternal supplementation of rumen-protected methionine as a methyl donor during the last trimester of gestation, lactation, and calves after early weaning in performance parameters, blood metabolites, and gene expression of *PPARg* pathway, oxidative stress, and DNA methylation; b) to understand the effects of rumen-protected niacin supplementation on cows and heifers during mid-gestation and their offspring consuming endophyte-infected tall fescue assessed through performance parameters, complete blood count analysis, blood metabolites analyses, and RNA-sequencing of offspring's liver.

Chapter 1
Literature review

Fetal programming

Maternal plane of nutritional and fetal growth

Maternal nutrition has a critical role in fetal growth, and recent studies have focused on investigating the effects of diet disturbance during gestation. Previous studies reported that body weight (BW) at birth could be related to the energy and protein content in the maternal diet. For example, the inclusion of energy sources, such as dried distiller grains with solubles (DDGS) and corn during late gestation diet, could lead to greater BW at birth (Radunz et al., 2010; Bohnert et al., 2013). Similarly, the inclusion of crude protein supplementation in beef cows during late gestation improves offspring's postnatal growth and fertility (Funston et al., 2010). Another study found that protein supplementation on beef cows grazing low-quality pasture changes the amino acid profile, increasing maternal BW and reducing maternal tissue mobilization (Lopes et al., 2020).

The effect of the nutritional intervention on gestation development is not time independent. Offspring whose dams experience nutrient deficiency during early pregnancy may present reduced postnatal performance (Micke et al., 2010); however, growth and postnatal survival and performance of fetuses can be recovered if dams return to an adequate diet during mid-gestation (Camacho et al., 2014; Maresca et al., 2018). Interestingly, visceral organs present reduced size in fetuses experiencing maternal nutrient restriction, decreasing postnatal growth compared with offspring born to cows without nutritional impairments during gestation (Meyer et al., 2010). Nevertheless, the critical period determining offspring's postnatal development and future carcass quality occurs during the last trimester of pregnancy. During this phase, muscle cells experience hypertrophy and lengthening, directly impacting birth and weaning weight

(Thornton, 2019). Contrary to muscle cells, intramuscular adipocytes begin to develop hyperplasia immediately before birth (Du et al., 2013). Du et al (2015) reviewed the timelines for skeletal muscle and tissue development for beef cattle, emphasizing the importance of a correct maternal nutrition on the future carcass quality of the offspring (Du et al., 2015)

Placenta effects

The developmental programming in a beef production system arises as one of the most important aspects to control because it shapes the offspring's physiological characteristics for their lifetime. The primary objective in a cow-calf system is to produce one calf per cow per year, and its achievement depends on the good nutrition, health, and management of the herd. Once conception is achieved, the process of the offspring development occurs in a synergistic manner between the embryo and the dam (Reynolds & Redmer, 1995).

The placenta is a temporal maternal organ that originates in the endometrium of the uterus after blastocyst implantation in cattle. It supports embryo and fetal growth through the exchange of nutrients, removal of wastes, respiratory and immune maintenance, and hormone secretion necessary for survival (Reynolds and Redmer, 1995; Furukawa et al., 2014). Compared to other eutherian mammals, ruminants present an epitheliochorial placenta, in which the trophoblast cells are attached to the uterine epithelial cells. In addition, ruminant placenta contains structures called placentomes. Each of them presents two different compartments: the caruncle and the cotyledon. The maternal portion of the placentome is called caruncle, and they are easily observed in a non-pregnant endometrium; whereas the cotyledon is the portion of the placentome exposed to the fetal side and surrounded by caruncle (Carter and Enders, 2013). The placenta presents two different and independent blood circulations; first, the umbilical circulation, which is in charge of the blood exchange between the fetus and the placenta, and the

uterine circulation, capable of providing nutrients from the maternal body to the placenta (Battaglia, 2007). In effect, disturbances of the normal function of one of these blood circulations lead to fetal growth restriction. Different factors determine uterine blood flow and placenta performance, such as plane of nutrition, maternal and fetal genotype, dam age, and gestation period.

Regarding the genotype, Ferrell (1991) showed the effects of fetal and maternal genotype on different parameters in the placenta and fetal growth. Fetus breed was the most determinant factor in body size because Charolais fetuses' body weight was greater than Brahman fetuses. However, the maternal uterine environment also plays a role in fetuses' growth. For example, this study showed that Charolais fetuses in Brahman cow were smaller than Charolais fetuses in Charolais cows. Furthermore, the number and size of placentomes were higher in Charolais compared with Brahman. In other words, maternal genotype is an essential factor to consider in fetal development since the anatomical structure of the placenta (i.e. number of placentomes) shapes the fetus' growth (Ferrell, 1991).

The redistribution of nutrients in the fetus body is tightly coordinated with the vital importance of the tissue, for example, the impact of intrauterine growth restriction (IUGR) is lower in the brain compared with other less vital organs (Ke et al., 2006). However, under IUGR, the brain can suffer changes in the expression of genes related to energy metabolism, as shown in rats (Lane et al., 2000; Venhoranta et al., 2013).

Understanding the complexity behind placenta physiology, the timing of fetal development, and IUGR occurrence provide strategies for mitigating the impact of low-quality nutrition or enhancing offspring development and post-natal growth.

Dystocia

Dystocia is usually defined as the difficulty of calving, in which a certain level of human assistance is required to complete the birth process (Meijering, 1984). Dystocia is the major cause of neonatal calf death, and it produces a high economic impact in cow-calf operations because it affects both the dam and offspring. Calves that survive dystocia present lower passive immune transfer and an increased risk of morbidity and mortality postnatally. Consequently, calves born in that condition experience lower post-natal growth and development (Rice, 1994). The occurrence of dystocia can be determined by factors related to the dam or the fetus. The disproportion between the fetus' body size and the dam's pelvic area is the most critical cause of dystocia. Calf's birth weight is one of the most important factors that cause dystocia. For example, a previous study indicates that offspring born with dystocia to primiparous beef dams had 4.2 kg greater BW at birth compared with calves born without dystocia, indicating the importance of low BW at birth for diminishing dystocia (Gregory et al., 1991). The calf sex also affects calving, being that the male calves are the ones that present more significant risks at birth. It has been shown that the dystocia rate of cows and heifers giving birth to male calves is greater compared to female calves (Bellows et al., 1971). The occurrence of multiple calving also affects dystocia. In beef breeds, parturition with dystocia is more frequently present in twins due to abnormal presentation than single parturition birth (Echternkamp and Gregory, 1999). Unusual presentation of fetuses affects normal calving and posterior survival of the calf. The calf's delivery in the backward or breech presentation negatively affects offspring survival rate and subsequent maternal pregnancy rate (Patterson et al., 1987).

Furthermore, in order to avoid dystocia, other production parameters need to be managed correctly, such as dams' body condition score (BCS) and body weight, parity of dams, and

adequate sire genetics for breeding. A correct and balanced maternal nutrition during gestation is a fundamental condition for reducing dystocia occurrence. Mortality at birth can be directly affected by maternal BCS. A previous study reported that 1% of calves mortality in beef cows with a BCS of 6 (on a scale from 1 to 9), compared with 11% of mortality in beef cows with BCS of 4, due to malnutrition or lack of supplementation during the last trimester of gestation (Bohnert et al., 2013). Additionally, the pregnancy stage (first, second or third trimester) in which the cows suffer nutritional restrictions may affect calf body weight at birth. For example, there is no effect on body weight at birth in calves born from beef cows receiving a diet with nutrient restriction during the first 100 days of gestation. This lack of increase in body weight at birth is related to the priority of the calf to differentiate and develop its internal organs, which require low energy from the dam (Long et al., 2010). However, enhanced maternal diets during the last trimester of gestation may lead to dystocia due to greater offspring body weight at birth. During the last trimester of gestation, fetuses' nutrient utilization is aimed towards tissue hypertrophy, and consequently, a significant increase in BW is observed (Bohnert et al., 2013). Another study showed that fat heifers usually present high percentage of calf losses at birth due to low delivery or abnormal presentation (Rice, 1994).

The number of parities of the dam is another crucial factor involved in dystocia, being more frequent in primiparous dams compared with multiparous (Nix et al., 1998). The pelvic area is the primary determinant trait of dystocia in primiparous heifers due to its high association with mismatch between the maternal calving canal and fetus' size. Johnson et al (1998) reported that pre-breeding and pre-calving pelvic area and pelvic width were negatively correlated with calving difficulty score (-0.20 and -0.24, respectively) in Hereford heifers (Johnson et al., 1988). In addition, Berger et al. (1992) showed that calves born from Angus heifer had lower

survivability during the first 24 h of life compared with mature Angus cows. The increase in calves' mortality rate with heifers can be directly associated with maternal size. For example, young and small heifers give birth to weak calves, which are more prone to die early in life, whereas larger heifers may have higher odds of dystocia due to greater calf body weight and calving difficulties (Berger et al., 1992).

Finally, not only the maternal effects may impair calving procedure but also paternal performance characteristics; therefore, knowing the genetic potential of the sire is a key regulator of dystocia occurrence. The utilization of bulls with high mature BW is usually positively correlated to BW at birth. Consequently, selecting bulls with lower birthweight might help to avoid dystocia (Rice, 1994). Currently, several breeds have their own expected progeny difference (EPD) for the trait 'calving ease', which refers to the inherited expected ability of the fetus to be born without delivery assistance. For avoiding dystocia, the selection of bulls with birthweight EPDs below the average and calving ease EPDs above the average is the most accurate choice (Funnell and Hilton, 2016).

Rumen-protected methionine

General description of rumen-protected methionine

Methionine (Met), $C_5H_{11}NO_2S$, is a neutral, essential amino acid that belongs to the universally called sulfur-containing amino acids group together with cysteine, homocysteine and taurine. However, only methionine and cysteine can be incorporated into protein. Methionine has an essential role in sulfur-containing amino acids since it is the only capable to be converted into cysteine and cystine (Mastrototaro et al., 2016). Furthermore, because Met is a nonpolar amino acid, it is primarily located in the hydrophobic interior core of proteins (Brosnan and Brosnan, 2006).

In ruminants, Met is one of the three most limiting essential amino acids along with lysine and threonine (Nimrick et al., 1970; Richardson and Hatfield, 1978). Ruminal degradation of Met takes place thanks to the action of bacteria and protozoa species (Scheifinger et al., 1976; Onodera and Ushijima, 1982). A previous experiment using ruminal microbes from steers consuming a diet composed of 60% of concentrate showed that Met apparent degradation occurs at a rate of 82 %/h (percentage of 1.6 mM of Met apparently degraded during a 6-hr incubation period). In addition, the same experiment reported that *in vitro* degradation is slower when only Met is present compared with being in combination with other essential amino acids (82 vs. 44 %/h) (Chalupa, 1976).

Once the limiting amino acids were identified in ruminants (Nimrick et al., 1970; Fenderson and Bergen, 1975), one of the most successful strategies for ensuring an adequate supplementation was the encapsulation of nutrients, also called "rumen-protected". The utilization of rumen-protected nutrients aims at avoiding ruminal microbial degradation (Kung and Rode, 1996). Therefore, coated Met was designed to supply the required amount of Met at the intestinal level through ruminal by-passing. A study conducted by Papas et al. (1984) provided one of the first insights on the effects of Met provided post-ruminally. Authors reported a greater feed intake and average daily gain in Holstein cows compared with control. The utilization of Smartamine® (Adisseo NA, Alpharetta, GA) on cattle has been under research since the 1990s showing promising results (Armentano et al., 1997; Nichols et al., 1998). Two naturally occurring isomers of Met coexist: D- and L- Met. However, Met can only be utilized as L-Met by the organism at a greater extent; therefore, D-Met must be converted into L-Met. Moreover, D-Met is also metabolized, probably in the liver, but at a lower rate compared with L-Met (Lapierre et al., 2012). DL-Met represents a minimum of 70% of Smartamine composition,

and it is coated with a pH-sensitive copolymer called poly(2-vinylyridine-co-styrene). It has been previously shown that a pH-sensitive coat results in the lowest rumen degradation compared with Ca-soap and hydrogenated fatty acids (Rossi et al., 2003). Interestingly, the same study also showed that ethyl-cellulose coating, another type of coat that is widely used, release lower amounts of Met at the small intestine in comparison to pH-sensitive coat (Rossi et al., 2003). Based on this information, it is possible to infer that pH-sensitive coating allows the greatest Met bioavailability at the small intestine level compared with other types of coats currently available in the market.

The effects of RPM have been widely investigated in dairy cattle due to its ability to cope against metabolic disorders and to improve overall animal performance. For example, several studies indicate that the administration of RPM increases milk and fat protein in Holstein cows (Rulquin and Delaby, 1997; Kowalski et al., 2003; Misciatteilli et al., 2003). In addition, high-producing dairy cattle usually suffer a metabolic misbalance in the liver during the transition period which leads to fatty liver condition. It has been recently shown that supplementation with RPM improves the immune-metabolic status on cows maintaining liver health when compared with cows receiving rumen-protected choline, which is another important compound in the one carbon metabolism (Zhou et al., 2016). Lastly, maternal administration of RPM has a positive impact on reproductive parameters and lasting effects on the offspring. Even though the female reproductive tract presents adequate Met for embryo survival (Bonilla et al., 2010), the supplementation with RPM improves embryo implantation and development in multiparous Holstein cows (Acosta et al., 2016; Toledo et al., 2017). Regarding offspring performance, (Alharthi et al. (2018) reported that maternal supplementation with RPM during the last 28 days of pregnancy increase in utero and postnatal growth of offspring. Interestingly, the authors also

analyzed the impact of maternal supplementation with RPM on colostrum; however, there was no significant difference in calves fed colostrum from a cow supplemented with RPM compared with those without receiving RPM supplementation.

As mentioned, most of the studies investigating the effects of RPM supplementation have been performed using dairy cattle as the animal model; however, a growing area of research has been recently focused on beef species. For example, Silva et al. (2021) have shown that maternal supplementation with RPM tended to produce greater energy-corrected milk and milk protein values, whereas the offspring tended to improve apparent total tract fiber digestibility and feed efficiency. Similarly, another experiment revealed the lasting effect of maternal RPM supplementation during preconception and early pregnancy on beef offspring's transcriptome. More specifically, offspring born to cows receiving RPM presented modified gene expression patterns associated with myogenesis, adipogenesis, and the Wnt/β-catenin pathway (Liu et al., 2020).

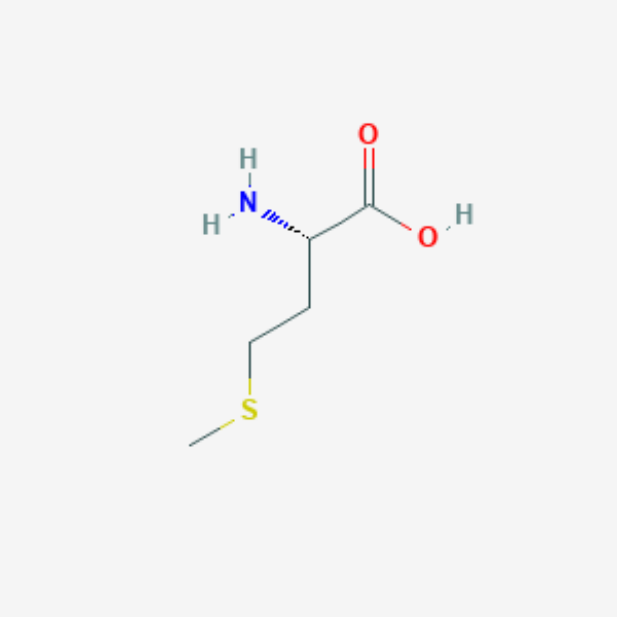


Figure 1.1. Depiction of the chemical structure of methionine. Source: PubChem.

Methionine metabolism

Methionine, in the non-ruminally protected form, suffers metabolic transformation along the gastrointestinal tract in ruminants. More specifically, in the rumen, Met can be reversibly converted to methionine sulfoxide by reductase enzymes from different microorganism species, to methanethiol for further incorporation into microbial protein, or converted into 2-ketobutyrate. Interestingly, 2-ketobutyrate in conjunction with ammonia can form propionate, which can be converted into glucose thanks to its glucogenic properties; or 2-amino butyrate, due to the action of protozoa. Furthermore, it has been shown that 2-amino butyrate can play an important role in glutathione homeostasis in myocardium; however, the effect of 2-aminobutyrate in other tissue remains to be investigated (Irino et al., 2016). At the same time, dimethyl sulfide, a compound found in milk, can be formed from methanethiol (Clark and Salsbury, 1980). At the intestinal level, microbial protein is digested and absorbed to be transported to different tissues. Finally, at the tissue level, methionine can follow different paths before being incorporated into protein, depending on the tissue or organ. For example, hepatocytes and renal cells can reduce methionine sulfoxide into Met. Consequently, the Met available at the tissue level is incorporated into protein or converted to cysteine, which also can be part of proteins (Figure 1.2) (Onodera, 1993).

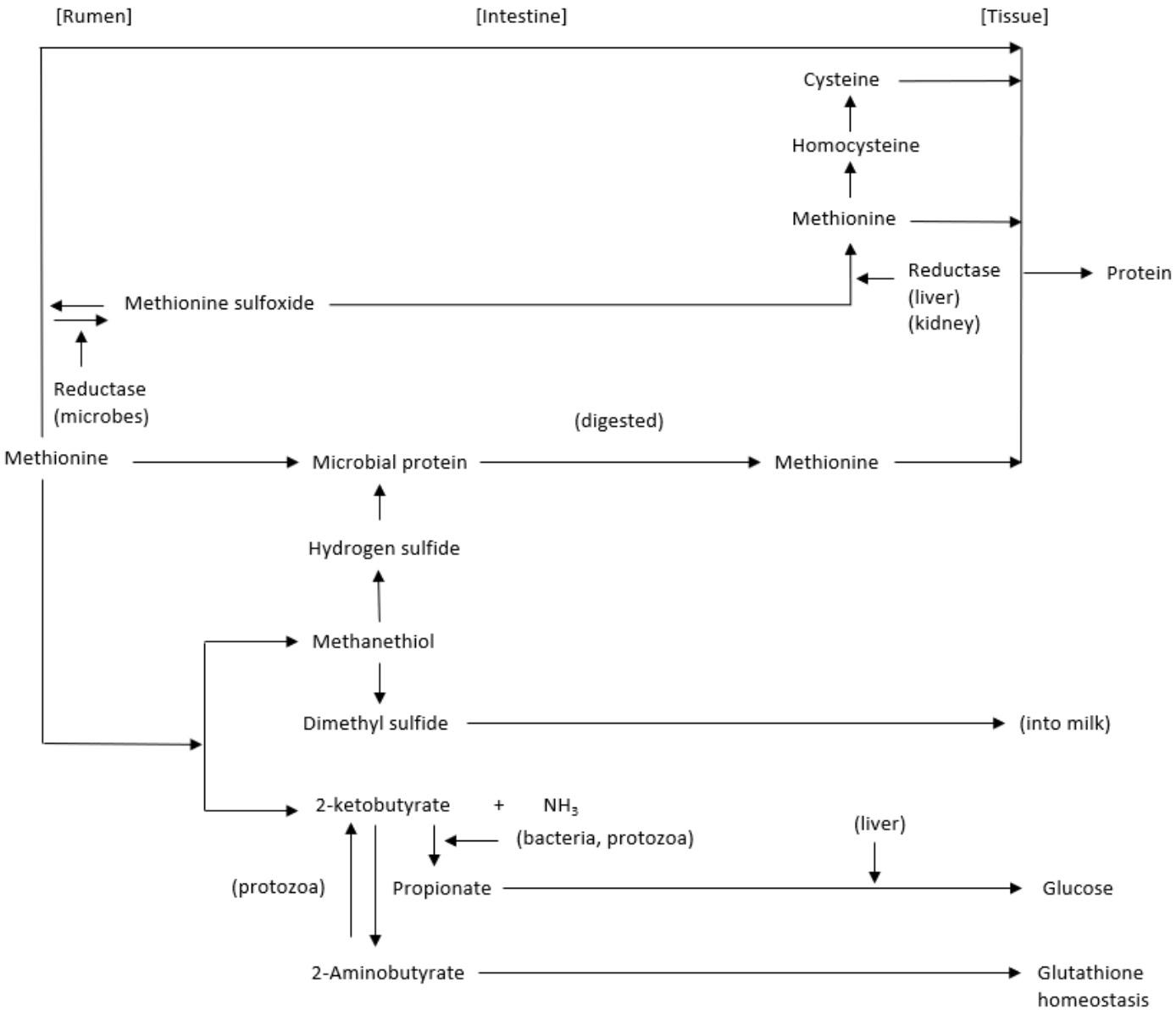


Figure 1.2. Methionine metabolism in the rumen, intestine, and tissue level on ruminants. Adapted from (Onodera, 1993; Lobley et al., 2003)

Biological functions of methionine

Glucogenic path and methylation

In ruminants, most of the carbohydrates from the diet are fermented into short-chain fatty acid in the rumen, and most of the required glucose is not absorbed at the gastrointestinal level

(Bensadoun et al., 1962; Knowlton et al., 1998). Consequently, gluconeogenesis is responsible for accounting for up to 90% of the glucose requirement (Young, 1977)

Methionine is a glucogenic amino acid that can be converted into cystathionine, through homocysteine, for further conversion into Succinyl-CoA, which is part of the TCA cycle. In addition, methionine is also part of gluconeogenesis since cystathionine can be converted to cysteine. Finally, pyruvate can be formed from cysteine, ending in glucose formation (Jacometo et al., 2017). In its path to conversion to glucose, methionine plays a crucial role in one-carbon metabolism, trans-methylation, and trans-sulfuration pathways, where it is part of the S-Adenosyl Methionine (SAM) formation, known as the universal methyl donor (Cantoni, 1975). The transfer of an adenosyl group from ATP to the sulfur group atom of methionine results in the formation of SAM. This process is regulated by an enzyme called methionine adenosyltransferase 1A (*MAT1α*), which is present primarily in the liver of mature mammals (Ramani and Lu, 2017). A previous study conducted by Ikeda et. al (2008) has found that CCAAT/enhancer binding protein-β (*C/EBP* β) plays a key role in *MAT1α* gene expression in mouse. The transcription factor *C/EBP* β binds to the promoter region of *MAT1α*, and this process is regulated by DNA methylation and histone acetylation. Authors reported a correlation between DNA hypermethylation and deacetyl-histone H3 in *MAT1α* gene promoters (Ikeda et al., 2008). S-Adenosyl methionine donates its methyl group to different substrates (i.e., cytosines, histones) for various biological processes and is converted to S-Adenosyl homocysteine (SAH). The enzyme that catalyzes the transfer of methyl groups from SAM depends on the acceptor molecule. For example, the transferase enzymes that act on SAM for the DNA methylation process are DNA Methyltransferase 1 and 3a (DNMT1 and DNMT3a, respectively) (Fatemi et al., 2002). The next step is the hydrolysis of SAH to form homocysteine,

catalyzed by the hydrolase enzyme called S-adenosyl homocysteine hydrolase (SAHH), also known as Adenocylhomocysteinase (*AHCY*) (Belužić et al., 2018). Cai et al. (2014) studied the effect of supplementing betaine, another compound involved in the one-carbon metabolism, to gestating sows. Authors found an upregulation in hepatic *AHCY* gene expression in the offspring, indicating that the increase of methionine concentration, through an increase in the concentration of a precursor as betaine, led to the upregulation of *AHCY* (Cai et al., 2014). Once homocysteine is formed, it is further converted into cystathionine by the action of an enzyme called Cystathionine beta-synthase (CBS). A previous study conducted by Zhao et al. (2012) suggested that *CBS* gene expression may be subject to epigenetic regulation through methylation (Zhao et al., 2012). Finally, cystathionine can follow two gluconeogenic routes: through α-ketobutyrate-Succinyl-CoA or Cysteine-Pyruvate (Jacometo et al., 2017).

Epigenetics and methylation

Even though the metabolism of methionine has been explained in previous pages, it is important to remark that one of the main objectives of this present thesis is to assess the effect of methionine as a methyl-donor compound on beef cattle genome. The epigenetic regulation by methylation has been widely studied in the literature among different species. Methylation can be defined as the covalent modification of DNA or histones by the addition of a methyl group. As mentioned, SAM is the chemical compound that donates the methyl group to DNA or histones. In DNA, methylation occurs mostly at CpG islands. CpG islands are clusters of dinucleotides (i.e., cytosine, and guanine nucleotides, located in CG-rich regions (>200 bp and GC pairs > % 50 content) along with the DNA that play a key role in epigenetic regulation of the genome (Janitz and Janitz, 2011). The methylation level of CpG islands leads to activation or repression of gene expression. For example, when methylation occurs in CpG sites located in a gene body,

gene expression is activated (Jones, 1999); however, when CpG sites are methylated in the promoter region, gene expression is repressed (Jang et al., 2017). Usually, approximately ~40% of genes in mammals present CpG islands on the promoter region, and they generally remain unmethylated. Therefore, methylation of CpG islands in the promoter region is used to ensure transcription initiation inhibition (Jones, 2012). Methylation of CpG islands takes place in response to the action of DNA Methyltransferase 1 (*DNMT1*), which is involved in the maintenance of DNA methylation, and *DNMT3a* and *DNMT3b*, enzymes related to DNA methylation and de novo DNA methylation (Fatemi et al., 2002; Jacometo et al., 2017).

Oxidative stress

Oxidative stress has been defined as the imbalance of the redox status of a cell due to the greater concentration of free radical elements such as superoxide (O_2^-) and nitric oxide (NO^-) that disrupt redox signaling and molecular damage (Sies, 2015). The main damaging compounds are reactive oxygen species (ROS) that are formed by the reduction of oxygen, such as hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-); and reactive nitrogen species (RNS), which are a result of nitrogen reduction, such as nitric acid ($HNO3^-$), and proxynitrite ($ONOO^-$) (Ferreira and Reid, 2008).

The cell has different mechanisms of defense for scavenging free radicals. For example, through a dismutation process, in which specific enzymes such as superoxide dismutase (SODs) cause spontaneous dismutation of O^- forming H_2O_2, and inhibits the formation of $ONOO^-$ by preventing the association of O^- and NO^-. In addition, glutathione pathway and glutathione peroxidase are responsible of generating H_2O and O_2 from H_2O_2 molecules (Powers et al., 2011). Previous studies indicate that rumen-protected methionine (RPM) supplementation affects the expression of genes related to dismutase enzymes, glutathione pathway, and glutathione

peroxidase. According to Dai et al. (2020), bovine mammary epithelial cells *in vitro* exposed to methionine resulted in a lower expression of *SOD1*. These findings support the results of an investigation conducted by our lab in which the expression of *SOD1* and *SOD2* in skeletal muscle were lower for heifers receiving RPM during 45 days compared with heifers without RPM supplementation (Alfaro et al., 2020). Even though, the lower expression of *SODs* was not a true activation due to RPM supplementation, it is possible to suggest that the greater availability of methyl groups may cause a decrease in free radical ions production.

Methionine is involved in the transsulfuration pathway due to its role as cysteine precursor. Cysteine and glutamate are the precursors of glutathione (Jacometo et al., 2017). The glutathione pathway and glutathione peroxidase (GPX) are characterized by protecting the cells from free radicals. Glutathione is generally in the reduced form (GSH) and is converted to glutathione disulfide (GSSG) after exposure to free radical ions. The reduction of GSSG takes places thanks to the action of a reductase enzyme called glutathione-disulfide reductase (GSR) (Jones, 2002). The site of expression of *GSR* is greater on liver tissue compared with skeletal muscle (Gsr (glutathione reductase)|Gene Report|BioGPS; http://biogps.org/). However, glutathione can be exported from the liver and transported by endosarcoplasmic reticulum membrane (Csala et al., 2003). A study conducted by Jacometo et al. (2017) reported an upregulation in hepatic glutathione synthase (*GSS), GSR*, and *GPX1* on calves born to cows receiving RPM supplementation. These results may indicate that greater glutathione levels may be produced in the liver of animals exposed to methionine, which potentially reach the skeletal muscle cells for balancing the redox status.

Fescue toxicosis

Tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort.) is a cool-season, perennial bunchgrass widely used in the southeast region of the United States thanks to its unique forage characteristics that provide high biomass productivity and quality. However, the outstanding production and quality rely on the symbiotic relationship that the plant present with a fungal endophyte (*Epichloë coenophiala*) (Chai et al., 2020). This mutualistic relationship benefits the endophyte by allowing an adequate environment for proliferation, and the plant also is benefited by a greater resistance to external factors, such as environmental or herbivores. The endophyte produces ergot alkaloids as second metabolites, which are detrimental for cattle. Fescue toxicosis includes a variety of negative symptoms as a result of ergot alkaloids consumption that will be fully described in a following session.

Effects of ergot alkaloids on the plant

The symbiotic relationship between the endophyte and tall fescue plants provides more nutrients to the fungus and greater pest, drought resistance and yield to the plant.

First, regarding herbivory resistance, it has been proposed that different anti-herbivory mechanisms are involved depending on the species consuming the plant. The most important alkaloids with anti-herbivory function in tall fescue can be divided in ergot and loline alkaloids (Bush et al., 1997). Loline alkaloids play a role in the anti-herbivory mechanism mainly against insects, acting as metabolic toxins and feeding deterrents, depending on the species. In contrast, ergovaline is toxic mainly to grazing animals. Loline alkaloids found in grasses infected with Epichloë present different isoforms due to changes in the 1-amino group, namely norloline, loline, N-methylloline (NML), N-acetylloline (NAL), N-acetylnorloline (NANL), and N-formylloline (NFL). The presence of different loline isoforms allows a broader number of

herbivore's species to target and reduce the possibilities to resistance by the herbivore species. For example, *Spodoptera frugiperda* larva growth can be affected by NFL and NAL, but loline and NML had no effect on the same parameter (Riedell et al., 1991). The diversification of loline is not only conducted by the endophyte itself but also the host plays an essential role in this process. A previous study conducted by Pan et al. (2014) has shown that the host plant is able to diversify Ergot metabolites. Authors found that an acetyltransferase of the plant is responsible enzyme to catalyze the conversion of NAL from loline. Therefore, the synthesis of NAL is host dependent. Meadow fescue (*Lolium pretense*) was used as the plant model in this study (Pan et al., 2014). Furthermore, the photosynthetic rate on infected plants has also been studied, showing that plants infected with *Epichloë* increase their photosynthetic rate, mainly due to the greater energy required to maintain both the plant and the fungus. A study using *Dactylis glomerata* infected with Epichloë as the plant model reported a three-fold increase in NADPH malate dehydrogenase (MDH), an enzyme that reversibly catalyzes the oxidation of malate to oxaloacetate, compared with control plants. Authors suggested that the greater expression of MDH may be related to an increase in energy demand of the plant due to the symbiotic relationship with the fungus; however, further research is needed to confirm this idea. Interestingly, the same study showed that enzymes involved in photosynthesis, such as Rubisco LSU, presented greater expression in orchard grass plants infected with *Epichloë* (Rozpądek et al., 2015). Temperature also plays an important role in the photosynthetic rate. Endophyte-infected tall fescue plants have greater photosynthetic activity at an ambient temperature higher than 25 ºC (Clay, 1990). Marks & Clay (1996) reported that E+ tall fescue plants photosynthesized at 20-25% greater rate than non-infected tall fescue plants (Marks and Clay, 1996). With the arrival of novel techniques, such as RNA-sequencing, transcriptome changes

due to temperature could be analyzed. A study conducted by Wang et al. (206) investigated the transcriptome changes due to heat and cold stress in Tall Fescue and Perennial Ryegrass. Authors found upregulations in the expression of genes that codify for heat stress shock protein in response to heat stress (40°C) and cold stress (-10°C) in both plants, indicating a possible role of these proteins in homoeothermic regulation (Wang et al., 2016).

Another benefit of the endophyte infection on the plant is drought resistance. Endophyte-infected tall fescue presents different physiologic responses to drought than endophyte-free varieties, such as stomatal closure and turgor pressure (Richardson et al., 1993) or an increase in root hair length (Malinowski et al., 1998). A previous study conducted by Nagabhyru et al. (2013) reported the greater adaptability of E+ tall fescue under drought conditions. Authors found the tiller number could persist under water-deficient conditions during the first three days after water removal from the soil compared with endophyte-free tall fescue under the same condition, in which tiller number dropped during that period mainly due to tiller death. Interestingly, this study also showed that proline, a precursor of loline, is present in greater levels for plants under water-deficiency conditions than watered plants. Furthermore, the loline levels were greater for E+ plants under drought conditions than well-watered E+ plants (Nagabhyru et al., 2013). These results may suggest that E+ tall fescue plants could activate their anti-herbivory mechanism under droughts conditions even to a greater extent when compared to normal, adequate environmental conditions. In order to obtain a deeper understanding of the mechanism of drought resistance, Dinkins et al. (2019) investigated the effects of water deficit at the transcriptome level of different tall fescue genotype varieties infected with different *Epichloë coenophiala* strains.

Interestingly, approximately 30% of the transcriptome was upregulated in plants (E+ and endophyte-free) under a water-deficiency scenario. The main pathways affected were related to oxidative stress, water stress, heat stress, osmotic stress, and the biosynthesis of proline. These findings support the previous study performed by (Nagabhyru et al., 2013), in which a greater proline level was observed. However, there was no pathway significantly affected either in an upregulated or downregulated manner when E+ plants under drought conditions were compared with E+ control (adequate water availability), except for a reduced number of genes that were differentially expressed. The main differentially expressed genes in unstressed E+ compared with endophyte-free tall fescue were related to dehydrin, a set of proteins that activate under cold or drought stress, and heat shock protein genes, indicating another factor that may prevent water-deficient physiological impairments (Dinkins et al., 2019).

The symbiotic relationship between the endophyte and the host enhances the survivability of both by an improved biotic and abiotic stressor resistance. The utilization of novel techniques offers a deep understanding of the defense mechanisms involved depending on the stressor agent.

Effects of ergot alkaloids on the animal

The consumption of ergot alkaloids causes numerous harmful effects on cattle health and performance. Ergot alkaloids, especially ergovaline, bind to monoamine neurotransmitters receptors (i.e., dopamine, serotonin, etc.) in the anterior pituitary. The mimicking effect of ergovaline on monoamine receptors can cause the inhibition of prolactin, adrenocorticotropic hormone (ACTH), and follicle-stimulating hormone (FSH). Prolactin synthesis is controlled mainly by the action of dopamine. This neurotransmitter can bind different types of dopamine receptors, which are different depending on the tissue. The inhibition of prolactin occurs by dopamine through binding the dopamine receptors located in the lactotropic cells of the anterior

pituitary. Dopamine-receptor 2 (*DRD2*) is present in the anterior pituitary and is coupled to a Gα protein that inhibits cAMP after dopamine coupling (Beaulieu et al., 2015). A previous study showed that ergovaline not only can bind to *DRD2* but also is able to inhibit cAMP production in a similar manner compared to dopamine *in vitro* (Larson et al., 1995). These findings were confirmed by Li et al. (2017) in a study that showed a lower serum concentration and a downregulation in prolactin and *DRD2* for steers consuming high-endophyte tall fescue compared with steers consuming low-endophyte fescue. Authors suggested that the lower expression of *DRD2* may be associated with a preventive mechanism of lactotropic cells to counter the lower production of prolactin (Li et al., 2017). In addition, prolactin also plays a role in hair growth in cattle, usually delaying or inhibiting the process (Littlejohn et al., 2014). During periods of increase in day length, cattle consuming E+ tall fescue present low prolactin levels leading to winter hair coat retention (Campbell et al., 2014). Like prolactin, ACTH synthesis is reduced by the inhibitory action of ergot alkaloids. Li et al. (2017) also found that the canonical pathway for glucocorticoid production was downregulated in steers consuming E+ tall fescue due to the downregulation of corticotropin-releasing hormone gene (*CRHR1*), which stimulates ACTH production. Finally, a previous study showed a reduction in circulating LH in steers due to the injection of the ergot alkaloid ergotamine (Browning and Leite-Browning, 1997). The decrease of LH levels may cause reproductive performance impairments associated with E+ tall fescue intake.

In mammals, the liver acts as the main detoxification organ. Liao et al. (2015) performed a comparative study in which beef steers were exposed to high vs. low endophyte-infected tall fescue. Authors found that genes related to ATP synthesis (*NDFC1*, *NDUFV2*, *ATP5D*, among others), proline and serine (*PYCR1* and *PSPH*, respectively), and pyruvate formation (*ALT2*)

were upregulated in steer consuming high-endophyte fescue. These results indicate that the exposure to high-toxic diets upregulates genes involved in energy metabolism (Liao et al., 2015). Similarly, mice receiving E+ diets had an upregulation of hepatic expression of ATP synthase H^+ transporting gene (*ATP5b)* which may be related to a feedback mechanism of hepatocytes due to a reduction in cholesterol levels in animals exposed to ergot alkaloids (Bhusari et al., 2006). However, this increase in ATP capacity by the liver could be a compensatory response to the greater need for meeting energy demands in the condition of a reduction of liver size due to fescue toxicosis occurrence (Brown et al., 2009). Similarly, a study conducted using rats as the animal model reported an upregulation of CYP isoforms, a set of genes that codify for proteins involved in the cytochrome P450 system, and a downregulation of genes that codify for antioxidant enzymes (*SOD1*, *SOD2*, *GPx*, and *GST*) (Settivari et al., 2008).

Finally, the vasoconstrictive effect of ergot alkaloids affects both peripheral and gastrointestinal vessels. The mechanism of action behind this symptom is also related to the inhibitory effect of ergot alkaloids on amine receptors, in this case, on adrenergic and serotonergic receptors. It was widely reported that ergovaline interacts with serotonergic receptors (serotonin receptors, 5-TH). A previous study conducted by Klotz et al. (2012) showed the effect on Angus steers grazing E+ tall fescue from 89 to 105 days. Authors reported lower serotonin and α-methylserotonin (5-TH2 receptor inhibitor) concentrations on animals exposed to high E+ tall fescue. Consequently, they had a greater contractile response of lateral saphenous veins. However, during the second experiment of this study, authors also confirmed that ergot alkaloids, such as ergonovine, ergocriptine, and ergotamine have greater affinities for the 5HT2A receptor in a reversible manner since the removal of E+ tall fescue for a period of three months caused the return to normal contractile response. Similarly, ergovaline may influence the normal

activity of serotonin receptor 5HT2A in ruminal and mesenteric vasculature (Trotta et al., 2018). Furthermore, α2-adrenergic receptor presents a higher affinity with ergopeptines and more participation in vasoconstriction than the α1-adrenergic receptor (Oliver et al., 1998).

From a biological standpoint, ergovaline presents an excellent mechanism of defense against mammal herbivory species due to its unique biochemistry structure that impairs normal animal metabolism by inhibiting amine receptors in the brain, smooth muscle, and internal organs vasculature. Based on this evidence, caution must be exercised when E+ tall fescue varieties are included in the forage system planning. Farmers need to have a basic knowledge about the negative effects of grazing E+ tall fescue, especially on gestating dams, which leads to offspring's growth and development impairments (Greene et al., 2020).

Rumen protected niacin

General description of niacin

Niacin, $C_6H_5NO_2$, is a water-soluble, essential B vitamin. Two forms of niacin exist: nicotinic acid and nicotinamide, also known as vitamin B_3. The term niacin refers to a broad description of pyridine 3-carboxylic acid and derived compounds with similar chemical structures and biological functions. Niacin is a pyridine monocarboxylic acid, in which a carboxyl group is the replacement of the hydrogen at position 3. Most niacin forms are part of pyridine nucleotides, more specifically nicotinamide ribonucleotide (NMN), and the nicotinamide adenine dinucleotides in their unphosphorylated (NAD) and phosphorylated (NADP) forms (Nakamura et al., 2012). Cattle can be fed with nicotinamide or nicotinic acid. As an example, ANEVIS™ (Qualitech, Chaska, MI) is composed by 70% of nicotinic acid, 20% of castor oil, and 10% of calcium.

In mammals, niacin can be synthesized from tryptophan in the liver. Non-essential amino acids, such as tryptophan, are important niacin precursors (Fukuwatari and Shibata, 2013). However, niacin deficiency, usually known as “pellagra”, occurs in humans from poor countries without access to sufficient dietary intake of niacin and protein. Pellagra was first recognized in 1735 by the Spanish physician Gaspar Casal, who associated the disease with poverty and low-quality corn consumption (Segula et al., 2012). The condition of pellagra is characterized by dermatitis, dementia, diarrhea, and in some cases death (Meyer-Ficca and Kirkland, 2016).

Interestingly, ruminal microbiota synthesis of B vitamins has been widely accepted among scientists (NRC Dairy Cattle, 2001). For example, research in the 1980s found that the amount of niacin present in the duodenum was greater than that provided in the diet, suggesting its synthesis by the microbial population (Miller et al., 1986; Zinn et al., 1987). Since B vitamins play an important role as a cofactor in lipid, protein, and carbohydrates synthesis, which increase dramatically during lactation, there may exist a need for supplementation, especially when high forage levels are included in the diet (Seck et al., 2017). Fiber length and maturity at harvest of ensiled forages are factors affecting niacin apparent synthesis by the rumen microbiome of dairy cows (Castagnino et al., 2016; Castagnino et al., 2017). Even though the supplementation with niacin on high-productive dairy cows was evaluated, showing positive effects (Schwab et al., 2005), little information is available of niacin supplementation on beef cattle. Since an increment in milk production is observed in lactating dairy cows supplemented with niacin, especially during early lactation reducing fat mobilization (Flachowsky, 1993), including niacin to gestating beef dams in the periparturient period may arise as a potential enhancer of milk yield, improving weaning weight of the offspring in cow-calf systems.

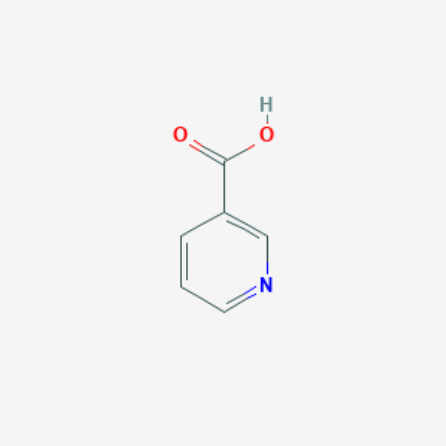


Figure 1.3. Chemical structure depiction of nicotinic acid. Source: PubChem

Niacin metabolism

Niacin synthesis

Microorganisms and plants differ from mammals regarding niacin synthesis due to the ability to synthesize the pyridine ring of NAD from aspartic acid and dihydroxyacetone phosphate. Hence, mammals obtain the pyridine ring structure from dietary nicotinic acid, nicotinamide, pyridine nucleotides, quinolinate and tryptophan (Foster and Moat, 1980; Henderson, 1983). In monogastric animals, the synthesis of niacin takes place thanks to the action of the microbiota present at the large intestinal level; however, its production is apparent insignificant because niacin is easily eliminated in the feces (Coates et al., 1968). On the contrary, ruminant animals are capable of synthesizing niacin by the action of microorganisms present at the ruminal level. Tryptophan can be converted into Kynurenine by the action of two enzymes which are organ-dependent: Tryptophan 2,3 dioxygenase, occurring in hepatic cells; and indoleamine 2,3-dioxygenase, which catalyze the conversion extrahepatically (Thackray et al., 2008). The conversion of 2-Amino 3-carboxymuconate-6 semialdehyde (ACMS) into quinolinate takes place spontaneously. In order to synthesize nicotinic acid mononucleotide, quinolinic acid phosphoribosyl transferase add a phosphoribosyl group to quinolinate. Also,

nicotinic acid, which could be provided by the diet, can receive a phosphoribosyl group thanks to the action of nicotinic acid phosphoribosyltransferase in order to be converted into nicotinic acid mononucleotide (Terakata et al., 2012). Furthermore, nicotinic acid mononucleotide receives an extra nucleotide by the action of nicotinamide mononucleotide adenyltransferase for the synthesis of nicotine adenine dinucleotide. Glutamine and ATP interact with nucleotide adenine dinucleotide, resulting in NAD^+ and glutamate, AMP, and a phosphate group as subproducts. NAD^+ can be converted into nicotinamide by the removal of ADP-ribose. Furthermore, nicotinamide can follow two paths: re-conversion into NAD^+ through nicotinamide nucleotide; or nicotinic acid mononucleotide through nicotinic acid (Zempleni et al., 2014).

At the intestinal level, the primary route for niacin absorption is passive diffusion. In the early 1960s, Turner & Hughes investigated the passage mechanisms of B vitamins, including nicotinic acid, using rats as the animal model. Authors in this study concluded that passage across the intestine occurs without energy expenditure (Turner and Hughes, 1962). Similarly, Erickson et al. (1991) evaluated the absorption of nicotinic acid and nicotinamide at the ruminal level *in vivo* using cannulated Holstein cows in mid-lactation. Interestingly, nicotinamide was absorbed at a rate of 0.98 g/h. In contrast, nicotinic acid did not show absorption in a 1-hour period, probably because it is ionized at the pH (~7) of the buffer, and its pKa is lower compared with nicotinamide (4.85 vs 14). Therefore, most of the nicotinic acid could be ionized and not ready for absorption (Erickson et al., 1991).

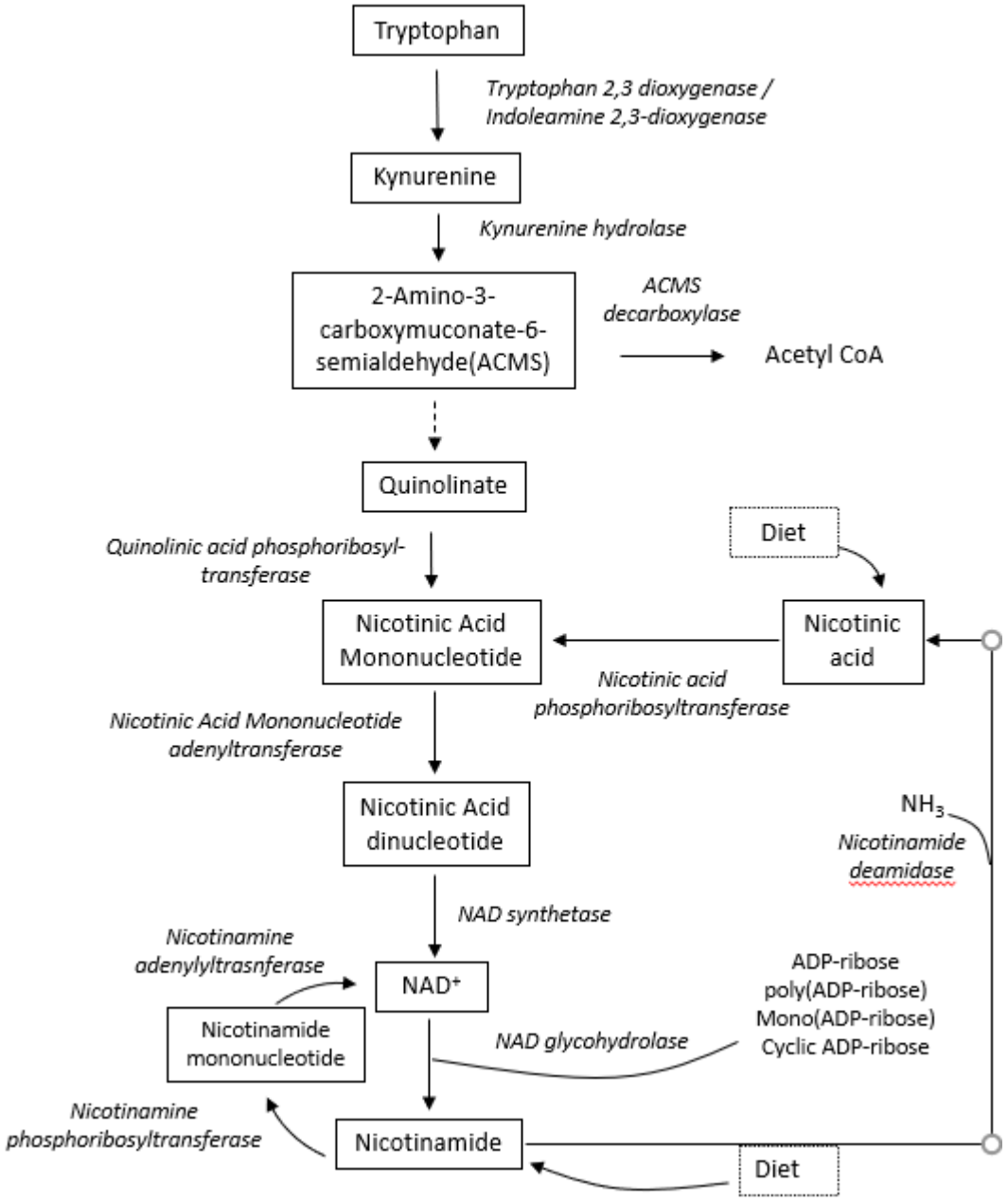


Figure 1.4. Niacin synthesis pathway. Adapted from (Henderson, 1983; Zempleni et al., 2014)

Niacin in coenzymes

Niacin is part of the cofactors NAD^+ and $NADP^+$ in the oxidized form and NADH or NADPH in the reduced form. Enzymes that depend on niacin are numerous and represent an important factor in metabolism in all organisms. For example, NAD^+ participates in approximately 400 reactions, whereas $NADP^+$ in 30 (Zempleni et al., 2014). The major role of NAD^+ and $NADP^+$ is to regulate cellular electron transfer reactions. Thus, NADH and NADPH are strong electron donors, participating in the detoxification of the cells through maintenance of the redox status of the cell (Ying, 2008). Furthermore, NAD^+ and $NADP^+$ play a key role in the metabolism of carbohydrates, fatty acids, and amino acids. For instance, the conversion of pyruvate to lactate via lactate dehydrogenase (LDH), causes regeneration in $NAD^{+,}$ which is critical for the glycolysis process since the NAD^+/NADH redox balance needs to be stable (Lunt and Vander Heiden, 2011). Likewise, NAD^+ is involved in the citric acid cycle. For example, NAD^+ acts as a cofactor for Pyruvate dehydrogenase, Isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and Malate dehydrogenase in the formation of Acetyl-CoA, α-ketoglutarate, Succinyl-CoA, and Oxaloacetate, respectively. Lastly, cytochrome P-450 (CYP) enzymes are group of detoxification proteins highly conserved in all organisms, from bacteria to mammals. In the liver, the primary organ for detoxification in mammals, CYP enzymes depends on the action of NADPH for their regeneration by a continuous re-reduction that takes place thanks to the action of NADPH-dependent cytochrome P450 reductases (Agledal et al., 2010).

Biological functions of niacin

Vasodilation

Niacin presents an important pharmacological use to counterattack dyslipidaemia, a condition characterized by high levels of low-density cholesterol. The administration of niacin increases high-density lipoprotein cholesterol due to its capacity to raise HDL cholesterol levels (Capuzzi et al., 1998; Guyton et al., 2000). However, long-term treatments with niacin leads to a side effect called flushing, in which cutaneous vasodilation occurs as result of the activation of the niacin receptor G protein-coupled receptor (GPR109A) located in dermal Langerhans cells (Benyó et al., 2006). In humans, niacin administration enhances prostaglandin D_2 release and stimulates cutaneous vasodilation (Morrow et al., 1992).

According to the physiological benefits of vasodilation on animals exposed to high ambient and humidity temperatures that allow heat dissipation, previous studies have shown a positive effect of rumen-protected niacin supplementation. For example, a lower average vaginal temperature and rectal temperature on moderately heat-stressed Holstein cows (Zimbelman et al., 2010). Similarly, Di Constanzo et al. (1997) reported a lower skin temperature for Holstein cows receiving niacin supplementation under mild or severe heat stress (Di Costanzo et al., 1997). In addition, a study conducted by Xiao et. al. (2017) found a greater expression of genes that codify for heat-shock proteins *HSP70* and *HSP27* in mammary alveolar cells, greater in mammary epithelial cells, but lower in endometrial cells *in vitro* (Xiao et al., 2017). Furthermore, the consumption of ergot alkaloids cause vasoconstriction (Klotz et al., 2016), which is the opposite to the vasodilatory effect of niacin. A recent study trying to alleviate the vasocontrictive effect of endophyte-infected tall fescue in uterine blood flow of pregnant Angus × Simmental cows and heifers consuming endophyte-infected tall fescue seeds (Gard Schnuelle et al., 2021) did not

show any significant effect on blood flow after administration of rumen-protected niacin. However, little is known about the impact of niacin supplementation on beef breeds exposed to heat-stress scenarios.

Niacin as a signal molecule

The maintenance of redox status acts on gene expression regulation. One example of this phenomenon occurs in mammalian's brain by the activity of NPAS2:BMAL1 heterodimer, a regulator of the circadian clock. A study performed by Rutter et al. (2001) found that the circadian clock is controlled by the coenzymes NADH and NADPH by enhancing the binding of transcription factors to the DNA; whereas the oxidized forms, NAD^+ and $NADP^+$, inactivate the binding process (Rutter et al., 2001). Similarly, NAD^+ is also involved in DNA repair pathway of mammalian's cells. The proteins participating in the coordination, regulation, and execution of base excision repair processes are NAD^+-dependent (Saville et al., 2020).

Oxidative stress

Antioxidants are regenerated and maintained by the presence of NADPH for sustaining a normal cellular redox balance. For example, glutathione plays an essential role in the antioxidant defense system, being the core of glutathione peroxidase and glutathione S-transferase. The conversion of glutathione disulfide into two molecules of glutathione is catalyzed by the enzyme glutathione reductase, in which NADPH acts as the coenzyme (Agledal et al., 2010). In addition, glutathione reductase and peroxidase, another enzyme called catalase breaks down the toxic compound hydrogen peroxide (H_2O_2). Catalase requires the electron donation of NADPH in order to be activated. The inactive form of catalase, also known as compound II, requires the reduction of the heme core for achieving protection against H_2O_2 (Kirkman et al., 1999).

Summary and conclusion

Pregnancy is a complex process in which several factors can have negative or positive effects on both the dam and the offspring. Understanding the mechanism behind the growth and development of the fetus could help cow-calf producers to implement strategies to enhance productive, desirable characteristics while reducing detrimental events affecting animal's performance. As discussed previously, planning and decisions before breeding such as parental genetic and time and type of nutritional supplementation directly affect gestation and calving. For example, the occurrence of dystocia is tightly related with the selection of inappropriate sires. The maternal supplementation with rumen-protected nutrients (i.e., methionine), could also represent a feasible strategy for enhancing offspring post-natal growth and development. Furthermore, RPM is involved in the one-carbon metabolism and plays an essential role in DNA methylation. These changes at the transcriptome level may benefit traits with high importance in terms of productivity (e.g., milk production, fat production).

Additionally, many cow-calf operation systems located in the Southeast region of the US are based on tall fescue pastures, and consequently, fescue toxicosis arises as one of the major problems in cattle production systems. The symptoms associated with fescue toxicosis also impairs fetus' growth and development, with a carry-over effect in calf's postnatal life. The consumption of ergot alkaloids usually causes vasoconstriction in animals. Moreover, niacin is widely known as a vasodilator; therefore, the utilization of RPN could help to cope with the vasoconstrictive effect of E+ tall fescue.

In conclusion, even though most of the previous research related to the supplementation with rumen-protected nutrients (i.e., methionine and niacin) was focused on dairy cattle, the utilization of new techniques, such as RNA-sequencing, could reveal metabolic changes in beef

cattle. More investigation is needed to fulfill the lack of knowledge regarding supplementation of rumen-protected nutrients on fetal programming on beef cattle and its effects on health performance under exposure to E+ diets.

Chapter 2
Fetal programming effect of rumen protected methionine on Angus × Simmental cows and heifers offspring's performance and skeletal muscle gene expression

Introduction

The utilization of protected amino acids has been rising in the dairy industry during the last several decades. High-producing dairy cows are usually deficient in methionine, and dietary supplementation improves overall health and milk production (Rulquin and Delaby, 1997; Kowalski et al., 2003; Misciatteilli et al., 2003). Nevertheless, the benefits of maternal supplementation with rumen-protected methionine (RPM) also extends into the gestating offspring. In addition, methionine plays an essential role in methylation because it is a precursor for S-Adenosyl Methionine, known as the major methylator compound (Cantoni, 1975). Due to the positive results obtained in investigation on dairy cattle, a novel area of research analyzes the effects of RPM on beef cattle. In the Southeast region of the US, the cow-calf operation system represents the predominant method of production. Thus, identifying novel, beneficial dietary strategies for improving both the dam and the offspring could result in positive productive and economic outcomes.

Gestation differs vastly between primiparous and multiparous mammals. Consequently, the postnatal growth and development of the offspring is tightly related to the maternal age.

To the best of our knowledge, no previous evidence showed the effects of maternal RPM supplementation on beef cattle dams on adipogenic, oxidative stress, and DNA methylation on offspring's skeletal muscle. Therefore, the objectives of our study were: 1) to identify changes in body weight, body condition score, and milk production on dams receiving RPM during the last trimester of gestation and the first ~85 days of lactation; 2) to asses changes in body weight, average daily gain, blood metabolites related to liver health on calves supplemented with

methionine; and 3) to analyze changes in adipogenic, oxidative stress, and DNA methylation-related genes of calves born to supplemented methionine dams.

Material and methods

Animals, dietary treatments, and experimental design

All procedures were approved by the Auburn University Animal Care and Use Committee (IACUC; PRN# 2017-3154). This study involved two calving season periods from October 2017 to May 2019.

A group of Angus, Angus × Simmental, and Simmental sired dams (n = 44) were located at North Auburn Research Unit, Auburn University, AL (32°41'N, 85°30'W) and divided into two groups based on maternal parity in order to identify effects regarding maternal age: primiparous (PRIM) and multiparous (MULT; Figure 2.1). Primiparous dams were artificially inseminated with semen from a mature, pure bred Simmental bull; whereas MULT dams were inseminated with a 5/8 Simmental, 3/8 Angus bull. Pregnancy pre-checking was performed before the beginning of the study to confirm pregnancy status. Calves' sex was identified by pre-check using trans-rectal ultrasound where the pregnant uterus and fetus can be identified. Primiparous dams calved from January to March, 2018; whereas MULT dams calved from September through October, 2018. Primiparous dams averaged 449 ± 32 kg, and MULT 515 ± 41 kg. In addition, each group was split into two treatment groups based on rumen-protected methionine inclusion in the diet: Rumen Protected Methionine treatment (RPM; n = 11) and Control (CTRL; n = 11). A successful adaptation to individual feeders separated with fences used for better control of nutritional treatments was performed in 5 days. After the adaptation period, dams received bermudagrass (*Cynodon dactylon*) hay *ad libitum* in combination with an individual, daily supplementation of 2.06 kg soybean hulls and corn gluten pellets in a 1:1 ratio (Table 2.1). In addition, RPM dams received 8 g/hd/day of top-dressed RPM at a fixed rate (Smartamine®, Adisseo NA, Alpharetta, GA; 0.07% DMI). Smartamine® presents a minimum

of 70% of Met, coated with a pH-sensitive polymer with a Met bioavailability of 80% (Schwab, 2007). Therefore, per 8 gr of RPM, animals received a minimum of 4.48 gr of metabolizable Met. Body condition score (BCS) was visually estimated by the same trained person on each dam at calving and 60 days after calving. Body condition was scored in increments of 1 unit, using a scale ranging from 1 to 9 (1 = thin and 9 = fat; Renquist et al., 2006). Pelvic area was determined using a sliding caliper. Pelvis height was obtained by measuring the linear distance between the approximate midpoint of the symphysis pubis to the bottom mid sacrum, whereas the pelvic width was estimated by measuring the linear distance between the shafts of the ilia. The pelvic area (cm^2) was determined by the measured length multiplied by the measured width (Laster, 1974).

At calving, birth date and body weight (BW) of calves were recorded only in male calves, whereas female calves were not included in the study. Calves had free access to maternal milk, bermudagrass hay, and Rough-N-Ready™ creep feeder starter (Archer Daniels Midland Company, Quincy, IL) to stimulate solid feed intake (Table 2.2). Calves were at least 45 days old at weaning. After early weaning (84 days of life on average), calves were relocated to Sugg Laboratory Pens for Animal Health Research pens (32°36'N, 85°31'W), College of Veterinary Medicine, Auburn, AL, where they received *ad libitum* bermudagrass hay in addition to 1% of BW of early weaning feed. Furthermore, calves born to RPM dams continued their maternal nutritional treatment post-weaning (Table 2.3). The inclusion rate of RPM in calves was adjusted after each BW measurement, and it ranged from 1.5 to 3 g/hd/day (0.07% DMI).

Both WCS dams and calves had *ad libitum* access to mineral salt supplement Stockman's complete (Sweetlix, Mankato, MN) in PRIM containing salt (NaCl, 11-13%), Ca (23-27.5%), Mg (4%, min), Mn (3,950 ppm, min), Cu (2500 ppm, min), Zn (6,000 ppm, min), I (150 ppm,

min), Se (26 ppm, min), Co (40 ppm), Vit A (660,000 IU/kg, min), Vit D3 (66,000 IU/kg, min), and Vit E (440 IU/kg, ppm); whereas MULT had *ad libitum* access to AMPT-A™ Mineral (ADM, Chicago, IL) containing salt (NaCl, 21%), Ca (15%), P (4%), Mg (3%), Co (150 ppm), Cu (1,200 ppm), Mn (3,600 ppm), I (200 ppm), Se (25 ppm), Vit A (90,900 IU/kg), Vit D (11,000 IU/kg), and Vit E (220 IU/kg).

Body weight from PRIM dams was obtained on 1/10/2017 (beginning of the study) and 5/3/2018 (weaning); whereas MULT dams' body weight was obtained at 7/26/2018 (beginning of the study) and 1/7/2019 (weaning). Calves BW was obtained at weaning, 25, 50, 80, and 100 days after weaning. Additionally, milk production was estimated by the weight suckle weight method (Beal et al., 1990). Briefly, at approximately 60 days of lactation, calves were separated from dams at 0800h for a period of 24 hours and relocated to a contiguous pen with *ad libitum* access to water. The following day, calves were weighed at 0800h and relocated with dams for a period of 30 minutes. After ensuring that all calves nursed properly from their dams, calves' BW was obtained and the difference between the initial and posterior BW was considered the estimation of maternal milk production per day.

A traditional knife castration was performed at 35 d and 39 d after weaning in PRIM and MULT calves, respectively. Immediately after castration, calves were implanted with Synovex® C (Zoetis, Parsippany, NJ), containing 100 mg progesterone and 10 mg estradiol benzoate. Simultaneously, calves received vaccination with Vision®7 (Merck, Kenilworth, NJ).
At 84 days after weaning in PRIM and 82 days after weaning in MULT, calves were orally dewormed using Safe-Guard® suspension 10% (Kenilworth, NJ), which contains Fenbendazole as the main active ingredient, at the recommended rate of 5 mL/100 kgs.

Muscle biopsies

Skeletal muscle biopsies of *Longissimus dorsi* were performed at weaning, 25, 50, and 100 days after weaning for gene expression analysis using real-time quantitative PCR (RT-qPCR). Each muscle sample was obtained from the left side with the incision side positioned progressively more caudal with each sequential biopsy for at least 5 cm. Anesthesia injections of 5 mL of lidocaine 2% (VetOne®, Boise, ID) were injected before each biopsy incision. A biopsy core of muscle (600 - 800 mg) was removed using a sterile skeletal muscle biopsy needle, placed in sterile 2 mL tubes, and immediately stored in liquid nitrogen for transportation and storage at -80°C until further analysis. No signs of infection, swelling or external bleeding were detected on the biopsy site after each procedure or in the following days. Biopsy procedures did not affect feed and water intake during the consequent days (Alfaro et al., 2020).

Serum analyses

Ten mL of blood were collected via jugular venipuncture on calves at 1 (weaning), 25, 50, and 100 days after weaning into blood collection tubes (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ). Serum was separated by centrifugation at 1,500 × g for 15 min, and an aliquot was stored frozen at -20 °C until analyzed for glucose, albumin, aspartate transaminase, alanine transaminase, and alkaline phosphatase at the Auburn University Endocrine Diagnostic Lab, Auburn, AL. All serum analyses were performed using a Roche/Hitachi Cobas C® c 311 analyzer (Roche, Basel, Switzerland) for clinical chemistry (Bowling and Katayev, 2010).

Glucose concentration was measured using the following working solutions: Reagent 1 (MES buffer: 5.0 mmol/L, pH 6.0; Mg2+: 24 mmol/L; ATP: $\geq$ 4.5 mmol/L; NADP: $\geq$ 7.0 mmol/L; preservative), and Reagent 2 (HEPES buffer: 200 mmol/L, pH 8.0; Mg2+: 4 mmol/L; HK (yeast): $\geq$ 300 μkat/L; G6PDH (E. Coli): $\geq$ 300 μkat/L; preservative).

Albumin concentration was analyzed using: Reagent 1 (Citrate buffer: 95 mmol/L, pH 4.1; preservatives, stabilizers), and Reagent 2 (Citrate buffer: 95 mmol/L, pH 4.1; bromcresol green: 0.66 mmol/L; preservatives, stabilizers).

Aspartate transaminase levels were analyzed using the following solutions: Reagent 1 (TRIS buffer: 264 mmol/L, pH 7.8 (37°C); L-aspartate: 792 mmol/L; MDH (microorganism): $\geq$ 24 µkat/L; LDH (microorganisms): $\geq$ 48 µkat/; albumin (bovine): 0.25%; preservative), and Reagent 2 (NADH: $\geq$ 1.7 mmol/L; 2-oxoglutarate: 94 mmol/L; preservative).

Alanine transaminase was analyzed with the following solutions: Reagent 1 (TRIS buffer: 224 mmol/L, pH 7.3 (37°C); L-alanine: 1120 mmol/L; albumin (bovine): 0.25%; LDH (microorganisms): $\geq$ 45 µkat/L; stabilizers; preservatives), and Reagent 2 (2-oxoglutarate: 94 mmol/L; NADH $\geq$ 1.7 mmol/L; additives; preservative).

Alkaline phosphatase concentration was measured using Reagent 1 (2-amino-2-methyl-1-propanol: 1.724 mol/L, pH 10.44 (30°C); magnesium acetate: 3.83 mmol/L; zinc sulfate: 0.766 mmol/L; N-(2-hydroxyethyl)-ethylenediamine triacetic acid: 3.88 mmol/L), and Reagent 2 (p-nitrophenyl phosphate: 132.8 mmol/L, pH 8.50 (25°C); preservatives).

Creatine kinase levels were assessed using the following solutions: Reagent 1 (Imidazole buffer: 123 mmol/L, pH 6.5 (37°C); EDTA: 2.46 mmol/L; Mg^{2+}: 12.3 mmol/L; ADP: 2.46 mmol/L; AMP: 6.14 mmol/L; diadenosine pentaphosphate: 19 µmol/L; $NADP^{+}$ (yeast): 2.46 mmol/L; N-acetylcysteine: 24.6 mmol/L; HK (yeast): $\geq$ 36.7 µkat/L; G6PDH (E. coli): $\geq$ 23.4 µkat/L; preservative; stabilizers; additives), and Reagent 2 (CAPSO buffer – 3-(cyclohexylamine)-2-hydroxy-1-propanesulfonic acid: 20 mmol/L, pH 8.8 (37°C); glucose: 120 mmol/L; EDTA: 2.46 mmol/L; creatine phosphate: 184 mmol/L; preservative; stabilizers).

Lactate dehydrogenase levels were assessed using the following solutions: Reagent 1 (N-methylglucamine: 400 mmol/L, pH 9.4 (37°C); lithium lactate: 62 mmol/L; stabilizers), and Reagent 2 (NAD: 62 mmol/L; stabilizers, preservatives).

RNA extraction

Approximately 100 mg of skeletal muscle tissue from each calf at each biopsy date was immersed in 1 mL of QIAzol Lysis Reagent (Qiagen, Hilden, Germany; Cat. #: 79306), homogenized for 1 minute, cooled on ice for 1 minute, and finally homogenized for another extra 1 minute. After 5 minutes of incubation on ice, each sample received 0.2 mL of chloroform and was vigorously shaken by hand for 15 seconds. Then, after 2 minutes of incubation, samples were centrifuged for 10 minutes at 12,000 × g at 4°C. After centrifugation, each tube presented a lower red phenol-chloroform layer, and interphase, and a colorless upper aqueous phase, which contains the RNA. Consequently, the upper phase was transferred to a new tube, and 0.5 mL of isopropanol was added and then incubated for 10 minutes. The following step consisted of centrifugation for 10 minutes at 12,000 × g at 4°C in which total RNA is precipitated as a white pellet at the bottom of the tube. The supernatant was discarded, and the pellet was re-suspended in 1 mL of 75% ethanol and centrifuged for 5 minutes at 7,500 × g at 4°C. Afterward, the tubes containing RNA pellets were opened for 10 minutes under the hood in order to be exposed to air drying. Finally, 20 µL of RNAase-free water were added directly to resuspend the RNA pellet. The RNA concentration was obtained using Nanodrop™ One^{C} (ThermoFisher, Waltham, MA). Samples were cleaned if they presented lower 260/280 (>1.90) and 260/230 (>1.70) ratios using RNA Clean & Concentrator kit according to manufacturer's protocol (Zymo Research Catalog # R1015). The integrity of RNA was assessed by electrophoresis gel, and samples showing two clear, parallel ribosomal bands were considered acceptable.

Primer design

The cDNA sequences for genes used were found at National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) or at University of California-Santa Cruz's Genome Browser (https://www.genome.ucsc.edu/). The sequences obtained were entered into Primer Express 3.0.1 software (ABI). The default settings (TaqMan® MGB quantification) were used; however, amplicon size was modified to 100 base pairs. The designed primer sequences were uploaded in the blast tool of NCBI Nucleotide Blast and ordered from Integrated DNA Technologies (https://www.idtdna.com).

cDNA synthesis

Complementary DNA (cDNA) was transcribed from clean RNA at a concentration of 100 ng/µL. First, Master Mix 1 (MM1) was prepared by mixing 9 µL of RNase-free water to 1 µL of Random Primers (Roche Diagnostics, Indianapolis, IN). Later, 1 µL of 100 ng total RNA was added. The mixture was incubated at 65ºC for 5 minutes. Then, the samples were incubated on ice for 3 minutes. For each sample, Master Mix 2 (MM2) was prepared by mixing 1.625 µL RNase-free water, 4 µL 5X First-Strand Buffer, 1 µL Oligo dT18, 2 µL 10 mM dNTP mix (10 mM), 0.25 µL of Revert aid (200 U/µL), and 0.125 µL of RNase inhibitor (20U/µL). Then, MM2 was mixed with MM1 + RNA (final volume of 20 µL and multiplied by total genes analyzed). The incubation protocol was as follows: 25ºC for 5 minutes, 42ºC for 60 minutes, and 70ºC for 5 minutes followed by 4ºC. A pooled sample was obtained from all samples to design the standard curve. Then, the pooled sample was diluted to a 1:2 ratio with RNase-free water for the first standard curve point. The subsequent standard curve points were diluted to a 1:4 ratio.

Preliminary primer testing

The testing of primers was performed as follow: 1 µL of Forward primer, 1 µL Reverse primer, 8 µL of pooled cDNA, and 10 µL of Perfecta SYBR Green through PCR. Samples were placed in an Eppendorf nexus gradient thermocycler (Hamburg, Germany) for 2 minutes at 50°C, 10 minutes at 95 ºC, 40 cycles of 15 seconds each at 95ºC and 1 minute at 60ºC for denaturation. Five µL of the PCR product were transferred to a new 0.2-mL PCR tube for agarose gel electrophoresis and mixed with 2 µL of loading dye. The ladder was prepared by mixing 0.6 µL of ladder (25 bp, from Invitrogen, Carlsbad, CA) with 2 µL of loading dye. In addition, 3 g of OmniPur® agarose (Calbiochem, San Diego, CA) were dissolved in 150 mL of TAE Buffer (Invitrogen, Carlsbad, CA; Cat # 15558-026). The agarose mix was heated for 1 minute in a microwave or until being completely disolved. Two µL of SYBR Safe were added to the agarose mix before cooling and then placed in the agarose gel apparatus. Ladders were added to the first well of each row, and the samples were added to the remaining empty wells. The gel ran at 80 mV until samples and ladder reached ¾ of the gel path.

Bio-Rad Chemi Doc XRS+ (Bio-Rad, Hercules, CA) apparatus was used to analyze the gel, utilizing Image Lab software (Bio-Rad, Hercules, CA). The accepted primers had a clear and single band at 100 bp. Finally, the accepted PCR products were cleaned with QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany; Cat. # 28104), before sending them for Sanger sequencing analysis to the University of Illinois Core Sequencing facility. Sequencing results were blast in NCBI website. The sequencing results that matched the primers blast were utilized. The following genes were selected as internal controls: In PRIM, Mitochondrial Ribosome-Associated GTPase 1 (*MTG1*), Ribosomal Protein S15a (*RPS15A*), and Ubiquitously Expressed

Prefoldin Like Chaperone (*UXT*); whereas in MULT, *UXT*, *RPS15A*, and Beta-2-Microglobulin (*B2M*).

Furthermore, Peroxisome proliferator-activated receptor gamma (*PPARg)*, Lipoprotein lipase (*LPL*), CCAAT/Enhancer Binding Protein (C/EBP) Gamma (*CEBPG*), CCAAT/Enhancer Binding Protein (C/EBP) delta (*CEBPD*), DNA Methyltransferase 1 (*DNMT1*), and Superoxide dismutase 2 (*SOD2*) were the selected target genes for this study.

Real time-Polymerase Chain Reaction (RT-PCR)

The last step in the gene expression analysis was RT-PCR. In PRIM, 8 µL of diluted cDNA sample, negative control, and standard curve were pipetted into their respective wells of a MicroAmp™ Optical 96-well (ThermoFisher, Waltham, MA) reaction plate in duplicates. Later, 10 µL of SYBR Green Master Mix, composed by 8 µL of PerfeCTa SYBR Green (Quanta Biosciences INC., Beverly, MA), 0.6 µL Forward Primer, 0.6 µL Reverse Primer, and 0.4 µL of water was pipetted into each well. The PCR reaction was executed in an ABI Prism 7500 HT SDS machine set to 2 minutes at 50ºC, 10 minutes at 95ºC for holding stage; 40 cycles of 15 seconds each at 95ºC and 1 minute at 60ºC for cycling stage; and 15 seconds at 95 ºC, 1 minute at 60 ºC, 30 seconds at 95 ºC, and 15 seconds at 60 ºC for dissociation curve stage. The data obtained were analyzed using the 7500 HT Sequence Detection Systems Software (version 2.3, Applied Biosystems, Foster City, CA). In MULT, 2 µL of diluted cDNA sample, negative control, and standard curve were pipetted into their respective wells of a LightCycler RNAse/DNAse 96-well (VWR, Radnor, PA) reaction plate in duplicates. Later, 8 µL of SYBR Green Master Mix, composed by 5 µL of PerfeCTa SYBR Green, 1 µL Forward Primer, 1 µL Reverse Primer, and 1 µL of water was pipetted into each well. The PCR reaction was performed

with a Roche LightCycler 480 Real-time qPCR machine (Basel, Switzerland) with the same settings than in PRIM.

Statistical analysis

The response variables analyzed included qPCR data, BW, BCS, and blood metabolites at different timepoints. Quantitative PCR data were analyzed using the GLIMMIX procedure of SAS (SAS 9.4 Institute, Cary, NC). Prior to statistical analysis, qPCR data was normalized using the geometric mean of housekeeping genes (*UXT*, *MTG1*, and *RPS15A* in PRIM; *UXT*, *B2M*, and *RPS15A* in MULT). Statistical analyses were performed using Ct values of targeted genes; whereas graphs were designed based on quantity values (2^ΔΔCt). Blood metabolites concentrations, BW, and BCS were analyzed using the MIXED procedure of SAS. Time and treatment were considered the fixed effect in the statistical model. The random effect in all models was animal within treatment. Mixed procedure of SAS included a repeated-measure statement with an unstructured covariate structure.

Fixed effects in the statistical model for each variable analyzed (i.e., genes, blood metabolites) included treatment (RPM or CTRL), time (Day 1, 25, 50, 100) on experiment and treatment × time on experiment interaction when appropriate (e.g., mRNA expression over time). Statistically significant differences were declared at $P < 0.05$ and tendencies at $P > 0.05$ and < 0.10. The statistical model used was: $Y_{ijl} = \mu + C_i + T_j + S_l + (C \times T)_{ij} + \varepsilon_{ijl}$; where, Y_{ijl} is the background-adjusted normalized parameter value (i.e, Ct or blood data value); μ is the overall mean; C_i is the fixed effect of time (4 or 5 levels); T_j is the fixed effect of treatment (2 levels); S_l is the random effect of heifer nested within treatment; C × T is the interactions of time by treatment and ε_{ijl} is the random error $(0, \sigma_e^2)$ associated with Y_{ijl}.

Milk production data was analyzed using the general lineal models (GLM) procedure of SAS.

Results

In order to avoid confounding effects due to differences in dams age at calving and different calving seasons, the data for each maternal parity was analyzed separately.

Primiparous

Animal performance

Body weight did not have a treatment × time interaction ($P = 0.885$; Table 2.4), although it presented a time effect ($P < 0.001$). At the beginning of the study, dams in RPM had a BW of 454 ± 32 kg and their BW decreased to 376 ± 41 kg at weaning; whereas CTRL dams had an initial BW of 442 ± 33 kg which decreased to 362 ± 41 kg at weaning. The inclusion of rumen-protected methionine (RPM) did not affect BW in dams ($P = 0.445$).

There was no treatment × time interaction effect ($P = 0.457$; Table 2.4) in BCS, however, there was a slight reduction in dam's BCS, represented by a time effect ($P < 0.001$). Body condition score decreased from 5.44 ± 0.52 to 4.89 ± 0.62 in RPM, and 5.63 ± 0.52 to 4.88 ± 0.62 in CTRL dams. Supplementation with RPM did not affect BCS ($P = 0.769$).

Calves born to PRIM dams did not present a significant treatment × time interaction effect in BW ($P = 0.218$) nor average daily gain (ADG; $P = 0.562$; Figure 2.3). Similarly, neither maternal nor offspring supplementation with methionine impacted calves' BW (treatment effect, $P = 0.176$). However, BW of calves increased significantly the first 100 days after weaning showing a time effect ($P < 0.001$).

Blood analyses

Plasma concentration of glucose significatively decreased from Day 1 to Day 25, from 92.3 ± 13.1 to 74.1 ± 10.1 mg/dL in RPM and 93.4 ± 4.6 to 76.4 ± 3.5 mg/dL in RPM and CTRL groups, respectively ($P < 0.001$; Figure 2.6). Plasma glucose concentration remained stable from

Day 25 to Day 100 after weaning. However, supplementation with methionine did not affect circulating glucose ($P = 0.962$). Furthermore, there was no treatment × time interaction effect for glucose ($P = 0.412$).

There was a tendency for treatment × time interaction effect in plasma albumin ($P = 0.082$; Figure 2.6). Albumin concentration decreased from Day 25 to Day 50 from 3.75 ± 0.27 to 3.27 ± 0.22 g/dL in the RPM group and 3.67 ± 0.27 to 3.30 ± 0.22 g/dL in CTRL group ($P < 0.001$). However, there was no difference between treatment groups ($P = 0.490$).

There was no treatment × time interaction effect ($P = 0.494$), treatment effect ($P = 0.406$), nor time effect ($P = 0.154$) for aspartate transaminase concentration in blood (Figure 2.6).

Alanine transaminase increased markedly from Day 1 to Day 25 from 13.8 ± 3.5 to 23.8 ± 3.3 U/L in RPM and 12.5 ± 3.5 to 25.37 ± 3.3 U/L in CTRL group (Figure 2.6). Similarly, the alanine transaminase concentration increased from Day 50 to Day 100 from 23.3 ± 5.2 to 28.0 ± 3.9 U/L in RPM and 23.2 ± 5.1 to 27.0 ± 3.9 U/L in CTRL group ($P < 0.001$). However, there was no treatment effect × time interaction effect ($P = 0.89$) nor treatment effect ($P = 0.323$).

Finally, plasma alkaline phosphatase decreased significantly from Day 1 to Day 25 from 374.4 ± 96.5 to 107.8 ± 24.0 U/L in RPM, and 255.7 ± 96.5 to 84.4 ± 24.0 U/L in CTRL group ($P < 0.001$; Figure 2.6). Furthermore, there was a treatment effect of supplementing methionine ($P = 0.047$). There was no treatment × time interaction effect for alkaline phosphatase concentration ($P = 0.553$).

Gene expression

There was a treatment × time interaction effect ($P < 0.001$), treatment effect ($P < 0.001$), and time effect ($P < 0.001$) for *PPARg* expression (Figure 2.8). Only RPM was upregulated from

Day 50 to Day 100. Furthermore, the *PPARg* expression of RPM group at Day 100 was markedly greater than CTRL group ($P = 0.003$).

Furthermore, there was a treatment × time interaction effect ($P = 0.016$) and time effect ($P < 0.001$), and a treatment tendency ($P = 0.078$) for *CEBPD* expression (Figure 2.8). In RPM, there was a downregulation from Day 25 to Day 50. At Day 100, RPM group had a greater *CEBPD* expression compared with CTRL group. Both *LPL* and *CEBPG* showed a treatment × time interaction effect ($P < 0.001$ and $p = 0.010$, respectively) and time effect ($P < 0.001$; Figure 2.8). There was a decrease in the expression of *LPL* in RPM group from Day 1 to Day 25 and from Day 25 to Day 50 ($P < 0.05$); however, there was a significant increase from Day 50 to Day 100. At Day 100, RPM group showed greater *LPL* expression compared with CTRL group. Expression of *CEBPG* was upregulated in RPM group between Day 25 to Day 50, and Day 50 to Day 100. Similarly, CTRL group had an upregulation between Day 50 to Day 100.

Finally, there was a treatment × time interaction effect ($P < 0.01$) and a time effect ($P < 0.001$) for *SOD2*, *NOS3*, and *DNMT1* expression (Figure 2.9). Furthermore, there was a treatment effect in *DNMT1* and *NOS3* expression ($P < 0.004$ and $P = 0.011$, respectively). There was downregulation in CTRL group of *NOS3* between Day 1 and Day 25; whereas there was an upregulation in RPM group from Day 50 to Day 100. At Day 100, calves in RPM group had a greater expression of *NOS3* compared with those in CTRL calves. Furthermore, there was an upregulation of *DNMT1* in RPM group from Day 1 to Day 25, Day 25 to Day 50, and Day 50 to Day 100. Similarly, calves in CTRL group also showed an upregulation of *DNMT1* from Day 25 to Day 50, and Day 50 to Day 100. Lastly, RPM group had a downregulation of *SOD2* between Day 25 to Day 50, and an upregulation from Day 50 to Day 100. At Day 100, calves in the RPM group had greater *SOD2* expression.

Multiparous

Animal performance

Body weight did not have a significant treatment × time interaction effect ($P = 0.959$; Table 2.4), although it presented a time effect ($P = 0.026$). At the beginning of the study, dams in RPM had a BW of 516 ± 43 kg, and their BW decreased to 499 ± 53 kg at weaning, whereas CTRL dams had an initial BW of 512 ± 43 kg, which decreased to 495 ± 53 kg at weaning. The inclusion of rumen-protected methionine (RPM) did not affect body weight (BW) in dams ($P = 0.865$).

There was no treatment × time interaction effect ($P = 0.878$) in BCS (Table 2.4); however, there was a slight reduction in dam's BCS, represented by a time effect ($P < 0.001$). Body condition score decreased from 6.11 ± 0.57 to 5.33 ± 0.55 in RPM, and 5.67 ± 0.57 to 5.00 ± 0.55 in CTRL dams. Supplementation with RPM did not affect BCS ($P = 0.169$).

Calves born to MULT dams showed a treatment × time interaction effect ($P < 0.001$), and a time effect ($P < 0.001$; Figure 2.4) in BW and a treatment × time interaction effect ($P < 0.001$), and time effect ($P < 0.001$) on ADG (Figure 2.4). Supplementation with methionine on maternal and offspring's diet did not impact calves' BW ($P = 0.206$). However, BW of calves increased the first 100 days after weaning significantly ($P < 0.001$).

Finally, there was no effect of maternal supplementation with methionine on milk production at approximately 60 days of lactation ($P = 0.823$; Figure 2.5).

Blood analyses

Plasma concentration of glucose, albumin, aspartate transaminase, alanine transaminase, and alkaline phosphatase did not present treatment × time interaction effect ($P > 0.10$); however, all variables showed a time effect ($P < 0.05$; Figure 2.7). Glucose concentration decreased from

98.7 ± 7.6 to 75.4 ± 6.8 mg/dL in RPM group and 99.1 ± 7.6 to 76.5 ± 6.8 mg/dL in CTRL group ($P < 0.001$) during the first 25 days after weaning.

In the contrary, aspartate transaminase concentration increased during the first 25 days after weaning from 55.6 ± 9.7 to 69.7 ± 11.2 U/L in the RPM group ($P < 0.001$). In addition, there was an increase in aspartate transaminase concentration from Day 50 to Day 100 from 69.4 ± 9.3 to 79 ± 9.8 U/L in RPM group and 67 ± 9.3 to 74.2 ± 9.8 in CTRL group ($P < 0.001$).

Lastly, alanine transaminase concentration increased during the first 25 days after weaning from 15.8 ± 3.8 to 24.2 ± 4.4 U/L in RPM group and 14.8 ± 3.8 to 23.7 ± 4.4 U/L in CTRL group ($P < 0.001$).

Gene expression

First, *PPARg*, *CEBPG*, and *CEBPD* did not show a treatment × time interaction effect ($P > 0.05$; Figure 2.10). There was a time tendency ($P = 0.052$) in *PPARg* expression, and a time effect ($P = 0.009$) in *CEBPD* expression. Furthermore, there was a treatment × time interaction effect ($P = 0.018$), and time effect ($P < 0.001$) in *LPL* expression. Calves in CTRL group had an upregulation between Day 1 to Day 25, and a downregulation from Day 25 to Day 50. Finally, there was no treatment × time interaction effect ($P > 0.05$) in *DNMT1*, and *NOS3* (Figure 2.11). There was a time effect ($P = 0.001$) in *NOS3*. Lastly, *SOD2* showed a treatment × time interaction effect ($P = 0.01$) and a time effect ($P < 0.001$; Figure 2.11). There was a significant upregulation in CTRL group from Day 1 to Day 25, and a downregulation between Day 50 to Day 100. In addition, calves in RPM had an upregulation of *SOD2* from Day 25 to Day 50.

Discussion

Animal performance

Maternal body weight and body condition score

The peripartal period is characterized by a reduction in maternal BW. During this period, ruminal capacity decreases due to the presence of the growing fetus, and it becomes more notable during the last trimester of gestation because of the exponential growth of the fetus (Stanley et al., 1993).

Body condition score is a visual estimation that indicates animal's body energy reserves and serves as a valuable tool to identify the overall nutritional status by producers. Preferably, BCS assessment must be performed by the same observer for maintaining evaluation consistency. The continual BCS estimation (i.e., estimations at different time points) allows close monitoring of the herd's nutritional status (Houghton et al., 1990). Furthermore, a correct BCS ensures an optimal postpartum interval until the return of estrus and greater pregnancy rates (Selk et al., 1988; Renquist et al., 2006). Therefore, BCS estimation plays an essential role in the nutritional planning and management of the cow-calf operation systems. During the peripartal period, BCS is reduced as a result of the reduction in BW. In the present study, the BCS scale from 1 to 9 was utilized, with 1 being an emaciated dam and 9 an over fat dam (Renquist et al., 2006). The reduction in BCS in both PRIM and MULT Angus × Simmental dams from late gestation to early weaning in the present study is a normal outcome due to the reduction in dry matter intake (DMI) during late gestation and the high nutritional demand during the lactation period. The lactation periods in PRIM differ from MULT, since MULT were mature cows and fully developed at the moment of calving, energy from the diet was redirected to recover BW and lactation (Linden et al., 2014). Most studies investigating the effects of Met on beef cattle

used a methionine hydroxy analog; however, a growing area of research is currently utilizing RPM in their experiments partly due to the vast information available on dairy cattle. The metabolism of methionine hydroxyl analog and protected DL-Met occurs differently in the animal's body (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012). The lack of effect of RPM supplementation on gestating cows has been recently reported. Silva et al. (2021) showed similar BW of gestating Angus crossbreed cows consuming 9.5 g/day/hd of 2-hydroxy-4(methylthio)-butanoic acid (10 g/hd/day; 3.7 g/hd/day of metabolizable Met) compared with control cows. The crude protein in this reported experiment was 8.2% of the diet (Silva et al., 2021). Similarly, another experiment in which Angus × Simmental cows received a diet with 12.1% CP and methionine hydroxy analog did not show differences in BW compared with cows without supplementation (Clements et al., 2017). Therefore, we suggest that supplementation with Met in diets with adequate CP does not alter animal performance on gestating beef dams.

Supplementing RPM during the periparturient period on dairy cows improved milk yield, milk fat and protein yield, and increased DMI and energy corrected milk (Osorio et al., 2013; Zhou et al., 2016; Batistel et al., 2017). Accordingly, the effects of RPM supplementation on milk production in beef species were also previously investigated. The utilization of methionine analogs has been used since early 1970's. For example, Varner (1974) reported an increase in milk yield and fat content in lactating beef cows receiving methionine hydroxy analog. In addition, this study also showed an improved weaning weight in animals born to cows receiving Met supplementation. The authors suggested that the greater weaning weight could be related to the greater milk and fat yield in supplemented cows (Varner, 1974). Contrary, other recent experiments did not show differences in milk yield due to the supplementation of RPM. For

example, Silva et al. (2021) showed that Angus crossbred cows receiving RPM supplementation tended to produce greater energy corrected milk, but there was no difference in milk yield compared with control cows (Silva et al., 2021). Similarly, mature Angus × Simmental cows receiving methionine hydroxyl analog supplementation did not differ from cows without methionine hydroxyl analog supplementation (Clements et al., 2017). Our study indicates that mature cows receiving RPM had similar milk production to CTRL. Since both treatments had an adequate amino acid profile in the diet and Met was not a limiting amino acid, the supplementation with RPM did not lead to improved milk yield. Furthermore, methyl donor requirements for lactation can be met by supplementation with methyl donor compounds involved in the one-carbon metabolism (i.e., choline, betaine, and Met). This practice is widely used in dairy cows, showing positive results (McFadden et al., 2020); however, evidence suggests that beef cows could not be deficient in methyl donor compounds during the peripartal period. In addition, another possible explanation of the lack of response in studies investigating the effects of RPM supplementation on beef cows could be related to the milk production estimation method. Weigh-suckle-weigh procedure is considered a reliable measurement, and the values obtained from this estimation can be used to compare treatments in ruminants. However, the lack of statistical differences in studies using this estimation method could be overcome by the greater accuracy of milking machines. Therefore, studies using milking machines will provide a more precise measurement of milk yield and could potentially show real, significant statistical differences among treatments.

Finally, we performed this study only with male calves in order to avoid sex biases during the analysis of data. Therefore, a total of 1 female calf born to PRIM CTRL dam, 3 female calves born to MULT CTRL dams, and 2 female calves born to MULT RPM dams were

excluded from the study. It is important to point out that PRIM dams experienced dystocia during calving in both treatment groups. More specifically, PRIM dams in CTRL group had 2 difficult calving (18% of total PRIM CTRL dams) with two stillborn calves (18% of total PRIM CTRL calves), whereas those in RPM had a total of 5 calves born from dystocia condition (55% of total PRIM RPM dams), with two stillborn calves (18% of total PRIM RPM calves). Since both treatments groups were inseminated with the same Simmental bull, and dams exposed to RPM during the peripartal period doubled dystocia events compared with dams in CTRL group, we speculate that RPM might cause a greater fetal growth in the womb, which led to greater BW at birth. Although, it is important to mention that other dams from the same herd that were out of our study had also calving difficulties and the veterinarian of the unit attributes it to health problems. Therefore, we cannot assure that the calving difficulties presented in this study are exclusively related to the administration of RPM. When running statistical analysis considering these calving difficulties, significant differences due to treatments applied were not detected (data not shown). Consequently, we cautiously recommend supplementing RPM on PRIM dams at a rate of 0.07% of DMI. Reducing the individual dose or supplementing with RPM during another stage of gestation may limit the occurrence of dystocia on Angus × Simmental PRIM heifers.

Offspring body weight and average daily gain

A normal, naturally occurring difference in BW between growing calves born to PRIM dams compared with MULT exists in the beef species (Linden et al., 2014). After calving, PRIM dams' nutrient partitioning is directed to growing and lactation. The greater energy requirement could be partly met by a greater DMI (as a percent of BW) compared with mature cows. Different nutritional and management strategies could be applied to ensure proper heifer growth,

return to cyclicity, and lactation. For example, protein and energy supplementation and early weaning are widely utilized in grazing cow-calf operation systems. However, milk yield is significantly lower in PRIM dams compared with MULT cows on the same breed (Fiss and Wilton, 1992). In the present study, early weaning and creep feeding strategies were applied in order to improve calves' growth. Creep feeding helps offspring's performance not only during the lactation period but also provides a carry-over effect, positively affecting production characteristics such as feedlot performance and carcass quality (e.g., marbling score) (Myers et al., 1999). Both PRIM and MULT dams had similar lactation length, weaning calves with a similar age in average (88 vs. 80 days). Since mature cows have superior milk yield, calves born to MULT cows had a greater weaning weight compared to calves born to PRIM dams (129.53 vs. 85.08 kg). Thus, calves born to MULT dams had greater ADG from birth to weaning compared with calves born to PRIM dams (1.05 vs. 0.63 kg/hd/day). This result suggests that, in accordance with previous evidence, male calves born to MULT cows are exposed to a greater amount of milk than male calves born to PRIM, positively impacting weaning weight in early weaning procedures (Ungerfeld et al., 2011). Interestingly, the maternal supplementation of RPM did not impact offspring performance during lactation in calves born to PRIM and MULT dams. In a previous experiment, PRIM beef dams receiving annual rye hay with ground corn, soybean meal, RPM and protected lysine showed positive results regarding milk production. However, offspring born to supplemented dams did not present differences in animal's performance (e.g., BW and ADG) compared to calves born to cows not receiving supplementation (Hess et al., 1998).

After weaning, calves born to RPM dams received RPM until the end of the study. Our results indicate that RPM supplementation does not alter growth on Angus × Simmental calves.

Offspring's diet was formulated to meet NRC requirements (NRC, 2016); therefore, Met was not limitin in the diet and could not have impacted body composition patterns of growth which led to changes in BW (i.e., skeletal muscle production).

Blood metabolites

Numerous metabolic, physiological, and endocrinal changes occur during weaning. Glucose is the main energy source for ruminants, and up to 50% of glucose is synthetized from propionate in ruminants. In addition, endogenous gluconeogenesis from glycerol could occur up to 40% in fasted animals (Oksbjerg et al., 2004). After weaning, circulating glucose concentration decreases significantly in ruminants due to the removal of liquid feed with high lactose content (e.g., maternal milk) from the diet, which presents a superlative nutritive value (Quigley et al., 1991). As expected, the present study showed that early weaning reduced serum glucose concentration in all calves, regardless maternal age, or supplementation with RPM. From Day 25 to Day 100, circulating glucose concentration remained stable at normal levels for beef cattle (~60-100 mg/dL) in all calves (Rumsey et al., 1999).

Albumin is the most abundant protein found in mammals' blood and is responsible for regulating blood volume and protein transport. It is mainly synthesized in the liver, usually serving as a liver health biomarker (Osorio et al., 2014b). In our study, maternal supplementation with RPM did not affect albumin concentration at early weaning. Previous evidence reported a tendency for a slight reduction in albumin concentration for Holstein calves born to dams supplemented with RPM compared with those without RPM supplementation from birth to Day 50 (Jacometo et al., 2016). Albumin concentration decreased in PRIM between Day 25 and Day 50 and there was a consistent reduction in MULT among timepoints; however, the supplementation with RPM did not affect albumin serum concentration. Likewise, Hill et al.,

2008 evaluated the inclusion of different levels of Met in young dairy calves. The authors reported no difference in albumin concentration among different Met inclusion in the diet. Changes in albumin concentration in both MULT and PRIM could be associated with the change of the diet, from lactation with creep-feeding access to a roughage and early wean feed supplement, as reported by (Lohakare et al., 2012). Most importantly, PRIM and MULT calves had circulating albumin within the normal range (2.7-3.9 g/dL) as reported by Keay and Doxey (1983) and Otter (2013), suggesting an adequate liver health status regardless of supplementation with RPM.

Furthermore, aspartate transaminase (AST) is the enzyme that catalyzes the transamination of aspartate and α-ketoglutarate into oxaloacetate and glutamate; whereas alanine transaminase (ALT) is the enzyme responsible for converting alanine and α-ketoglutarate into pyruvate and glutamate. The serum activity of AST and ALT are also analyzed for identifying hepatocellular injury (Vuppalanchi and Chalasani, 2018). Remarkably, a previous study showed that maternal supplementation with RPM resulted in a lower concentration of offspring AST at birth in Holstein calves. However after colostrum intake, AST concentration remained similar compared with control calves until 50 days of life (Jacometo et al., 2016). In PRIM and MULT calves, ALT significantly increase from Day 1 to Day 25, and from Day 50 to Day 100. In accordance with our results, a previous study conducted by Suzuki et al., (2016) found greater ALT concentration in early weaned Holstein calves compared with others of similar age that were lactating. These results indicate that AST and ALT concentration changes after weaning were not severe and were at normal levels as previous studies (Pavlík et al., 2009; Jacometo et al., 2016).

Finally, serum alkaline phosphatase (ALP) was analyzed for identifying liver health damage. Alkaline phosphatase is a plasma membrane-bound glycoprotein enzyme produced by the liver and bone, and its main function is to hydrolyze phosphate monoesters (Sharma et al., 2014). Calves born to PRIM receiving RPM showed a slight greater concentration of circulating ALP. This difference may be explained by a greater bone growth in PRIM-RPM calves compared with those in PRIM-CTRL from birth to weaning (Yamaguchi and Yamaguchi, 1986; Chiba et al., 2020). Most importantly, neither PRIM nor MULT calves exceed the maximum reference value of 1200 U/L (20 µkat/L)(Dresler et al., 2016). Consequently, based on the previous evidence and our results, we suggest that all calves were not exposed to severe stress during the experiments.

Gene expression

Adipogenic genes

Currently, the price of beef in the United States is driven mostly by the intramuscular fat content or marbling. Therefore, several genetic and nutritional strategies have been applied in beef production systems during the last several decades for improving this trait in fattening animals. In addition to the vast impact of stocker, backgrounding, and finishing phase management and nutrition on marbling (Owens and Gardner, 2000; Lancaster et al., 2014), there is growing evidence showing that maternal nutrition plays a key role in mature adipose tissue deposition (Du et al., 2013). More specifically, maternal nutrient restriction occurring during early to mid-gestation results in reduced skeletal muscle mass and increased fat content in the offspring (Zhu et al., 2006; Long et al., 2012). Conversely, intrauterine nutrient restriction during the last trimester of gestation causes reduced fetal adipose tissue (Symonds et al., 2004). For example, a report conducted by Underwood et al. (2010) showed that maternal plane of nutrition

during mid- to late-gestation affect carcass quality. In this study, Angus mature cows were exposed to native range pasture or improved pasture from 120 to 150 through 180 to 210 days of gestation. Steers born to cows on improved pasture had greater final BW, hot carcass weight, 12^{th} rib fat thickness, but there was no significant difference in marbling score. However, Larson et al. (2009) showed that steers born to Red Angus × Simmental mature cows receiving protein supplementation during late gestation had greater marbling content and quality grade, and a tendency to have higher empty body fat content compared with steers born to dams without protein supplementation.

Peroxisome Proliferator-Activated Receptor Gamma (*PPARg*) is the essential and primordial transcription factor for adipogenesis in mammals. Therefore, *PPARg* is considered the 'master regulator' of adipogenesis and lipogenesis, and can control the gene expression of other adipogenic factors (Wu et al., 1999). It has been demonstrated that *PPARg* plays an essential role in intramuscular fat accumulation, and its level of expression is associated with nutritional status and breed. For example, Moisá et al. (2014) reported the crucial role of *PPARg* in early stages of cattle growth and final carcass quality. In this study, authors showed greater number of carcasses with 'High choice' quality on early weaned steers compared with conventional weaned steers. Interestingly, there was a greater expression of *PPARg* and *CEBPA*, which are key regulators of adipogenesis, on early weaned calves, suggesting their important role in the process of intramuscular fat deposition that led to a greater percentage of 'High choice' carcass quality grade. Similarly, a previous study conducted by Duarte et al. (2013) compared the expression of adipogenic factors in Wagyu, a breed known by its high intramuscular fat content, vs. Angus steers. There was a greater expression of *PPARg* in Wagyu steers than Angus steers; nevertheless, authors reported that Wagyu steers had greater abundance of mesenchymal

progenitor cells from which adipocytes and fibroblasts are differentiated. Thus, adipogenic differentiation occurred in a greater extent in Wagyu compared with Angus cattle. Another factor associated with fatty acid composition is the lipoprotein lipase (LPL). More specifically, *LPL* regulates the lipolysis of triglycerides enhancing the accumulation of adipose tissue (Pethick et al., 2004; Oh et al., 2013).

Furthermore, CCAAT/enhancer-binding proteins (CEBP) are transcription regulators involved in adipogenic and lipogenic processes. The CEBP family comprises at least five proteins: CEBPA, CEBPB, CEBPD, CEBPE, and CEBPG. Each of these factors are present in almost all cell types as transcription factors, and they are involved in specific biologic mechanisms (Wedel and Lömsziegler-Heitbrock, 1995). In our study, we analyzed the expression of *CEBPD* and *CEBPG* due to their role on adipose tissue development on growing animals. It has been previously shown that *CEBPD* acts as an upstream regulator of *PPAR*g in mammals (Cao et al., 2015; Małodobra-Mazur et al., 2020). Similarly, *CEBPG* is highly expressed during early stages of adipocyte differentiation (Bachmeier and Löffler, 1997). In our study, there was an upregulation of *PPARg*, *CEBPD*, and *LPL* at the end of study in PRIM group receiving RPM compared with CTRL group. Maternal parity has been identified as major contributor to fat accumulation, being the progeny of primiparous dams who have the greater adipose tissue compared than those born to multiparous dams (Symonds et al., 2004). It was previously known that brown adipose tissue ablation causes increment in on total body lipid, increasing the probability of obesity (Lowell et al., 1993). A posterior study confirming this statement on ruminants reported that primiparous ewes' offspring present more fat mass than those born to multiparous ewes due to the loss of brown adipose tissue (Hyatt et al., 2010). Therefore, PRIM calves may be more prone to experience upregulation in adipogenic-related

genes (i.e., *PPARg*, *LPL*, *CEBPD*) than MULT. In addition, reports indicate *PPARg* expression can be altered by the diet received. For example, supplementation with methionine increases PPARg protein abundance on adipose tissue of Holstein cows 30 days after parturition (Liang et al., 2019). Similarly, a study conducted by Osorio et al. (2016) identified the upregulation of hepatic *PPARa*, another member of the peroxisome proliferator-activated receptor family, as a result of a greater methylation due to the supplementation of RPM (Smartamine®) on peripartal Holstein cows. Even though there may exist differences in adipogenic genes among tissues, Moisá et al. (2014) showed that dietary changes on beef steers can impact adipogenic gene expression in skeletal muscle of beef cattle. Since calves in our study were exposed to methionine through maternal feeding (e.g., womb and milk) and directly after weaning, supplementation of RPM might have a direct impact on adipogenic genes on PRIM-RPM; however, the overall lack of response of RPM on MULT remains to be elucidated. A possible explanation could be related to the greater brown adipose tissue content in MULT in which RPM supplementation may not exert any effect on adipogenic-related genes. In addition, calves born to primiparous dams could be more susceptible to experience methylation in their genome, including adipogenesis-related genes, as shown by the upregulation in *DNMT1* among time points. In contrast, the expression of *DNMT1* remained stable among time points in calves born to multiparous cows in both CTRL and RPM groups.

Oxidative stress and DNA methylation

Oxidative stress in a detrimental condition caused by an imbalance in the redox status of the cell. The compounds causing oxidative stress are superoxide (O_2^-) and nitric oxide (NO^-) that disrupt redox signaling and molecular damage (Sies, 2015). Reactive oxygen species (ROS) results from the reduction of oxygen into reduced oxygen (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH-). Superoxide dismutase (SOD) family play a key role in scavenging free radicals in the cell. Mammals present three isoforms of SOD and they differ in their composition and site of action: SOD1 is located mainly in the cytosol and the intermembrane space of mitochondria to a lesser extent and is formed mainly by Cu/Zn, SOD2 is formed by Mn and is located in the mitochondrial matrix, and SOD3 is located at the extracellular matrix (Fukai and Ushio-Fukai, 2011). The main function of SODs is to cause spontaneous dismutation of O^- resulting in the formation of H_2O_2. Furthermore, SODs inhibit the formation of $ONOO^-$. Since the major site of formation of O_2^- takes place in the mitochondrial respiration chain, measuring the expression of *SOD2* serves as a reliable indicator of ROS regulation in the cell (Hu et al., 2005). In addition, compounds involved in the trans-sulfuration pathway are also related to oxidative stress regulation, such as homocysteine, which is a product of methionine. One of the effects of homocysteine is to inhibit the production and release of nitric oxide (Li et al., 2007). Nitric oxide is synthesized by nitric oxide synthases isoforms, such as nitric oxide synthase 1 (NOS1), NOS2, and NOS3. First, the enzyme highly present in neural tissue is NOS1, which is also known as “nNOS”. Second, NOS2 is abundant in macrophages and it is known as inducible NOS (iNOS). Finally, NOS3 is involved in muscular and vascular tone. Since NOS3 is an endothelial nitric oxide isoform that also modulates muscular oxygen consumption and

microvascular blood flow, this enzyme is usally referred as “eNOS” (Kobzik et al., 1995; Harrison, 1997; Mattila and Thomas, 2014).

Interestingly, in accordance with a previous study from our lab (Alfaro et al., 2020), in after RPM supplementation on heifers for a period of 45 days, *SOD2* was downregulated. In this study, PRIM calves receiving RPM had a downregulation of *SOD2* from Day 25 to Day 50 suggesting a greater capability of the cell to cope against redox imbalance. Similarly, Osorio et al. (2014) showed a downregulation of hepatic *SOD1* but a stable expression of *SOD2* in peripartal Holstein cows receiving supplemented Smartamine. Authors suggested that methionine could be involved in the dismutation mechanism of the cell, lowering the oxidative stress status. However, contrary to previous results, our study indicates that the expression of *SOD2* and *NOS3* in skeletal muscle were upregulated between Day 50 to Day 100 in PRIM-RPM and had a greater expression at Day 100 compared with CTRL-PRIM. Similarly, MULT-RPM had upregulation of *SOD2* between Day 25 and Day 50. Since calves were growing during the experiment, our results may suggest a possible connection between ROS action on skeletal muscle hypertrophy. Accordingly, a previous report showed that nitric oxide regulates induced-hypertrophy in skeletal muscle by activating transient receptor potential cation channel, subfamily V, member 1 (TRPV1) on mice. Authors found that NO and $ONOO^-$ indirectly activate the mammalian target of rapamycin (mTOR), which is a kinase protein and the major factor for protein synthesis (Ito et al., 2013).

Supplementing one-carbon metabolism substrates enhance methylation processes in mammalian DNA. Methionine is an essential amino acid with critical importance in methylation processes because it is a precursor of S-Adenosylmethionine, widely recognized as the universal methyl donor (Niculescu and Zeisel, 2002). Maternal diet can promote epigenetic modifications

in the fetus and posterior offspring life. The epigenetic alterations can be enhanced by DNA or histone methylation. Therefore, increased dietary methionine directly affects methylation status in different tissues, including skeletal muscle (Chmurzynska, 2010). Recently, Liu et al. (2020) conducted a study where Brangus × Angus crossbred beef cows were supplemented with Methionine from days -30 to +90 relative to the beginning of breeding season. This study revealed that maternal supplementation of methionine during the peripartal period alters overall DNA methylation levels in offspring 30 days after birth. The enzymes capable of methylating DNA belong to the DNA-methyltransferase (DNMT) family. More specifically, DNMT1 plays an essential role in DNA methylation maintenance on daughter DNA strands and it is highly active during myogenesis; whereas DNTM3 enzymes are involved in *de novo* methylation (Liu et al., 2016). Even though, the process myogenesis and consequently the total skeletal muscle fiber number of all the calves in our study was expected to be completed at the moment of sampling, the supplementation with RPM upregulated the expression of *DNMT1* on PRIM-RPM from Day 50 to Day 100. A previous experiment conducted in humans identified changes in the methylation status of the epigenome due to changes in the diet. During this study, young men who were exposed to a high-fat overfeeding diet for 5 days had an upregulation in *DNMT1* and *DNMT3a* from skeletal muscle, suggesting that expression of *DNMT1* could be altered in growing and mature mammals by the diet consumed (Jacobsen et al., 2012). In addition, a recent experiment conducted by Iio et al. (2021) found a novel function of *DNMT1* during muscle regeneration in mature male mice. These results suggest that *DNMT1* may be involved in other methylation processes in addition to cell division. However, the upregulation of *DNMT1* in PRIM-RPM was not consistent in PRIM-MULT. Further research is needed to elucidate the

mechanisms behind the upregulation of *DNMT1* in calves born to primiparous dams, and if this greater expression has an impact on skeletal muscle hypertrophy.

Summary and conclusions

Herd management is essential in cow-calf operations, and more critically, during gestation. Management practices, including diet formulation, have a substantial impact on gestating dam and offspring's post-natal growth and development. Maternal supplementation with RPM may have different effects if applied on PRIM and MULT, affecting offspring metabolism in a different manner. Our study showed similar performance parameters (e.g., BW and BCS) on PRIM and MULT supplemented with RPM during the last trimester of gestation and the first ~85 days of lactation compared with those receiving a CTRL diet. Similarly, growth parameters did not differ in calves born to PRIM and MULT receiving RPM compared with CTRL during lactation and after weaning. Interestingly, RPM supplementation did not improve milk yield on MULT cows. Blood metabolites that serve as biomarkers for liver health status were in the normal range levels in all treatment groups. Remarkably, calves born to PRIM-RPM had an upregulation in *PPARg*, *CEBPD*, and *LPL* at the end of the study compared with those in PRIM-CTRL. These results suggest that calves that had greater exposure to methionine in-utero, milk, and direct supplementation after weaning with RPM may result in greater adipose tissue development, and potentially increase marbling during the finishing period. However, whether the upregulation of these genes are further translated into better carcass quality remains to be elucidated. Finally, PRIM-RPM calves had greater expression of *SOD2*, *NOS3* and *DNMT1* at Day 100 compared with PRIM-CTRL. Our results are in accordance with previous evidence that show alteration in DNA methylation due to the inclusion of supplemental RPM. Furthermore, the

greater expression of oxidative stress-related genes could be indicator of a greater hypertrophy process in skeletal muscle.

Tables and figures

Table 2.1. Chemical composition of maternal base diets fed to dams during the peripartal period. A) Diet fed to primiparous Angus dams. B) Diet fed to multiparous dams. Bermudagrass hay was fed *ad libitum* whereas soybean meal and corn gluten supplement was offered in a rate of 2.72 kg/hd/day.

A)

Ingredient[1]	DM	CP	NDF	ADF	TDN	Crude Fat
Bermudagrass hay	84.7	8.1	75.66	42.89	53.65	-
Soybean hull/corn gluten supplement	90.84	16.23	47	27.86	90.25	11.82

B)

Ingredient[1]	DM	CP	NDF	ADF	TDN	Crude Fat
Bermudagrass hay	88.5	12.5	72.2	43.9	52.7	-
Soybean hull/corn gluten supplement	90.84	16.23	47	27.86	90.25	11.82

DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDN = total digestible nutrients

[1]All ingredients are expressed on a DM basis.

Table 2.2. Chemical composition of offspring base diets fed to calves during the lactation period. A) Diet fed to primiparous dams' offspring. B) Diet fed to multiparous dams' offspring.. Calves had *ad libitum* access to maternal milk, bermudagrass hay, and creep feed starter.

A)

Ingredient [1]	DM	CP	NDF	ADF	TDN	Crude Fat
Bermudagrass hay	84.7	8.1	75.66	42.89	53.65	-
Creep Feed Starter	89.53	21.24	19.34	11.27	99.47	7.72

[1] All ingredients are expressed in a DM basis

B)

Ingredient [1]	DM	CP	NDF	ADF	TDN	Crude Fat
Bermudagrass hay	88.5	12.5	72.2	43.9	52.7	-
Creep Feed Starter[2]	-	16	22	-	-	2.6

DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDN = total digestible nutrients

[1]All ingredients are expressed in a DM basis

[2] Rough 'N' Ready, ADM, Chicago, IL.

Table 2.3. Chemical composition of offspring base diets fed to calves during the early weaning period. A) Chemical composition of early-wean feed. B) Diet fed to calves born to primiparous dams. C) Diet fed to calves born to multiparous dams. Calves were fed *ad libitum* access to bermudagrass hay and 1% of BW as DM basis of early wean feed.

A)

Ingredients [1]	**%**
Distiller grain	12.5
Limestone 38%	0.5
Corn	17.5
Cotton seed hulls, dry	10
Soybean hulls, dry	43.4
Molasses	5
Soybean meal, dry	10
Rumensin® 80[1]	0.02
Salt	0.5
Availa® 4[2]	0.1
J&R All Purpose[3] Mineral	0.5
Total	100

[1] Elanco Animal Health, Greenfield, IN

[2] Zinpro Corporation, Eden Prairie, MN

[3] Z&R Supply Inc., East Dubuque, IL

B)

Ingredients [1]	**DM**	**CP**	**NDF**	**ADF**	**TDN**	**Crude Fat**
Bermudagrass hay	92.35	8.27	71.61	39.34	57.70	-
Early-wean feed	89.30	15.93	37.93	26.24	68.26	4.22

C)

Ingredients [1]	**DM**	**CP**	**NDF**	**ADF**	**TDN**	**Crude Fat**
Bermudagrass hay	87.20	11.60	79.70	39.72	56.05	-
Early-wean feed	89.30	15.93	37.93	26.24	68.26	4.22

DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDN = total digestible nutrients.

[1]All ingredients are expressed in a DM basis

Table 2.4. Effect of rumen-protected methionine supplementation during the last trimester of gestation and lactation on body weight (kg) and body condition score of A) primiparous dams, and B) multiparous dams.

A)

Item	Treatment		SEM	*P* - value		
	CTRL	RPM		Trt	Time	Trt×Time
BW, kg						
Initial BW	442.33[a]	454.04[a]	28.76	0.445	0.001	0.885
BW Weaning	362.73[b]	376.46[b]				
BCS						
BCS Calving	5.63[a]	5.44[a]	0.22	0.769	0.001	0.457
BCS 60 days post-calving	4.88[b]	4.89[b]				

B)

Item	Treatment		SEM	*P* - value		
	CTRL	RPM		Trt	Time	Trt × Time
BW, kg						
Initial BW	512.5	516.4	21.7	0.865	0.026	0.959
BW Weaning	495.1	499.7				
BCS						
BCS Calving	5.67[a]	6.11[a]	0.23	0.169	0.001	0.878
BCS 60 days post-calving	5.00[b]	5.33[b]				

Statistical differences were declared at $p \leq 0.05$, and tendencies at $p > 0.05$ and < 0.1.
[a,b] Superscripts represent statistical differences between two time points for the same treatment.
Tables show *p*-values for Treatment effect (Trt), time effect (Time), or Treatment × Time interaction effect (Trt × Time)

Table 2.5. Overall least mean squares fold change values for expression of genes analyzed in *Longissimus dorsi* muscle of PRIM calves in CTRL and RPM groups.

Gene transcript	Day 1		Day 25		Day 50		Day 100		SEM	*p-value*		
	CTRL	RPM	CTRL	RPM	CTRL	RPM	CTRL	RPM		Trt	Time	Trt × Time
PPARg	1.0162	1.4869	1.0178	1.1206	1.0734	1.5037	1.0873	4.6592	0.9015	0.0003	0.0001	0.0005
LPL	1.0171	1.1382	1.1006	0.9925	1.0753	0.6329	1.1122	3.2893	0.4945	0.1781	0.0001	0.0009
CEBPG	1.0827	0.5881	1.0139	0.9083	1.1068	1.3933	1.3411	1.8002	0.3503	0.9648	0.0001	0.0104
CEBPD	1.0567	2.0379	1.1266	2.1079	1.1498	0.6127	1.094	2.705	0.471	0.0778	0.0001	0.0162
DNMT1	1.007	0.9351	1.0041	0.9978	1.044	1.582	1.1158	1.871	0.1587	0.0044	0.0001	0.0008
SOD2	1.0075	0.9125	1.0062	0.9269	1.0474	0.5917	1.1516	2.038	0.1796	0.8746	0.0001	0.0029
NOS3	1.0359	0.8697	1.0103	1.3847	1.0757	1.0018	1.2065	2.5812	0.2564	0.0115	0.0001	0.0005

Table 2.6. Overall least mean squares fold change values for expression of genes analyzed in *Longissimus dorsi* muscle of MULT calves in CTRL and RPM groups

Gene transcript	Day 1		Day 25		Day 50		Day 100		SEM	*p-value*		
	CTRL	RPM	CTRL	RPM	CTRL	RPM	CTRL	RPM		Trt	Time	Trt × Time
PPARg	1.0205	1.1645	1.0243	1.0018	1.0244	0.9701	1.2989	0.9218	0.2958	0.6376	0.0522	0.9073
LPL	1.0156	0.7085	1.0913	1.4485	1.0272	0.8976	1.026	0.9785	0.1472	0.3414	0.0001	0.018
CEBPG	1.0461	0.7258	1.0389	0.8412	1.0153	0.9055	1.1263	0.7239	0.1873	0.0001	0.1559	0.5197
CEBPD	1.1432	1.6573	1.1404	1.3896	1.1134	0.9599	1.055	1.2123	0.6146	0.7934	0.0099	0.4475
DNMT1	1.0049	1.1634	1.0456	1.1698	1.0087	1.0388	1.0292	1.0129	0.1207	0.1038	0.1589	0.3391
SOD2	1.0256	0.8179	1.0346	1.284	1.0148	1.4725	1.0111	0.9516	0.1508	0.1782	0.0001	0.0109
NOS3	1.0201	0.9474	1.1777	1.0614	1.0704	0.9832	1.067	0.8975	0.1675	0.5116	0.0014	0.8374

Table 2.7. Quantitative real time PCR performance among the 7 genes measured in skeletal muscle samples

Gene transcript	Median Ct[1]	Median ΔCt[2]	Slope[3]	(R^2)[4]	Efficiency (%) [5]	Efficiency[6]	Relative mRNA abundance[7]	1/EΔCt[8]	%
Winter Calving Season									
PPARg	29.375	6.339	-2.459	0.999	155.07	2.550	0.003	0.001	0.084
LPL	23.804	0.690	-2.513	0.999	150.78	2.500	0.531	0.170	16.955
CEBPG	25.300	2.341	-2.548	0.999	146.90	2.469	0.121	0.038	3.848
CEBPD	23.269	0.183	-2.610	0.999	141.73	2.416	0.851	0.272	27.157
DNMT1	26.948	4.044	-2.318	0.996	171.75	2.700	0.018	0.006	0.575
SOD2	22.668	-0.509	-2.568	0.999	145.29	2.452	1.579	0.504	50.388
NOS3	26.793	3.627	-2.407	0.998	160.45	2.603	0.031	0.010	0.993
							3.134	1.000	100.000
Fall Calving Season									
PPARg	29.874	6.294	-1.914	0.991	166.60	3.331	0.001	0.0004	0.036
LPL	25.234	1.654	-2.064	0.993	152.61	3.051	0.158	0.112	11.155
CEBPG	25.432	1.851	-2.501	0.999	125.06	2.511	0.182	0.128	12.836
CEBPD	25.199	1.618	-2.459	0.998	127.64	2.550	0.220	0.155	15.514
DNMT1	27.106	3.526	-2.463	0.993	128.16	2.547	0.037	0.026	2.615
SOD2	23.878	0.297	-2.494	0.998	126.58	2.518	0.760	0.536	53.649
NOS3	26.904	3.324	-2.711	0.994	117.80	2.338	0.059	0.042	4.194

1-The median is calculated considering all time points and all calves.
2-The median of ΔCt is calculated as [Ct gene – geometrical mean of Ct internal controls] for each time point and each calves.
3-Slope of the standard curve.
4-R^2 stands for the coefficient of determination of the standard curve.
5-Efficiency (%) is calculated as [10(-1 / Slope)]-1 x 100.
6-Efficiency is calculated as [10(-1 / Slope)].
7-Relative mRNA abundance = 1/ Efficiency Median ΔCt.
8-1/EΔCt = relative mRNA abundance/Σ relative mRNA abundance

Table 2.8. Gene ID, GenBank accession number, hybridization position, sequence and amplicon size of primers for *Bos Taurus* used to analyze gene expression by RT-qPCR.

Gene ID	Accession #	Gene	Primers[1]	Primers (5'-3')[2]	bp[3]	Category[4]
Internal Control						
509768	NM_001025327.2	*MTG1*	F.258	CAACAAAATGGACCTGGCAGAT	115	P
		MTG1	R.372	CTTGACATTTTCATCCTTCACACAGT		
337888	NM_001037443.2	*RPS15A*	F.230	GCAGGCTAAATAAGTGTGGAGTGA	90	P-M
		RPS15A	R.319	GGGATGGGAGCAGGTTATTCT		
525680	NM_001037471.2	*UXT*	F. 337	CTGGCAGAAGCTCTCAAGTTCA	100	P
		UXT	R. 436	GGATATGGGCCTTGATATTCATG		
525680	NM_001037471.2	*UXT*	F. 134	CAGCTGGCCAAATACCTTCAA	125	M
		UXT	R. 288	GTGTCTGGGACCACTGTGTCAA		
280729	NM_173893.3	*B2M*	-	CCGAGTGAAACACGTTACTTTG	-	M
		B2M	-	CCAAATGAAGAATGTTCAAATCTCG		
Genes of interest						
281496	NM_201527.2	*SOD2*	F 290	AGAAGGGTGATGTTACAGCTCAGATAG	93	P-M
		SOD2	R 382	GATTTGTCCAGAAGATGCTGTGAT		
281993	NM_181024.2	*PPARg*	F. 439	ACCCGATGGTTGCAGATTATAAG	140	P-M
		PPARg	R. 578	GGAGTTGGAAGGCTCTTCATGA		
280843	NM_001075120.1	*LPL*	F. 768	CAAGTCGCCTTTCTCCTGATG	105	P-M
		LPL	R. 872	CATGCCCTACTGGTTTCTGGAT		
281678	NM_001075676.1	*CEBPD*	F.1440	AGGAGATGGAAAGGACAGTCACA	100	P-M
		CEBPD	R.1539	AACGACTTTATTTATTCGTCCAGGTT		
617530	NM_001034315.1	*CEBPG*	F. 405	AGGAACAACATGGCTGTGAAAA	95	P-M
		CEBPG	R. 499	TCATTCTCTTCCTTGAGCTGATTG		
281119	NM_182651.2	*DNMT1*	F. 1323	TCTGGTTCAGCAAAGCCGATATAT	105	P-M
		DNMT1	R. 1427	ATCAAAACCAGCAATCCACCAT		
287024	NM_181037.3	*NOS3*	F. 3944	CTCCGGAAGTATCTTATCTTGAAACC	135	P-M
		NOS3	R. 4078	AACTGTAATTGACAGCACTGGCTTAG		

[1] Primer direction (F – forward; R – reverse) and hybridization position on the sequence.
[2] Exon-exon junctions are underlined.
[3] Amplicon size in base pair (bp).
[4] Maternal categories of calves in which a particular primer was used. P = primiparous, M = multiparous

Table 2.9. Sequencing results of PCR products from primers of genes designed for this experiment. Best hits using BLASTN (http://ncbi.nlm.nih.gov) are shown.

Internal control	
MTG1 (P)	CGACCGAAATACCACCCTTGGAAGAGAGGCATAAAACATGTTTGTTTTTCCAACCTGTGTGAAGGATGAAAATGTCAAGA
RPS15A (P-M)	GACTGATATGAGTGTCACTCATAGTATCCTAGGATAGTGGCAGAATAACCTGCTCCCAGTCCCAACTCCACACTATTATTACGC
UXT (P)	GTCTAAACGTCCCCTCCGGGGGCTCAGCGACAACCTTCACCAAGGGACTTCCATTGAATATTCAAGGGCCCATTATTCCAA
UXT (M)	Kadegowda et al. (2009)
B2M (M)	Chowdhury et al. (2007)
Genes of interest	
SOD2	CCGTTGCCCGGTCGTGGTAGTCTACGGGGTGCCATATCAATCACAGCATCTTCGTGACAAATCATAGAA
PPARg	CAGTGACTCAGAGTACAAGTGCATGCAAAGTGGAGCCGTGTATCCCCACCTTATTATTCGTGAAAAGACTCGAGTCATAGTAC
LPL	CGATGGATCAGTACCACATCACCAGGGGGTCACCAGGTCGAAGTATCGGAATCCAGGAAAACNCAGTAGGCGCATGA
CEBPD	GACTTACTGCTACTGTCAGTACTTGTATATTTTCAAAGATTAACTGGACGAATAAATAAAGTCGTCCTAGCA
CEBPG	GCAGGTAGCAGCAGAGCGCAGGATACGCGTGCAGAGAGGTCAATCAGCTCAAGGGGAAGAGGAATGGAAA
DNMT1	CGATACATCTCGCAGTGTATTATGGCAAAATTTTGGCATAAACGAATGGTGGATTGCTGGTTTCTGATAA
NOS3	GTATCTATCATTCATATTTATTATTGAGATACCATAAGAGACTGGACCAGAAGTTAGGAGACCTATCTAAGA

(P), (M), (P-M) represents the maternal category of calves in which a particular primer was used. P = primiparous, M = multiparous

Table 2.10. Sequencing results of genes using BLASTN from NCBI (http://ncbi.nlm.nih.gov)

Gene transcript	Best hit in NCBI
CEBPD	Bos taurus CCAAT enhancer binding protein delta (CEBPD), mRNA
CEBPG	Bos taurus CCAAT enhancer binding protein gamma (CEBPG), mRNA
B2M	Bos taurus beta-2-microglobulin (B2M), mRNA
DNMT1	Bos taurus DNA methyltransferase 1 (DNMT1), mRNA
LPL	Bos taurus lipoprotein lipase (LPL), mRNA
MTG1	Bos taurus mitochondrial ribosome associated GTPase 1 (MTG1), mRNA
NOS3	Bos taurus nitric oxide synthase 3 (endothelial cell) (NOS3), mRNA
PPARg	Bos taurus peroxisome proliferator activated receptor gamma (PPARG), mRNA
RPS15A	Bos taurus ribosomal protein S15a (RPS15A), mRNA
SOD2	Bos taurus superoxide dismutase 2 (SOD2), mRNA
UXT	Bos taurus ubiquitously expressed prefoldin like chaperone (UXT), mRNA

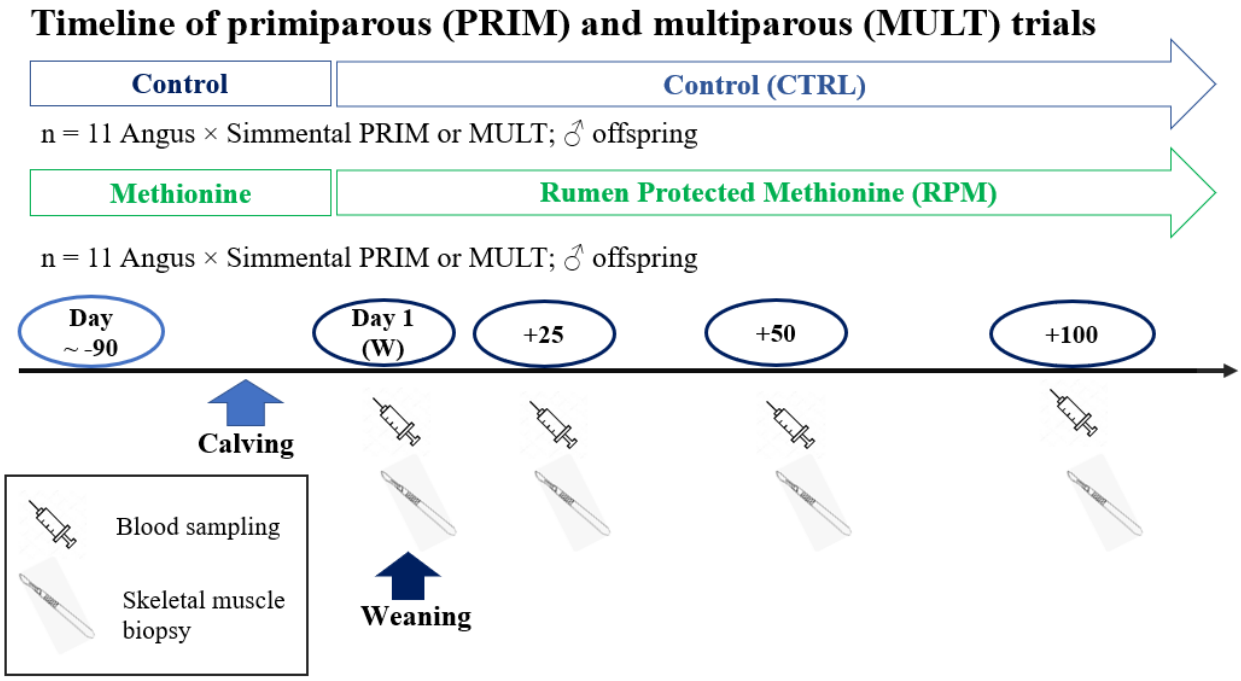


Figure 2.1 Experimental design and timeline of the study.

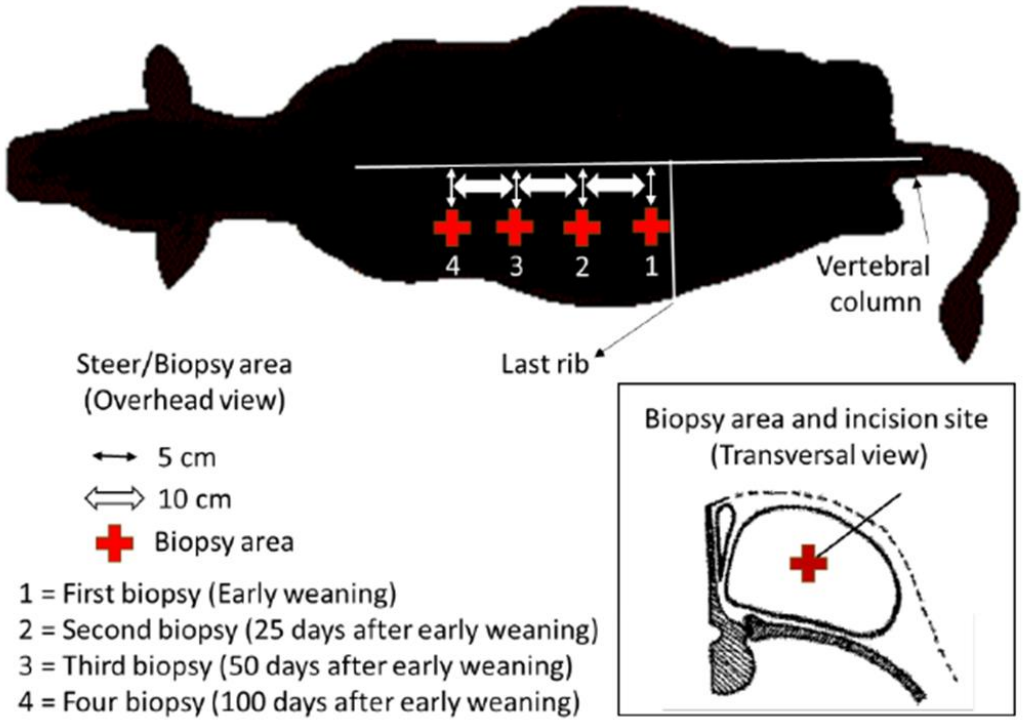
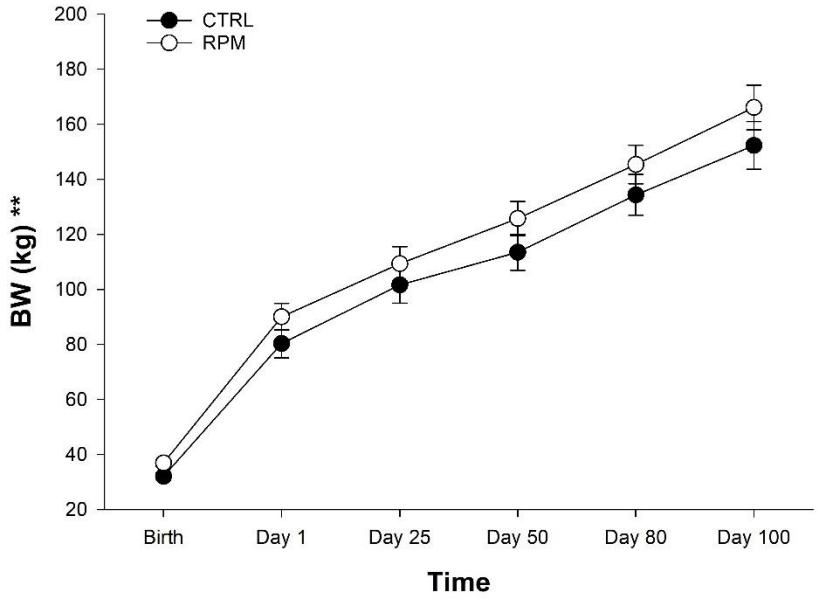


Figure 2.2. Overhead and transversal representation of skeletal muscle biopsy area of calves used in the study.

A)



B)

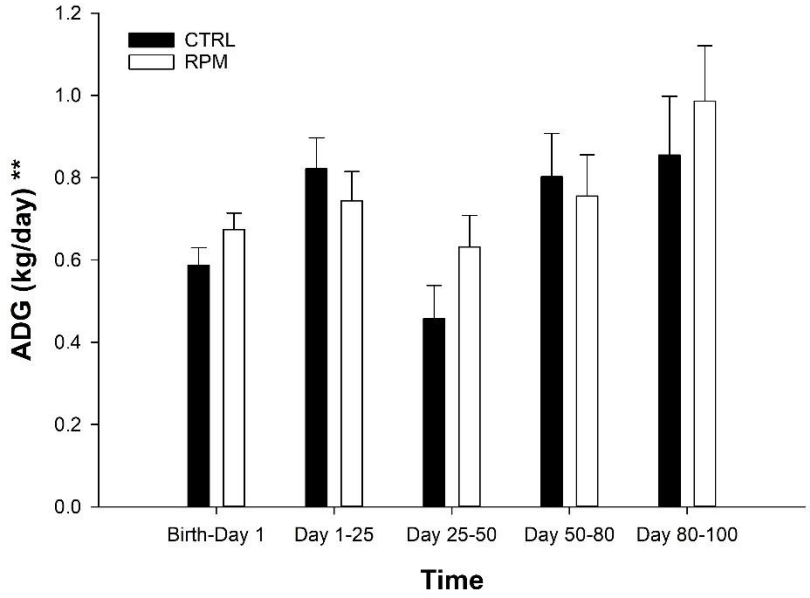
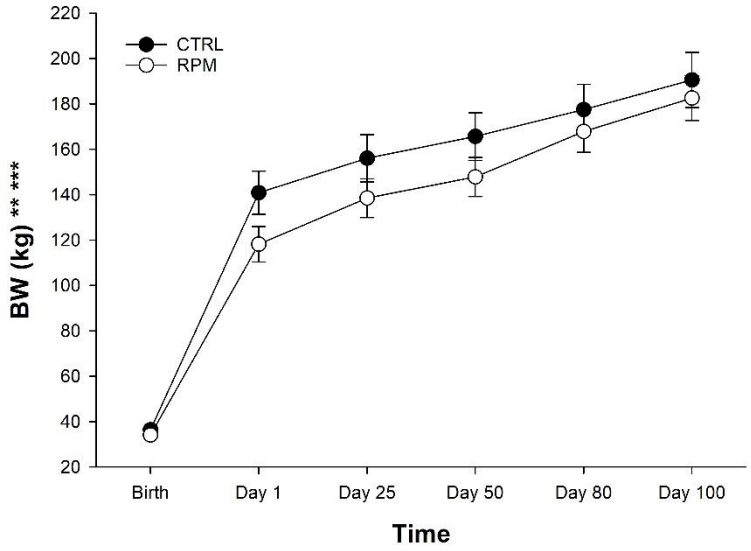


Figure 2.3. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to PRIM dams receiving RPM during the peripartal period, on A) body weight (BW), and B) average daily gain (ADG). Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Error bars represent SEM.

A)



B)

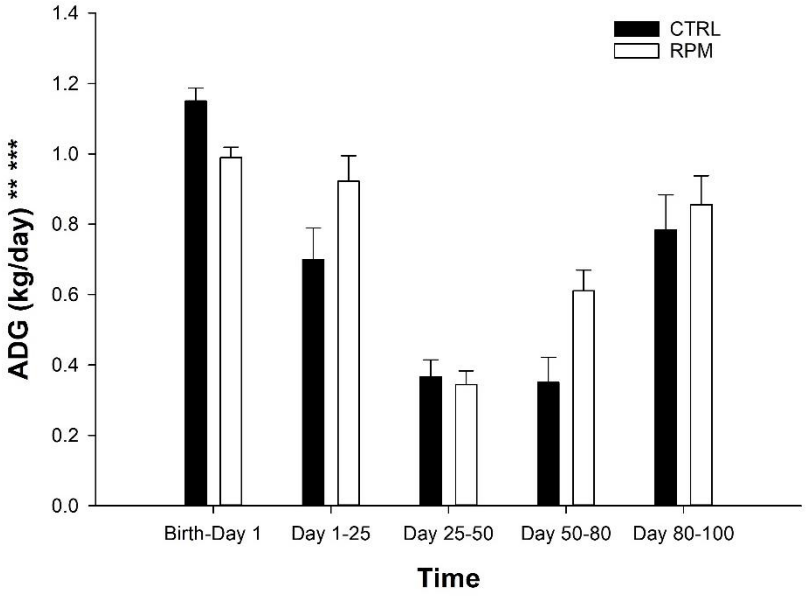


Figure 2.4. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to MULT dams receiving RPM during the peripartal period, on A) body weight, and B) average daily gain. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Error bars represent SEM.

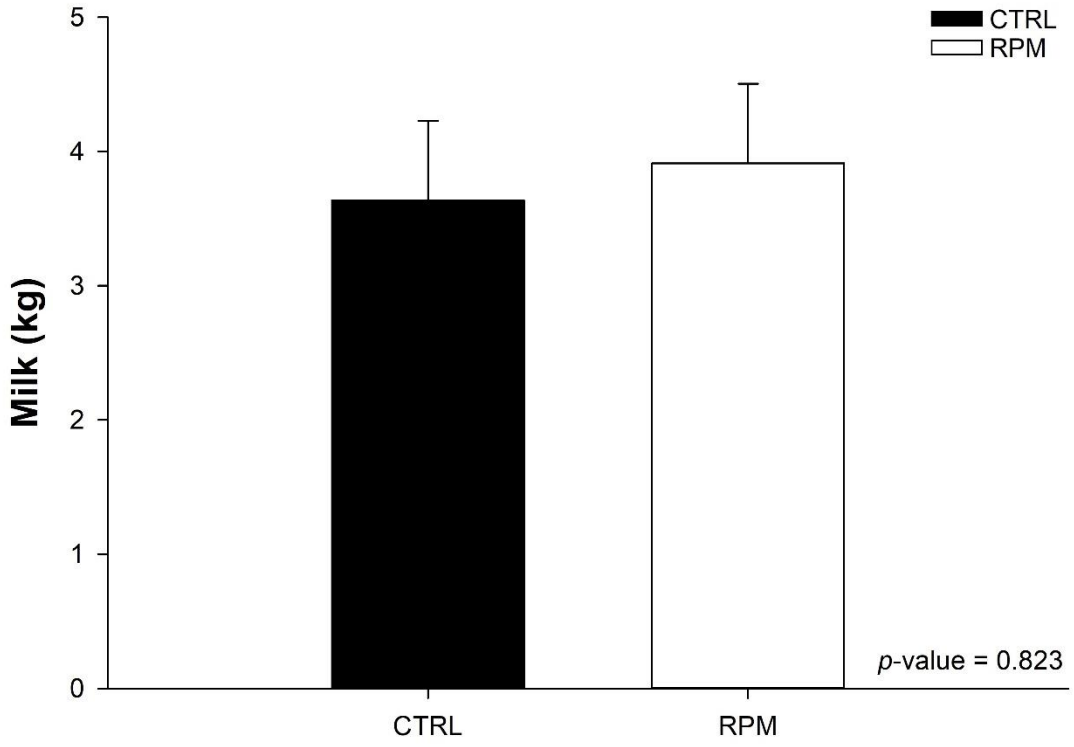
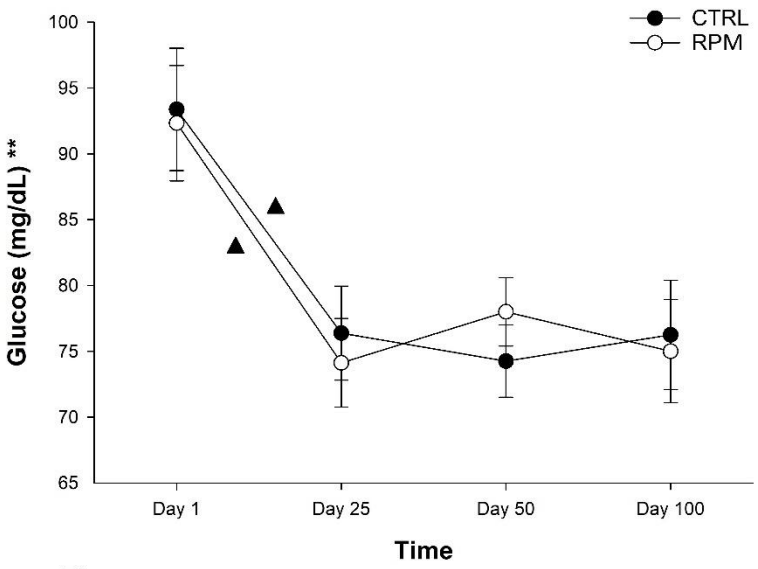
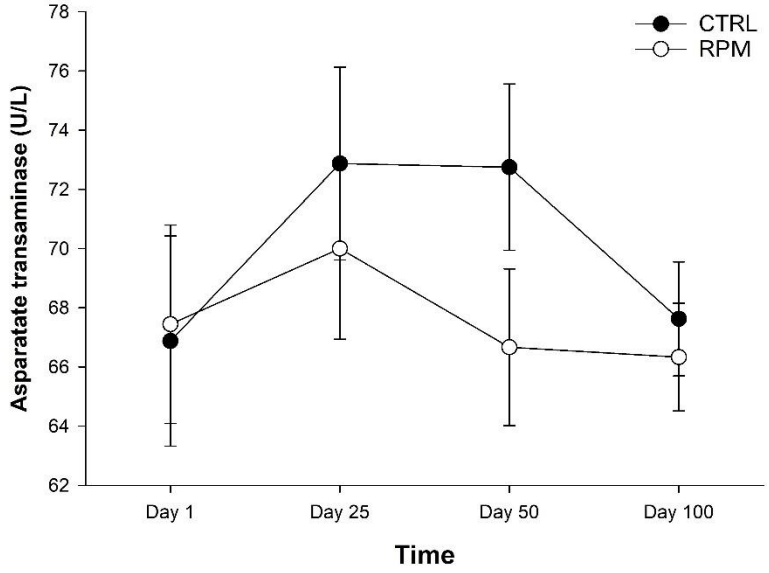


Figure 2.5. Effect of rumen-protected methionine supplementation on multiparous dams' estimated milk production at ~60 days of lactation using the weigh-suckle-weigh method. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

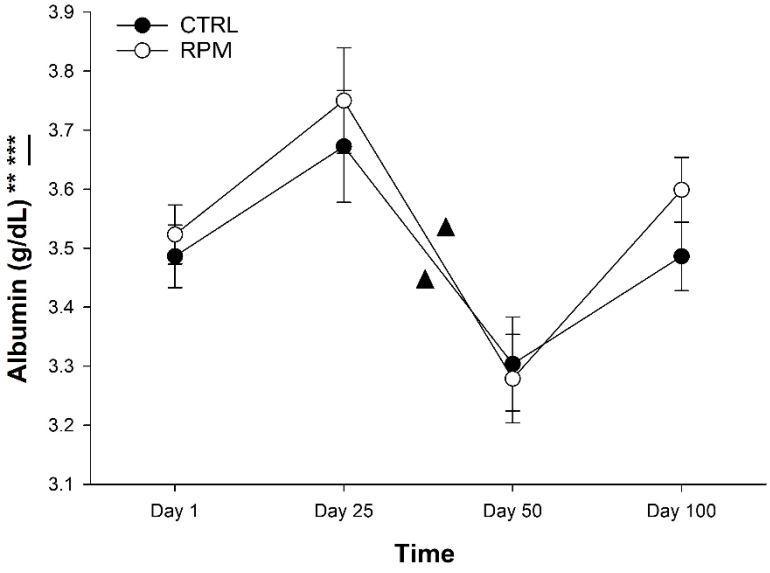
A)


CTRL
RPM
Glucose (mg/dL) **
100
95
90
85
80
75
70
65
Day 1
Day 25
Day 50
Day 100
Time

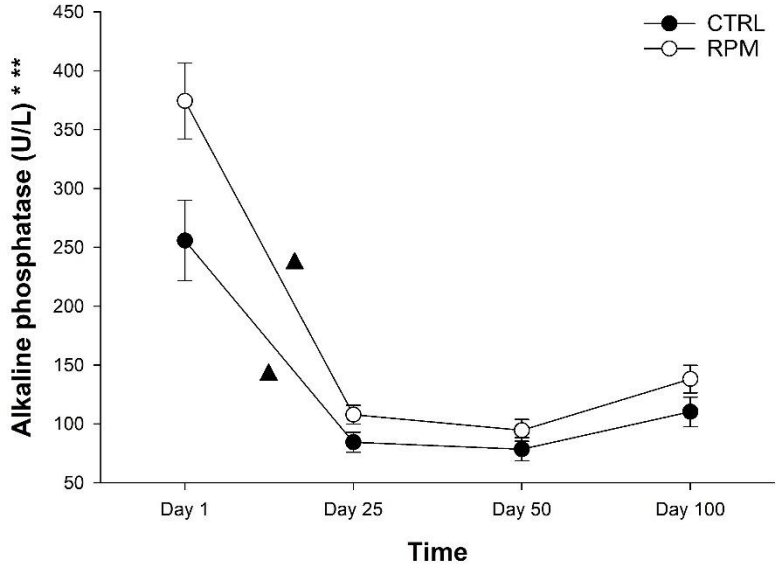
B)


CTRL
RPM
Asparatate transaminase (U/L)
78
76
74
72
70
68
66
64
62
Day 1
Day 25
Day 50
Day 100
Time

C)


CTRL
RPM
Albumin (g/dL) ** ***
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
Day 1
Day 25
Day 50
Day 100
Time

D)



E)

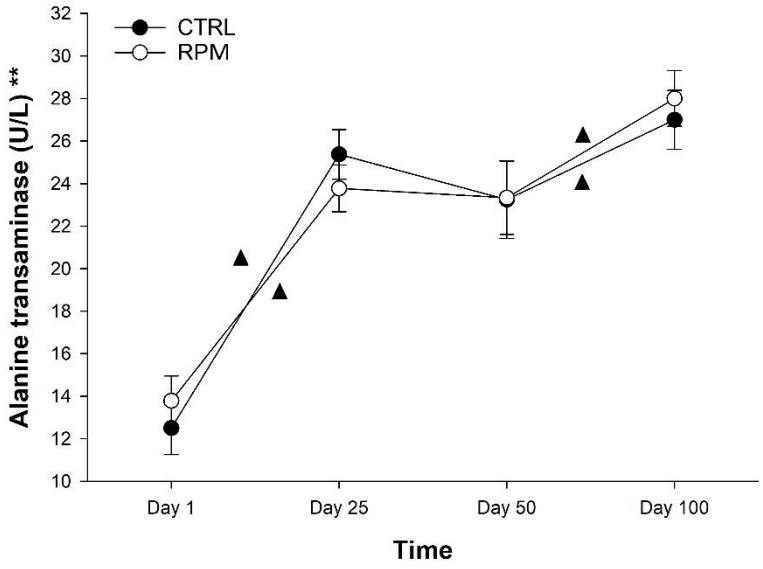
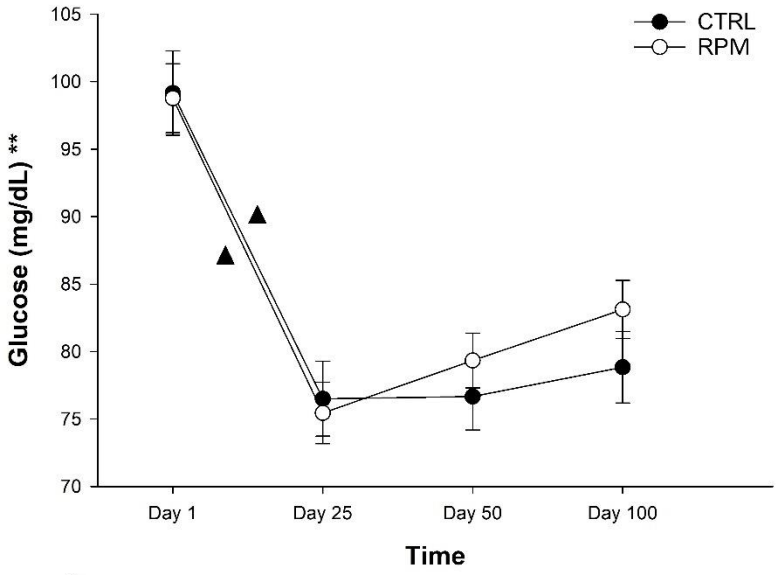
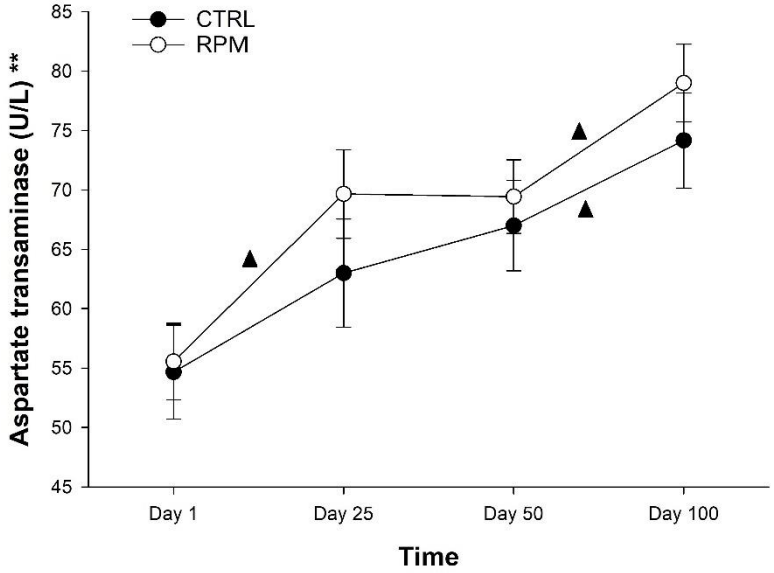


Figure 2.6. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to primiparous dams receiving rumen-protected methionine supplementation during the peripartal period on blood metabolites. Panels: A) Glucose, B) Aspartate transaminase, C) Albumin, D) Alkaline phosphatase, and E) Alanine transaminase. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($p < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($p < 0.05$) between treatments at the same time point. Error bars represent SEM.

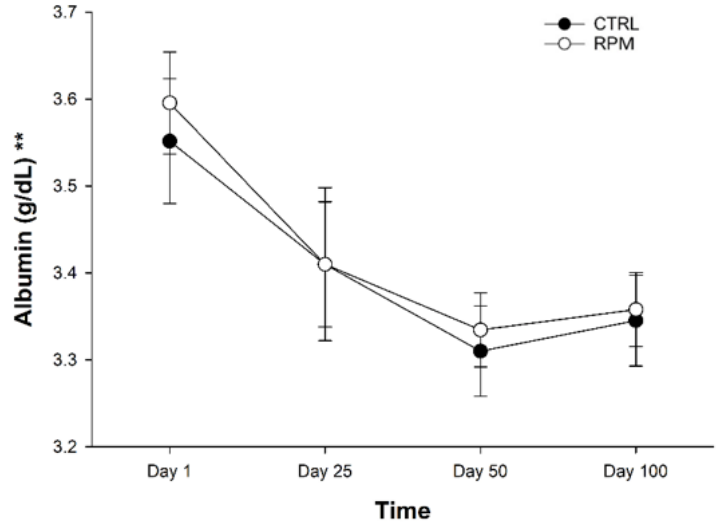
A)


CTRL
RPM
Glucose (mg/dL) **
105
100
95
90
85
80
75
70
Day 1
Day 25
Day 50
Day 100
Time

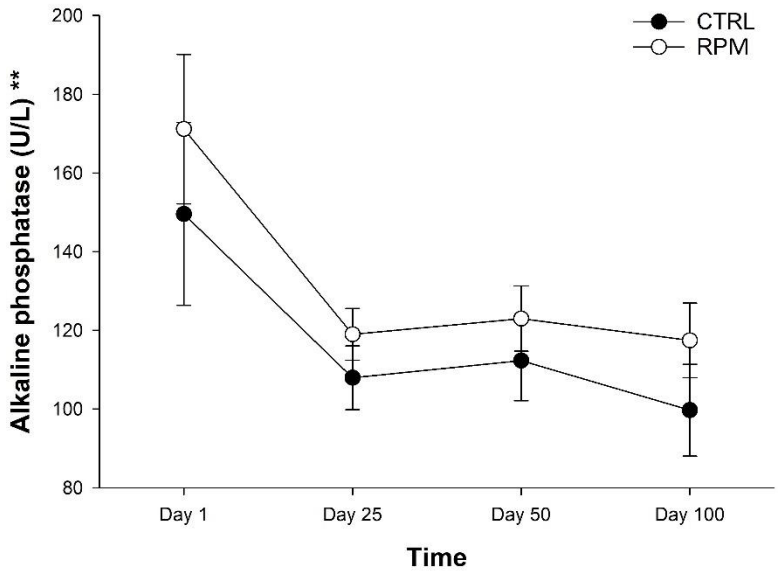
B)


CTRL
RPM
Aspartate transaminase (U/L) **
85
80
75
70
65
60
55
50
45
Day 1
Day 25
Day 50
Day 100
Time

C)


CTRL
RPM
Albumin (g/dL) **
3.7
3.6
3.5
3.4
3.3
3.2
Day 1
Day 25
Day 50
Day 100
Time

E)



F)

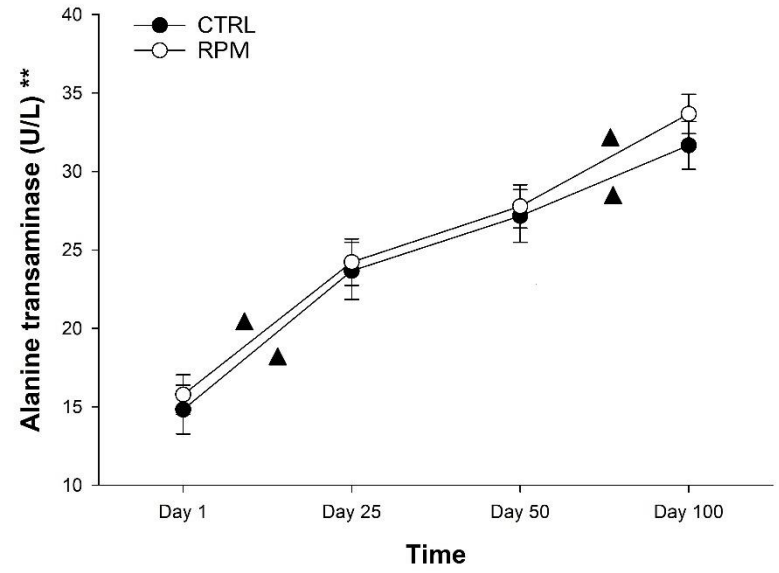
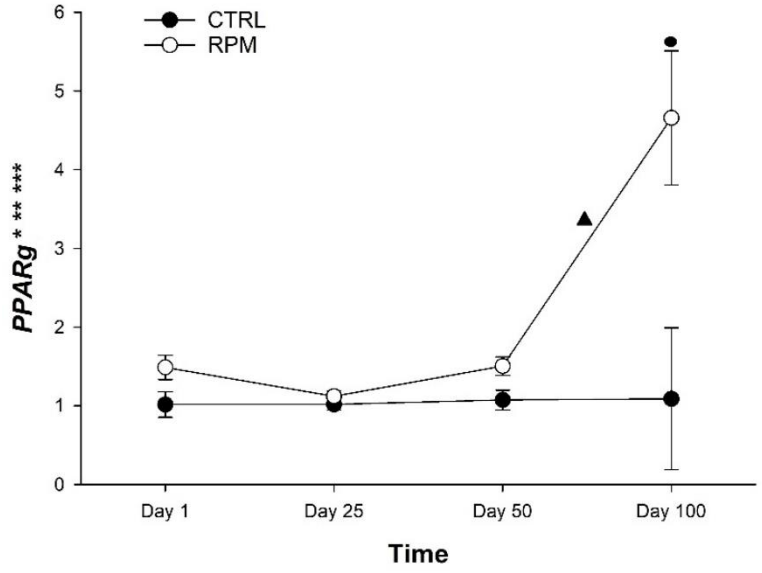
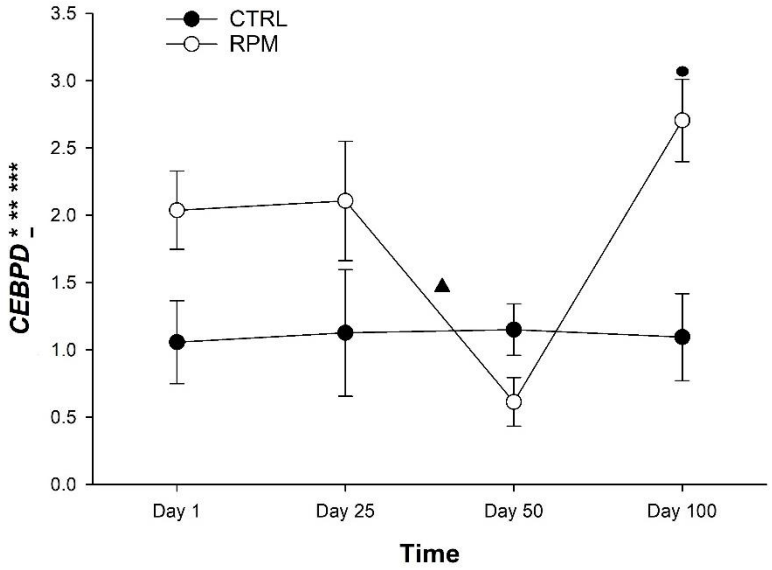


Figure 2.7. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to multiparous dams receiving rumen-protected methionine supplementation during the peripartal period on blood metabolites. Panels: A) Glucose, B) Aspartate transaminase, C) Albumin, D) Alkaline phosphatase, and E) Alanine transaminase Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($p < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($p < 0.05$) between treatments at the same time point. Error bars represent SEM.

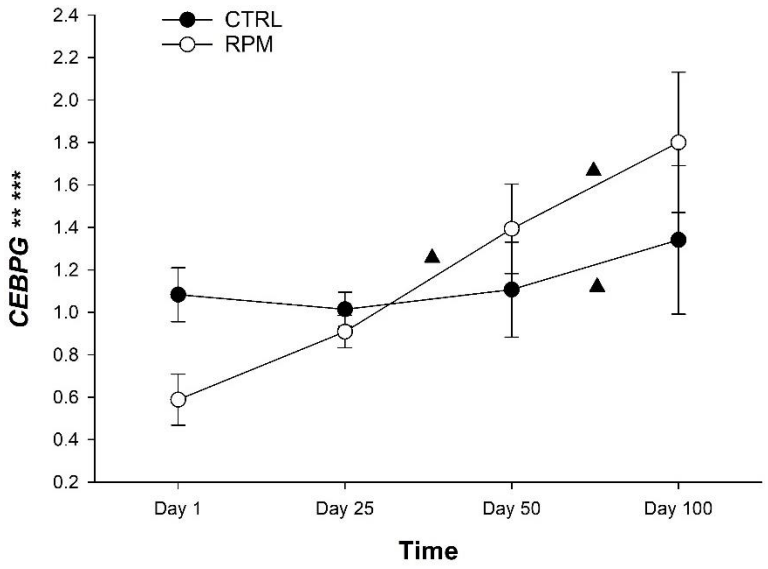
A)


CTRL
RPM
PPARg * ** ***
0
1
2
3
4
5
6
Day 1
Day 25
Day 50
Day 100
Time

B)


CTRL
RPM
CEBPD_* ** ***
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Day 1
Day 25
Day 50
Day 100
Time

C)


CTRL
RPM
CEBPG ** ***
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
Day 1
Day 25
Day 50
Day 100
Time

D)

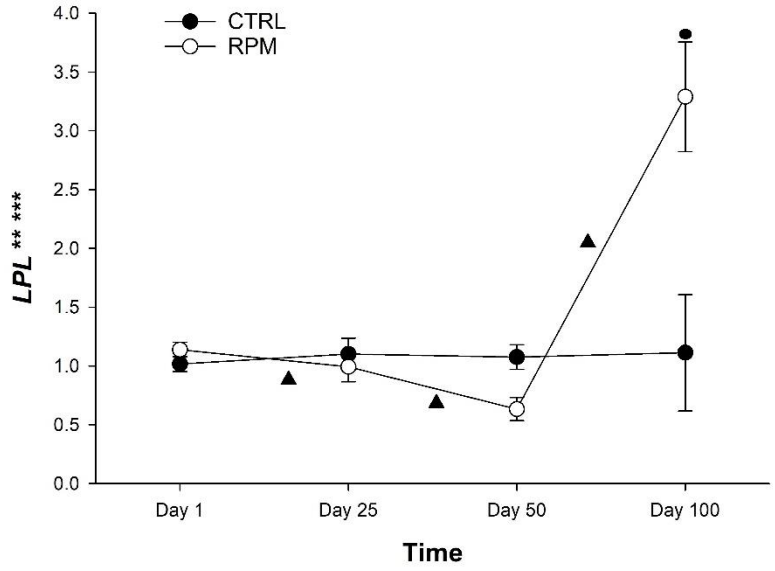
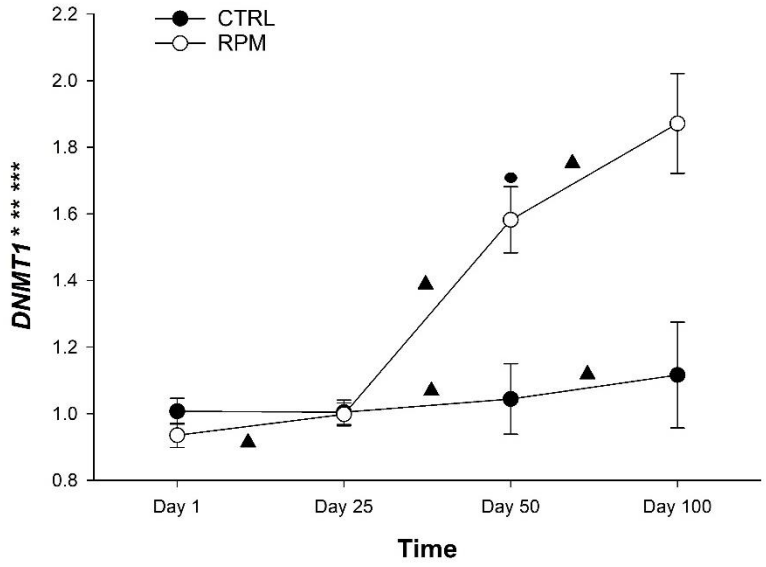
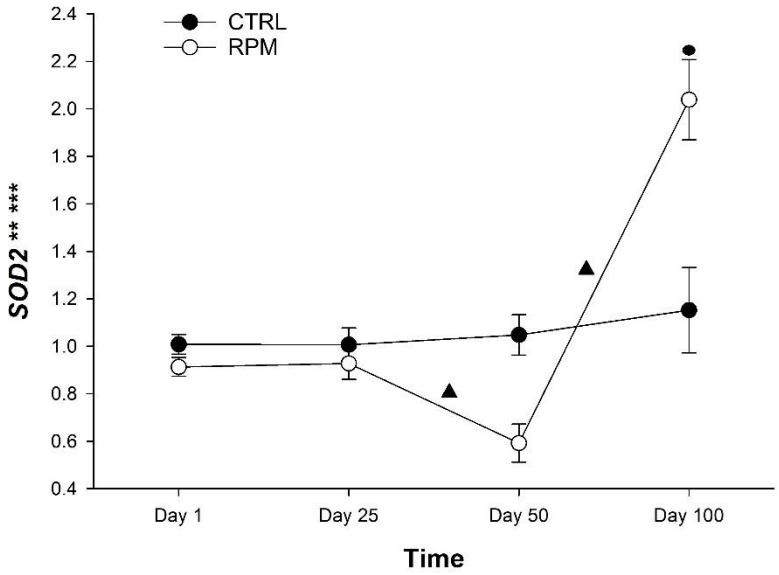


Figure 2.8. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to primiparous dams receiving rumen-protected methionine supplementation during the peripartal period on adipogenic gene network (fold change). Panels: A) *PPARg*, B) *CEBPD*, C) *CEBPG*, and D) *LPL*. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($p < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($p < 0.05$) between treatments at the same time point. Errors bars represent SEM.

A)


CTRL
RPM
DNMT1 * ** ***
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
Day 1
Day 25
Day 50
Day 100
Time

B)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2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
Day 1
Day 25
Day 50
Day 100
Time

C)

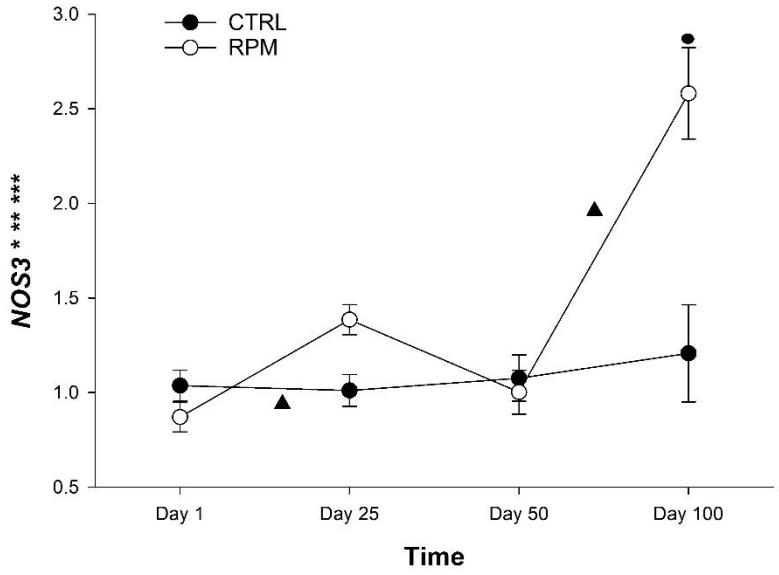
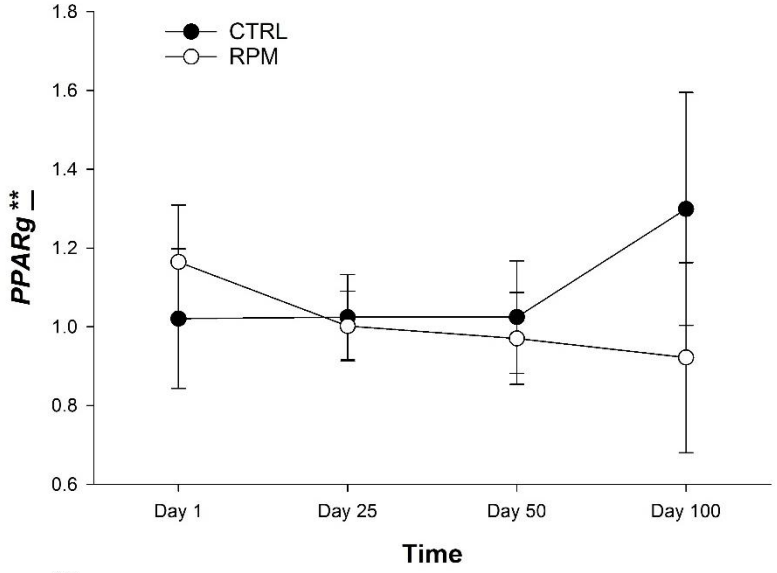

CTRL
RPM
NOS3 * ** ***
3.0
2.5
2.0
1.5
1.0
0.5
Day 1
Day 25
Day 50
Day 100
Time

Figure 2.9. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to primiparous dams receiving rumen-protected methionine supplementation during the peripartal period on DNA methylation and oxidative stress-related genes (fold change). Panels: A) *DNMT1*, B) *SOD2*, and C) *NOS3*. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($p < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($p < 0.05$) between treatments at the same time point. Error bars represent SEM.

A)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RPM
PPARg **
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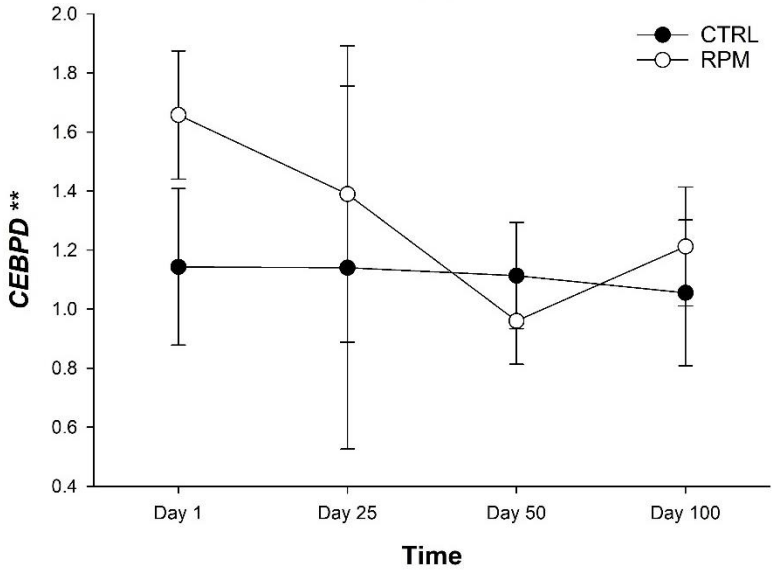
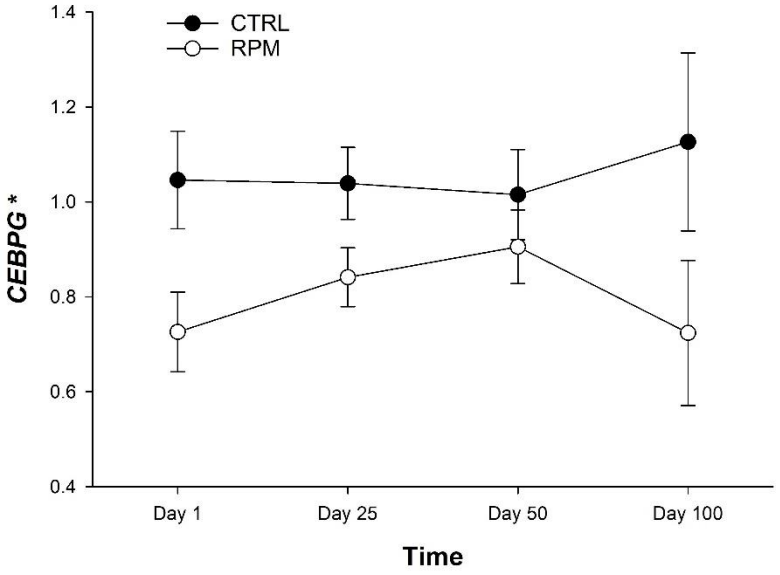
1.6
1.4
1.2
1.0
0.8
0.6
Day 1
Day 25
Day 50
Day 100
Time

B)


CTRL
RPM
CEBPD **
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
Day 1
Day 25
Day 50
Day 100
Time

C)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RPM
CEBPG *
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1.2
1.0
0.8
0.6
0.4
Day 1
Day 25
Day 50
Day 100
Time

D)

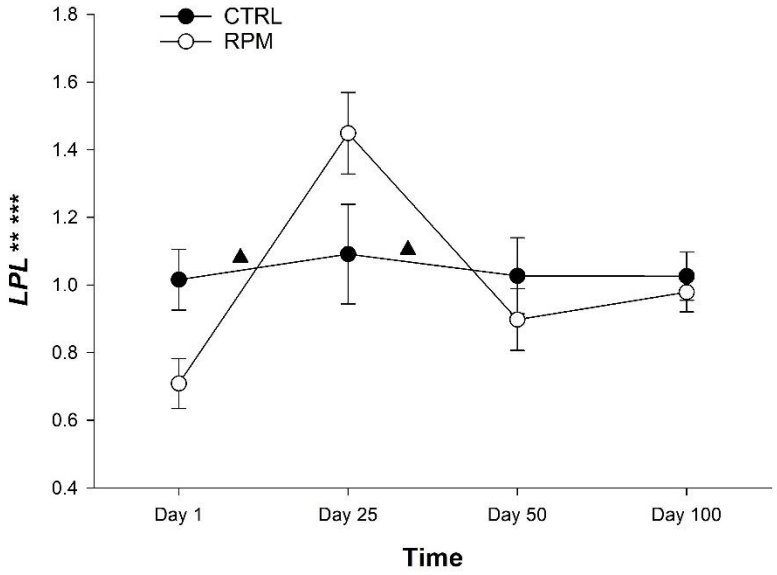
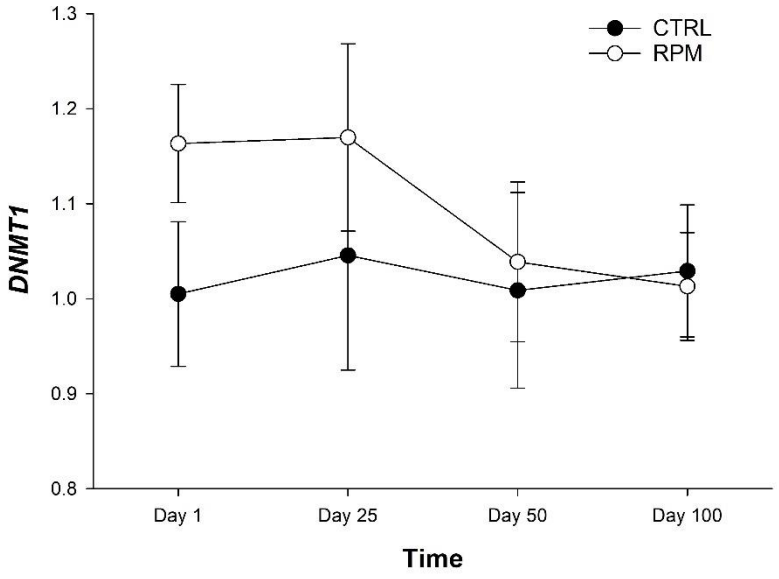
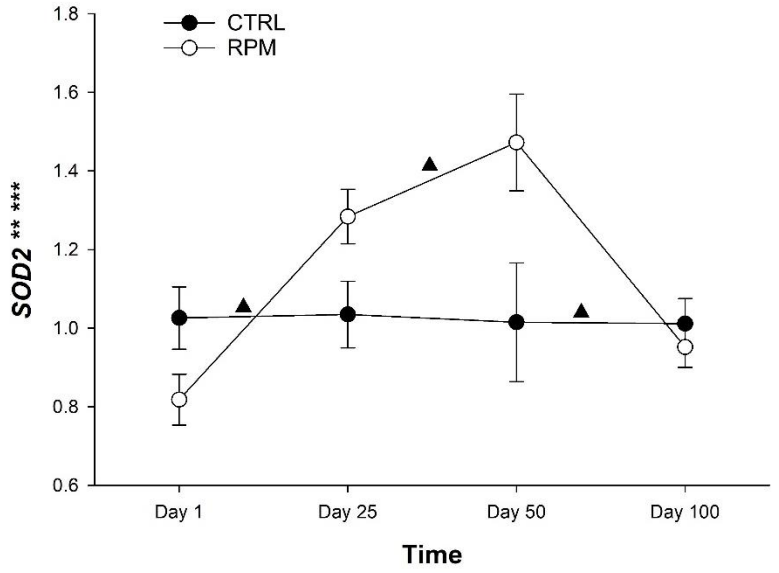


Figure 2.10. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to multiparous dams receiving rumen-protected methionine supplementation during the peripartal period on adipogenic gene network (fold change). Panels: A) *PPARg*, B) *CEBPD*, C) *CEBPG*, and D) *LPL*. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($p < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($p < 0.05$) between treatments at the same time point. Error bars represent SEM.

A)


CTRL
RPM
DNMT1
1.3
1.2
1.1
1.0
0.9
0.8
Day 1
Day 25
Day 50
Day 100
Time

B)


CTRL
RPM
SOD2 ** ***
1.8
1.6
1.4
1.2
1.0
0.8
0.6
Day 1
Day 25
Day 50
Day 100
Time

C)

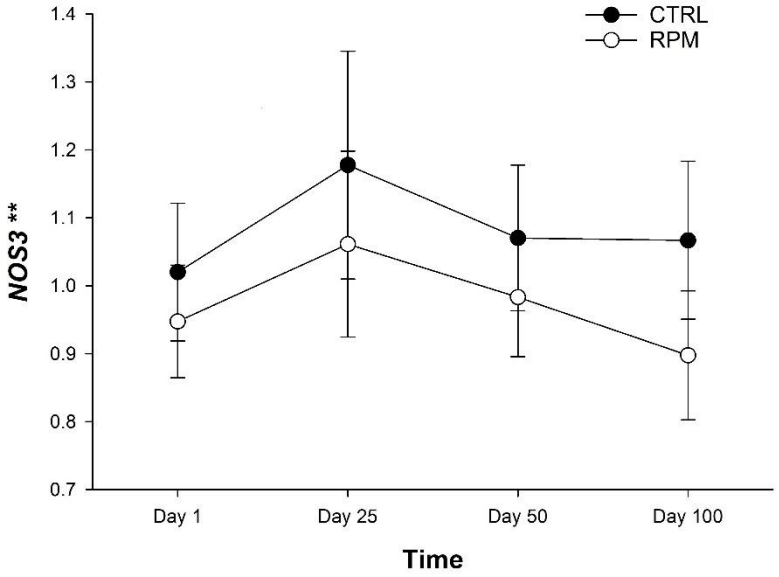

CTRL
RPM
NOS3 **
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
Day 1
Day 25
Day 50
Day 100
Time

Figure 2.11. Effect of in-utero, lactation, and post-weaning exposure to methionine in calves born to multiparous dams receiving rumen-protected methionine supplementation during the peripartal period on DNA methylation and oxidative stress-related genes (fold change). Panels: A) *DNMT1*, B) *SOD2*, and C) *NOS3*. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Treatment × time interaction effect (***), time effect (**) and treatment effect (*). Tendencies are denoted if symbols (*, ** or ***) are underlined. Symbols (▲) on lines denote significant differences ($p < 0.05$) between two time points for the same treatment, symbols (●) denote significant differences ($p < 0.05$) between treatments at the same time point. Error bars represent SEM.

Chapter 3
Effects of endophyte-infected tall fescue on performance of genotyped pregnant beef cows supplemented with rumen-protected niacin

Introduction

Tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort.) is a world-wide, extensively used cool-season bunch grass among cattle and horse production systems. Tall fescue originated in Western Europe and brought to the United Stated probably as a contaminant in other grasses from Europe. The first and current most common cultivar, "K31", was released in 1942 in Eastern Kentucky (Fergus and Buckner, 1972). Forage production, persistence, high nutritional quality, and pest resistance are some of the positive attributes that K31 tall fescue presents (Dillard et al., 2019). Interestingly, the superior forage characteristics by which producers prefer and utilize tall fescue result from a symbiotic relationship between the plant and the fungus *Epichloë coenophiala*. Ergot alkaloids are produced as secondary metabolites by the fungus (Leuchtmann et al., 2014). Furthermore, the endophyte is strategically present in greater concentration in reproductive organs than vegetative organs. The endophyte's inability to reproduce sexually outside the plant cause a relocation of the fungus on seeds in preference to other organs. The term "fescue toxicosis" has been extensively utilized for referring to the cumulative symptoms experienced by animals consuming endophyte-infected (E+) tall fescue, such as reduction in DMI, vasoconstriction, retention of winter coat, among others. From a toxicology standpoint, the most relevant alkaloid is ergovaline, which is responsible for herbivory resistance causing several impairments to animals consuming E+ tall fescue.

Endophyte-infected tall fescue consumption causes metabolic and endocrinologic changes as a result of fescue toxicosis. It was previously shown that ergovaline present in tall fescue seed extract was degraded in the rumen of dairy cattle by hyper-ammonia producers and

tryptophan-utilizing bacteria (i.e. *Clostridium aminophilum F, Clostridium sticklandii SR, Prevotella bryantii B14) in vitro* (Harlow et al., 2017). Once absorbed, the remaining ergot alkaloids can be removed by the bile in feces or metabolized by the liver, where they are converted into lysergic acid or smaller ergoline alkaloids and excreted in urine (De Lorme et al., 2007).

Niacin, known as a vasodilator agent, has also been supplemented in a rumen-protected form to cattle showing positive results with regard to body temperature regulation (Di Costanzo et al., 1997; Zimbelman et al., 2010). In addition, cattle selection based on genetic resistance to fescue toxicosis is a growing area of research for dampening the negative symptoms of this condition.

The objective of this study was to assess the effects of ergot alkaloids consumption at a pharmacological level for causing toxicosis on genotyped Angus × Simmental cows and heifer in mid-gestation supplemented with rumen-protected niacin on body weight, offspring performance, and blood metabolites.

Material and methods

Animals, dietary treatments, and experimental design

The experiment was conducted at the Beef Evaluation Center, Auburn University, Auburn, Alabama. All procedures were approved by the Auburn University Animal Care and Use Committee (IACUC; PRN# 2019-3484).

Hair samples were collected from a total of 153 Angus × Simmental cows and 53 replacement heifers from the Alabama Agricultural Experimental Station Black Belt Research and Extension Center (Marion Junction, Alabama, 36759). A total of 20-30 hairs were pulled from the animal's tail switch, ensuring that hair roots were present in the sample. Tolerant and susceptible animals were selected based on information obtained from a genetic test for tolerance for fescue toxicosis (TSnip™, Agbotanica, LLC, Columbia, MO), which provides a tolerance index. The genetic test results are presented to cattle producers in a six-point star rating scale for most susceptible animals (zero stars) or most tolerant animals (five stars). The genetic test identified animals with low (0–1 star, n = 14), or high tolerance (4–5 stars, n = 14) to fescue toxicosis. After receiving the results from the genetic test, a total of 28 pregnant animals (i.e., 11 cows and 17 heifers), between 6 and 7 months of gestation, were transported to the Auburn University Beef Cattle Evaluation Center (Auburn, AL). Afterwards, they were stratified by body weight (BW; 540 ± 88 kg) and genetic resistance to fescue toxicosis (susceptible, 0-1 stars; or tolerant, 4-5 stars); and randomly assigned to dietary treatments: 1) Susceptible Control (SC; n = 7); 2) Susceptible Niacin (SN; n = 7); 3) Tolerant Control (TC; n = 7); and 4) Tolerant Niacin (TN; n = 7). After a successful adaptation of 14 days to Calan Gate System (American Calan Inc., Northwood, NH), which was used to ensure individual supplementation, four treatment groups received twice per day, 1.16 kg of endophyte-infected tall fescue seeds, 1.16 kg of pellets

composed by soybean hulls (50%), corn gluten feed (50%); and 0.12 kg molasses in dry-matter basis per animal per day (Figure 3.1; Table 3.1) in combination with bermudagrass hay *ad libitum*. Additionally, TN and SN dams received 6 g/hd/day as-fed of top-dressed rumen-protected niacin (RPN; ANEVIS™, QualiTech Inc., Chaska, MI), following manufacturer's maximum dose recommendation. Ergovaline concentration in fescue seeds was on average 5,000 ppb on a DM basis, as measured by HPLC (detection limit = 25 ppb) (Chinchilla-Vargas et al., 2020) at the Veterinary Medical Diagnostic Laboratory of the University of Missouri. The daily dose of ergovaline required to produce characteristic signs of fescue toxicosis in beef cattle range from 10 to 20 µg/kg BW/day (Spiers et al., 2004; Holtcamp et al., 2019). Therefore, toxic fescue seed supplementation provided a maximum ergovaline concentration of 20 µg/kg of BW/day. During the study, the average temperature was 26.6 ºC; whereas the average relative humidity was 76.8% (National Oceanic and Atmospheric Administration, Washington, DC). The temperature-heat index (THI) was 71.20, and it was calculated as followed: "THI = ambient temperature + (0.55 - 0.55 × relative humidity / 100) × (ambient temperature - 58)", in which ambient temperature was expressed in Fahrenheit (Schlatter, 1987). After the experiment, dams were relocated to Alabama Agricultural Experimental Station Black Belt Research and Extension Center, where calving, lactation and normal weaning (at ~8 months) of the offspring occurred. Milk production was estimated by the weigh-suckle-weigh method (Beal et al., 1990). Briefly, at approximately 160 days of lactation, calves were separated from dams at 0800h for a period of 24 hours and relocated to a contiguous pen with *ad libitum* access to water. The following day, calves were weighed at 0800h and relocated with dams for a period of 30 minutes. After ensuring that all calves nursed properly from their dams, calves' BW was obtained and the

difference between the initial and posterior BW was considered the estimation of maternal milk production per day.

A mineral block (Compass Minerals America Inc, Overland Park, KS) containing salt (96%-99%), Mn (2400 ppm), Fe (2400 ppm), Cu (260 -360 ppm), Zn (320 ppm), I (70 ppm), and Co (40 ppm) was accessible to the animals during the total duration of the study.

Liver biopsies

Liver samples (0.5 – 1.0 g) were obtained on Day 1 and Day 30 using a sterilized bone marrow aspiration needle (Monoject™, Dublin, Ireland)(Coleman et al., 2019). An area surrounding the 11^{th} and 13^{th} ribs was scanned by ultrasound to identify the optimal area to perform the liver biopsy. Anesthesia injections of 5 mL of Lidocaine 2% (VetOne®, Boise, ID) were injected in the selected area. The incision on the skin was performed using a sterilized scalpel blade. The second liver sample, on day 30, was positioned in the same intercostal area as the incision side of the sample on Day 1, and they were separated by at least 5 cm. Each liver sample was rinsed with sterile saline, placed in sterile 2 mL cryotubes, and immediately stored in liquid nitrogen for further transportation and storage at -80°C at the Beef Nutriepigenomics Laboratory of the Department of Animal Sciences, Auburn University.

Blood metabolites

Blood samples were collected from dams on Day 1 and Day 30 via coccygeal vessel venipuncture (i.e., tail-head) into blood collection tubes. Blood analyses were conducted in all animals in all timepoints. Blood serum was separated by centrifugation at 1,500 × g for 15 min. Aliquots were stored frozen at -20 °C until analyzed for alkaline phosphatase, alanine transaminase, aspartate transaminase, glucose, albumin, creatine kinase, and lactate dehydrogenase and albumin at the Auburn University Endocrine Diagnostic Lab, Auburn, AL.

Creatine Kinase concentration was measured using a Roche/Hitachi Cobas C analyzer using Reagent 1 (Imidazole buffer: 123 mmol/L, pH 6.5 (37 °C); EDTA: 2.46 mmol/L; Mg2+: 12.3 mmol/L; ADP: 2.46 mmol/L; AMPR: 6.14 mmol/L; diadenosine pentaphosphate: 19 μmol/L; NADP+ (yeast): 2.46 mmol/L; N-acetylcysteine: 24.6 mmol/L; HK (yeast): > 36.7 μkat/L; G6PDH (E. Coli): > 23.4 μkat/L; preservative; stabilizers; additives), and Reagent 2 (CAPSO* buffer: 20 mmol/L, pH 8.8 (37 ºC); glucose: 120 mmol/L; EDTA: 2.46 mmol/L; creatine phosphate : 184 mmol/L; preservative; stabilizers). Serum lactate dehydrogenase concentration was analyzed using Cobas c 311 using Reagent 1 (N-methylglucamine: 400 mmol/L, pH 9.4 (37 °C); lithium lactate: 62 mmol/L; and stabilizer) and Reagent 2 (NAD: 62 mmol/L; stabilizers and preservatives)(Bowling and Katayev, 2010).

Prolactin was evaluated as per Bernard et al. (1993) with the following modifications: rabbit anti-bovine PRL antibody (National Institute of Diabetes & Digestive & Kidney Diseases; Torrance, CA) was utilized at 1:200,000 final dilution as per Dr. A.F. Parlow (Pituitary Hormones & Antisera Center, Harbor-UCLA Medical Center) and secondary antibody (goat anti-rabbit gamma globulin; Antibodies, Inc., Davis, CA) final concentration was determined empirically at the University of Tennessee at 1:55. Intra- and inter-assay coefficients of variation were 5.20% and 9.97 %, respectively.

The remaining metabolites were analyzed in serum according to the methods previously described in Chapter 2.

Statistical analysis

The response variables analyzed included maternal and offspring's body weight, and blood metabolites on Day 1 and Day 30. Maternal and offspring BW and blood metabolites were analyzed using the MIXED procedure of SAS (SAS 9.4 Institute, Cary, NC, USA). Mixed

procedure of SAS included a repeated-measure statement with an unstructured covariate structure. Time, genetic treatment and nutritional treatment were considered the fixed effect, whereas animal within genetical treatment and nutritional treatment was considered the random effect of the statistical model. The statistical model used was: $Y_{ijlk} = \mu + C_i + N_j + G_l + S_k + (C \times N \times G)_{ijl} + (C \times N)_{ij} + (C \times G)_{il} + (N \times G)_{jl} + \varepsilon_{ijl}$; where, Y_{ijlk} is the BW or blood metabolite value; μ is the overall mean; C_i is the fixed effect of time (2 levels); N_j is the fixed effect of nutritional treatment (2 levels); G_l is the fixed effect of genetic treatment; S_k is the random effect of heifer nested within genetic treatment and nutritional treatment; C × N × G is the interaction of time by genetic treatment by nutritional treatment; C × N is the interaction of time by nutritional treatment; C × G is the interaction of time by genetic treatment; N × G is the interaction between genetic treatment and nutritional treatment; and ε_{ijlk} is the random error (0, σ_e^2) associated with Y_{ijlk}. Milk production data were analyzed using the general lineal models (GLM) of SAS.

Results

Animal performance

There was a genetic × nutritional × time interaction tendency ($P = 0.055$), nutritional × time interaction tendency ($P = 0.052$), and a time effect ($P = 0.01$) for BW (Table 3.2). Tolerant dams receiving rumen-protected niacin (RPN) experienced a significant reduction in BW during the experiment ($P < 0.05$). However, there was no difference among treatments in calves at birth ($P > 0.10$). Calves' BW increased markedly between birth and weaning ($P < 0.001$; Table 3.3). In addition, there was no difference in milk production at approximately 160 days of lactation among treatments ($P = 0.668$; Figure 3.2).

Blood metabolites

Circulating glucose, albumin, aspartate transaminase, alanine transaminase, alkaline phosphatase, creatine kinase, and lactate dehydrogenase did not present a nutritional treatment × genetic treatment × time interaction effect ($P > 0.10$; Table 3.4). Glucose and alkaline phosphatase decreased at the end of the study and had a time effect ($P < 0.001$). Circulating albumin concentration was lower at Day 30 compared with Day 1 for TC and TN groups, whereas lactate dehydrogenase concentration was lower at Day 30 in all treatment groups. Albumin and lactate dehydrogenase showed a genetic treatment × time interaction effect ($P = 0.01$, and $P = 0.01$, respectively), and a time effect ($P < 0.001$).

In addition, aspartate transaminase showed a genetic treatment × time interaction effect ($P = 0.008$) and a time effect ($P = 0.051$). Plasma alkaline phosphatase concentration was lower at Day 30 compared with Day 1 in all treatments ($P < 0.05$). Alanine transaminase showed a genetic treatment × nutritional treatment interaction effect ($P = 0.024$), a time effect ($P = 0.001$), and a nutritional treatment effect ($P = 0.035$). Alanine transaminase concentration was lower at

Day 30 for all treatments; whereas SN had the greater concentration at Day 30 compared with the rest of the groups ($P = 0.05$).

Creatine kinase presented a nutritional treatment × time tendency effect ($P = 0.074$) and a time effect ($P < 0.001$). Creatine kinase concentration was lower at Day 30 compared with Day 1 in TN ($P < 0.05$).

Finally, there was no treatment × time interaction effect ($P > 0.10$) for prolactin concentration (Figure 3.3). However, there was a significant reduction for this hormone at Day 30 compared with Day 1 in all treatments, showing a time effect ($P < 0.001$).

Discussion

Animal performance and milk yield

Body weight is an essential parameter for estimating an animal's nutritional energy status during gestation. From early to mid-gestation, the main goal of achieving an optimal body condition score is to ensure a proper fetus development and a quick return to estrus after calving. Fluctuations in maternal BW caused by changes in the diet, if severe, could affect fetus growth, leading to reduced BW at birth or dystocia (Long et al., 2010). In our study, even though there was a significant decrease of BW in TN receiving E+ seeds, calves born to those dams did not show reduced BW at birth compared to the rest of the treatments. The reduction in BW could be partly explained by the consumption of E+ and the exposure to high temperatures typically occurring during early summer in Auburn, Alabama. Ergot alkaloid consumption is widely known for decreasing dry matter intake, which leads to BW loss (Hemken et al., 1981). Interestingly, a recent study conducted by Galliou et al. (2020) investigated the effect of T-snip™ genetic test for fescue toxicosis tolerance on pregnant Angus cattle. This study utilized a total of 150 multiparous purebred cows and was relocated to two different locations. Authors reported no effect in genetic tolerance on average BW. Still, there was a location × genetic tolerance interaction effect for the same parameter in a specific location with the greatest infection rate. Thus, there could be an association between total ergot alkaloids concentration of the plant and the tolerance to fescue toxicosis evaluated using a T-snip™ genetic test (Galliou et al., 2020). However, the length of this study was 14 weeks and differed from our experiment, which was conducted in a total of 4 weeks. Therefore, one possible explanation might be that the exposure to a high level of endophyte provided by E+ tall fescue seeds in a relatively short period (30 days) causes a differential effect on the animal compared with a lower dose of E+

during a longer time period. Furthermore, pharmacological use of niacin has been discovered to counter-attack obesity by inhibiting lipolysis and reducing total adipose tissue content in the body. Wanders et al. (2013) reported that niacin reduces adipose tissue inflammation by decreasing pro-inflammatory cytokine, chemokine, and macrophage concentration in blood. These findings could explain the significant reduction of BW in TN dams consuming E+ tall fescue seeds and RPN for 30 days.

In addition to offspring's BW at birth, the lack of difference in offspring's BW at weaning could also be explained by the similar milk yield among treatments. It is well known that the effects of E+ consumption on milk production have a negative impact in ruminant species. Previous studies reported that a reduction in milk yield exists in ruminants consuming E+, mainly due to prolactin suppression, which is a key hormone for the normal occurrence of lactation (Akers et al., 1981; Brown et al., 1993; Brown et al., 1996; Britt et al., 2020). However, to the best of our knowledge, our study is the first to assess the effect of animal genotyping for fescue toxicosis tolerance on milk yield. Although, further research is needed in this area. Furthermore, niacin effect on lactation performance has shown conflicting results in the literature. More importantly, most of the studies investigating the effect of niacin were conducted using dairy cattle breeds, showing an increment (Dufva et al., 1983) or no change in milk production (Di Costanzo et al., 1997; Zimbelman et al., 2010). Therefore, we believe that these results could not be extrapolated to beef breeds.

In conclusion, the inclusion of a control group consuming an endophyte-free diet might have revealed the negative impact of dams consuming E+ diets. Furthermore, research studies using a larger number of genotyped dams should be conducted to confirm that genetic resistance

to fescue toxicosis or niacin supplementation does not affect milk yield on Angus × Simmental dams in the southeastern region of the US.

Blood metabolites

Animals exposed to E+ tall fescue are prone to experience disturbances in liver metabolism because of the detoxification process. Research studies monitoring liver health utilize different blood metabolites as markers, such as glucose, albumin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), and lactate dehydrogenase (LDH). All the mentioned metabolites showed a time effect, except for AST, which had a tendency for time effect. Clearly, the reduction in the concentration of the different metabolites results from exposure to high levels of ergot alkaloids in the diet.

Briefly, the liver is the main glucogenic organ of ruminant's body, accounting for up to 90% of total glucose turnover. During E+ tall fescue consumption, a noticeable consequence of fescue toxicosis is the reduction of feed intake, affecting the levels of different blood metabolites, such as glucose. Previously, a fetal programming experiment conducted using Angus-cross cows as the animal model showed that nutrient restriction during early to mid-gestation caused a decrease in maternal circulating glucose (Taylor et al., 2018). Thus, animals with reduced feed intake due to exposure to E+ diets could experience a reduction in glucose levels. For example, Jackson et al. (2015) reported reduced circulating glucose levels for Angus crossbred steers grazing high-endophyte infected tall fescue compared with low-endophyte infected tall fescue during summer months. Even though we did not measure individual feed intake due to the nature of the diet offered (*ad libitum* bermudagrass hay was part of the diet), an evident reduction of hay was provided at the end of the study compared to the beginning, based on the animal's demand. Therefore, we believe that the decrease in DMI due to fescue toxicosis

was the primary cause of the reduced circulating glucose observed in all treatments (i.e., time effect) at the end of the study.

Albumin, the most abundant protein in circulating blood in an adult mammal, is produced in the liver and plays a vital role in regulating blood volume and as a transporter for fatty acids, steroids, and thyroid hormones. Furthermore, ALT is a hepatic enzyme responsible for converting pyruvate and glutamate from alanine and α-ketoglutarate. This transamination process occurs mainly in mammals' circulating plasma and liver. Similar to our results, a previous study showed a reduction in ALT and a tendency to a decrease in albumin levels on Angus and Angus × Hereford steers grazing E+ tall fescue compared with steers grazing E- tall fescue (Oliver et al., 2000). In their study, Jackson et al. (2015) also showed a reduction of circulating albumin. The authors proposed an impairment in albumin synthesis by hepatic cells due to a possible decrease in amino acid uptake by the gut as a result of a reduction of feed intake. Contrary to our expectations, animals genetically tested as susceptible to fescue toxicosis did not experience a significant suppression in albumin concentration as tolerant dams. The interaction effect of genetic treatment × time may indicate a possible association between genetic tolerance and albumin level, in which susceptible animals could present more stable albumin levels.

Furthermore, AST is the enzyme responsible for the transamination of aspartate and α-ketoglutarate to oxaloacetate and glutamate. Circulating AST levels show the mitochondrial turnover of hepatocytes, which indicates liver health status. Interestingly, previous studies have reported the same trends showing reduced circulating AST in beef cattle consuming E+ compared with those exposed to E- diets (Dougherty et al., 1991; Brown et al., 2009; Jackson et al., 2015). Even though numerous studies have revealed the suppressive effects of ergot alkaloids

on AST, the detailed mechanism by which toxic fescue inhibits AST was not fully explained in the literature to date.

Another clinical signal of fescue toxicosis is the suppression of ALP (Jackson et al., 2015), a critical dephosphorylating enzyme primarily found in the liver of mature cattle. Thus, ALP is widely utilized as a biomarker for assessing liver health. The results in the present study are congruent with previous evidence on beef species (Nihsen et al., 2004; Brown et al., 2009; Jackson et al., 2015). According to Schultze et al. (1999), animals exposed to E+ experience the loss of mucosal intestinal cells and reduction of osteoblast activity which might lead to suppression of ALP isoenzymes activity and decrease in BW.

Creatine kinase is an enzyme responsible for the reversible conversion of creatine and adenosine triphosphate (ATP) and adenosine diphosphate (ADP). Therefore, CK is associated with energy production, and it is most present in tissues with high energy demand, such as muscle or brain (Meyer et al., 1984). In cattle, three CK isoenzymes have been detected: CK-MM, present in skeletal muscle; CK-MB, specific for the heart; and CK-BB, located in the brain. During gestation, circulating CK levels increases due to the greater metabolic demand (Abramov et al., 1996). Analysis of CK in serum shows the amount of CK-MM; however, other isoenzymes, such as CK-MB and CK-BB, could be present, especially during gestation. Interestingly, a study found a high correlation between elevated CK and AST levels with endometritis incidence in dairy cows (Sattler and Fürll, 2004). In addition, the consumption of E+ causes suppression in CK in both growing beef steers (Jackson et al., 2015), and mature beef cows (Dougherty et al., 1991). In accordance with previous evidence, our study showed a reduction in creatine kinase levels in all treatments. This reduction could be associated with lower energy production, primarily due to the toxicosis effect of E+. Another factor causing the

consistent decrease in CK could be associated with thermal condition. Earlier reports showed a reduction in CK levels in beef cattle exposed to heat stress conditions (Nazifi et al., 2003; ZongGang et al., 2015). In our study, there was a greater reduction of circulating CK on TN dams compared with the rest of the treatments. In rats with ischemia (e.g., reduction in blood flow), the pharmacological use of niacin has been proven to reduce CK levels, improving heart recovery after perfusion (Trueblood et al., 2000). In our study, CK levels had a tendency for a nutritional treatment × time interaction effect. The supplemented RPN may play a role in creatine kinase synthesis and release, as shown in the previous evidence. Although, the mechanism of inhibition of CK synthesis by niacin remains to be elucidated.

Lactate dehydrogenase is an oxidoreductase enzyme found in most tissues. This enzyme converts lactate to pyruvate in a reversible manner with the reduction of NAD^+ to NADH (Schumann et al., 2002). It is also an important precursor for lipid synthesis (Whitehurst et al., 1981). The relevance of changes on circulating LDH relies on the alterations of liver health status. In ruminants, the main glucogenic substrate is propionate; however, lactate can also be an important glucogenic source. The site of action of LDH mostly occur at the cytosol level, and recent research showed that LDH can be found at the mitochondria (Farhana and Lappin, 2021). Therefore, LDH usually is associated with the first step of gluconeogenesis. It has been shown that LDH is a sensitive enzyme that is usually affected by the presence of toxic contaminants in mammalian metabolism. Mice exposed to a contaminant mixture (aldrin, DDT, endosulfan, among others) had a significant reduction of LDH in a dose-dependent manner. The authors suggested that the suppression of LDH could be caused by an inactivation process induced by oxidase reactions. In accordance to results in our study, previous evidence has shown a reduction in circulating LDH on cattle consuming E+ tall fescue (Oliver, 2005). For example, Jackson et

al. (2015), reported a suppression of 18% to 23% in Angus crossbred steers grazing high endophyte-infected tall fescue compared with steers grazing low endophyte-infected tall fescue. Similarly, a study using beef crossbred steers showed that animals grazing KY 31 (E+ tall fescue) had lower circulating LDH compared with animals grazing HiMag4 and HiMag9, both novel, E- tall fescue (Nihsen et al., 2004). One of the possible reasons of this reduction may be related to a lower utilization of pyruvate as a glucogenic precursor. For example, ergot alkaloids consumption also causes a suppression in glucose 6-phosphate, which is the immediate precursor of glucose in gluconeogenesis (Rosenkrans et al., 2000). The reduction of LDH at the end of the study is congruent with the lower circulating glucose in the present study.

Finally, and most importantly, circulating prolactin has been widely used as a biological marker to identify the occurrence of tall fescue toxicosis in different species, such as cattle (Porter and Thompson, 1992; Borba et al., 2018), sheep (Henson et al., 1987), horses (Breuhaus, 2003), and rats (Jackson et al., 1986). The mechanism of inhibition of ergot alkaloids is by mimicking monoamine neurotransmitters (e.g., dopamine) which has a key role in prolactin secretion. Dopamine inhibits lactotroph activity in the hypothalamus by binding dopamine receptors (D2) present on their cellular membrane. After the activation of D2 receptors, a downstream downregulation of *PRL* occurs, which leads to a decrease in prolactin excretion (Fitzgerald and Dinan, 2008). It has been previously shown that the potency of inhibition of cAMP is significatively greater by ergovaline binding to D2 compared with dopamine, suggesting the importance of the negative impact of consumption of tall fescue infected with ergot alkaloids (Larson et al., 1995). In our study, there was a clear, significant reduction in circulating prolactin level during the experiment for all treatments. These results are consistent with other previous experiments that observed reduced circulating prolactin levels in gestating

beef cattle (Shoup et al., 2016) and ewes (Britt et al., 2020) exposed to E+ tall fescue. Remarkably, the lower prolactin levels at the end of the study are a clear sign of fescue toxicosis on gestating dams. Interestingly, genetic treatment did not affect prolactin levels. The study conducted by Galliou et al. (2020) reported the effects of T-snip™ genetic test on performance data on gestating Angus cow-calf pairs (e.g., BW and weaning weight); however, authors did not show results related to prolactin levels. Therefore, genetic resistance to fescue toxicosis, as indicated by T-snip™, might not be associated with dopamine nor prolactin metabolism. However, further research is needed in order to confirm the mechanisms of genetic resistance, if present, on animals genotyped as tolerant by T-snip™.

Summary and conclusions

In the cow-calf production cycle, gestation is the most determinant period. On the one hand, it critically affects maternal current and post-parturient overall status. On the other hand, gestation determines offspring growth and development, having a carry-over impact on post-natal life. Therefore, nutritional alteration can positively or negatively impact maternal and offspring performance. A notorious example of this phenomenon occurs when beef cattle dams exposed to E+ during gestation disturb normal growth and development of the offspring. Our study, in accordance with previous evidence in the literature, clearly showed typical signs of fescue toxicosis in terms of BW loss and circulating prolactin levels. More specifically, the utilization of RPN may accelerate BW losses in beef cattle dams, as shown by TN whose experienced a significant reduction in BW compared with the rest of the treatments. However, and most importantly, neither birth weight nor weaning weight was affected in calves born to TN, suggesting that the statistical difference of maternal BW during gestation did not impact offspring's in-utero and post-natal growth and development.

Furthermore, the consistent reduction of blood metabolites that recall for liver health status could be noted as another sign that animals were exposed to fescue toxicosis. The previous evidence in literature indicates that, since the liver is the major detoxifying organ of mammals, the consumption of E+ negatively affects the concentration of critical metabolites for maintaining physiological homeostasis. We could feasibly confirm that dams were experiencing fescue toxicosis through the intake of E+ seeds.

Interestingly, and contrary to our expectations, there was no significant positive impact of the utilization of T-snip™ on beef cattle dams consuming E+ tall fescue in BW, blood metabolites, and milk production parameters. Similarly, we expected that niacin could improve

overall animal status through its vasodilatory capacity, potentially mitigating the vasoconstrictive effect of ergot alkaloids. However, the supplementation with RPN did not benefit the maternal and offspring parameters analyzed. More research utilizing more animals and different endophyte inclusions in the diet is needed to validate the results reported in our study.

Tables and figures

Table 3.1. Chemical composition of maternal base diet fed to dams during mid-gestation. bermudagrass hay was fed *ad libitum*, whereas 2.27 kg of fescue seeds and 2.27 kg of pellets were offered individually in daily basis. All ingredients are expressed in a DM basis.

Ingredients [1]	% DM	CP	NDF	ADF	TDN	Crude Fat
Fescue seeds [2]	90.55	16.12	48.49	16.03	64.11	-
Pellets [2,3]	90.93	29.96	10.68	4.92	78.83	3.47
Molasses [2]	84.00	5.80	-	0.40	72.00	-
Bermudagrass hay	90.73	10.37	74.48	39.27	56.15	-

DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDN = total digestible nutrients

[1] All ingredients are expressed on a DM basis

[2] Fescue seeds, pellets, and molasses were fed as supplement on a 48.5:48.5:3 ratios.

[3] Pellets were composed by 49% ground corn, 49% soybean meal, and 2% soybean oil.

Table 3.2. Effect of rumen-protected niacin supplementation on genetically tested Angus × Simmental dams exposed to endophyte-infected tall fescue during mid-gestation on body weight (kg).

Treatment	Time (d)	BW (kg)	SEM	*p* - value						
				GT	**NT**	**Time**	**GT×Time**	**NT×Time**	**GT×NT**	**GT×NT×Time**
Susceptible control	1	541.82	35.61	0.954	0.739	0.010	0.382	0.052	0.914	0.055
	30	528.25								
Susceptible niacin	1	540.13	35.61							
	30	527.14								
Tolerant control	1	541.04	35.61							
	30	532.73								
Tolerant niacin	1	537.79[a]	35.61							
	30	509.81[b]								

Statistical differences were declared at $p \leq 0.05$, and tendencies at $p > 0.05$ and < 0.1.
[a,b] Superscripts represent statistical differences between two time points for the same treatment.
Table shows *p*-values for Genetic Treatment (GT), Nutritional Treatment (NT), Genetic Treatment × Time interaction effect (GT × Time), Nutritional Treatment × Time interaction effect (NT × Time), Genetic Treatment × Nutritional Treatment effect interaction (GT × NT), and Genetic Treatment × Nutritional Treatment × Time interaction effect (GT × NT × Time).

Table 3.3. Effect of rumen-protected niacin supplementation on genetically tested Angus × Simmental dams exposed to endophyte-infected tall fescue during mid-gestation on offspring's body weight (kg)

Treatment	Time (d)	BW (kg)	SEM	*p* - value						
				GT	**NT**	**Time**	**GT×Time**	**NT×Time**	**GT×NT**	**GT×NT×Time**
Susceptible Control	Birth	28.4	5.00	0.955	0.677	0.001	0.540	0.322	0.911	0.114
	Weaning	289.8	23.00							
Susceptible Niacin	Birth	32.2	5.00							
	Weaning	247.7	23.00							
Tolerant Control	Birth	28.9	5.00							
	Weaning	269.2	23.00							
Tolerant Niacin	Birth	30	5.00							
	Weaning	294.5	23.00							

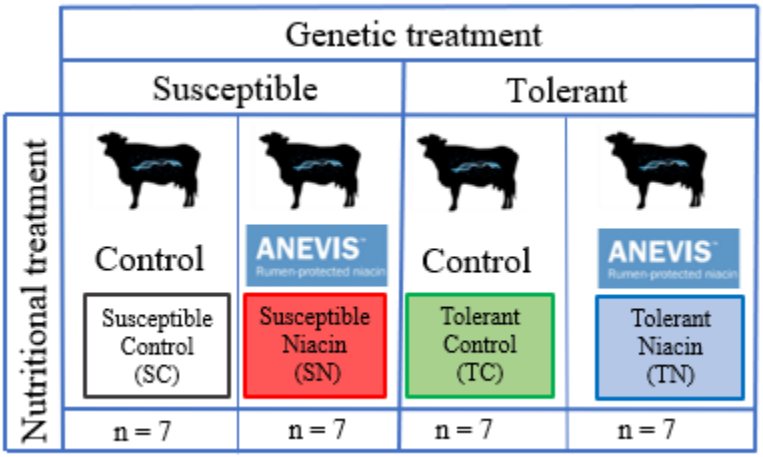
Statistical differences were declared at $p \leq 0.05$, and tendencies at $p > 0.05$ and < 0.1.
Table shows *p*-values for Genetic Treatment (GT), Nutritional Treatment (NT), Genetic Treatment × Time interaction effect (GT × Time), Nutritional Treatment × Time interaction effect (NT × Time), Genetic Treatment × Nutritional Treatment interaction effect (GT × NT), and Genetic Treatment × Nutritional Treatment × Time interaction effect (GT × NT × Time).

Table 3.4. Effect of rumen-protected niacin supplementation on genetically tested Angus × Simmental dams exposed to endophyte-infected tall fescue during mid-gestation on blood plasma metabolites.

Metabolite	SC		SN		TC		TN		SEM	p - value						
	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30		GT	NT	Time	GT*Time	NT*Time	GT*NT	GT*NT*Time
Glucose (mg/dL)	71.71	70.71	71.86	68.57	75.14	69.71	74.43	74.29	4.9	0.570	0.980	0.040	0.630	0.830	0.780	0.270
Albumin (g/dL)	3.57	3.51	3.53	3.44	3.63^{a}	3.43^{b}	3.53^{a}	3.31^{b}	0.05	0.474	0.133	0.001	0.010	0.595	0.606	0.920
Aspartate transaminase (U/L)	70.14	67.57	70.14	79.29	86.57	60.71	79.71	63.29	4.9	0.850	0.481	0.051	0.008	0.291	0.600	0.800
Alanine transaminase (U/L)	29.14^{a}	$20^{b,y}$	34.43^{a}	$25.57^{b,z}$	30.71^{a}	$20.57^{b,yz}$	30.86^{a}	$20^{b,y}$	1.07	0.147	0.035	0.001	0.250	0.868	0.024	0.698
Alkaline phosphatase (U/L)	63.67^{a}	34.59^{b}	76.53^{a}	51.50^{b}	98.57^{a}	65.31^{b}	75.21^{a}	41.73^{b}	21.46	0.238	0.729	0.001	0.951	0.542	0.430	0.330
Creatine kinase (U/L)	215.29	189.4	184.57	144.7	206.00	221	255.14^{a}	157.14^{b}	31.78	0.299	0.252	0.001	0.407	0.074	0.295	0.194
Lactate dehydrogenase (U/L)	1340.36^{a}	932.39^{b}	1294.9^{a}	933.07^{b}	1431.04^{a}	889.83^{b}	1448.51^{a}	911.6^{b}	47.46	0.494	0.870	0.001	0.011	0.639	0.685	0.909

Superscripts ^{a,b} represent statistical difference ($p \leq 0.05$) between time points for the same treatment
Superscripts ^{y,z} represent statistical difference ($p \leq 0.05$) between treatments for the same time point
Table shows *p*-values for Genetic Treatment (GT), Nutritional Treatment (NT), Genetic Treatment × Time interaction effect (GT × Time), Nutritional Treatment × Time interaction effect (NT × Time), Genetic Treatment × Nutritional Treatment interaction effect (GT × NT), and Genetic Treatment × Nutritional Treatment × Time interaction effect (GT × NT × Time).

A)



B)

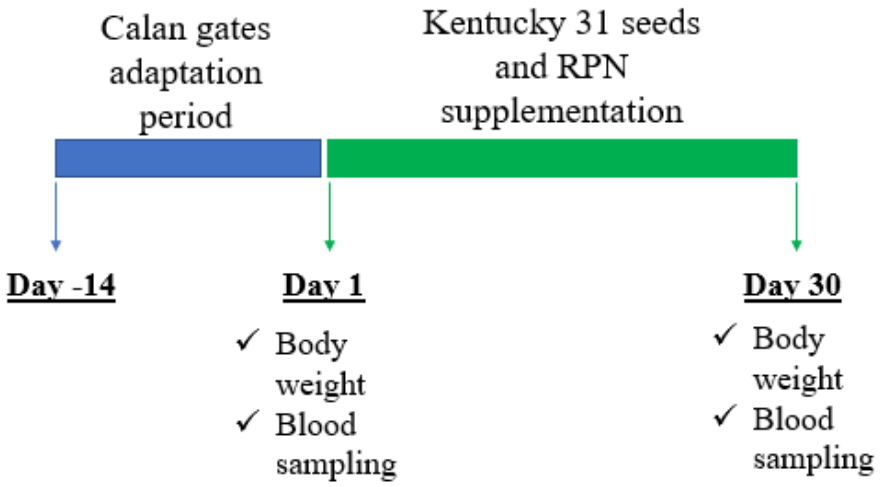


Figure 3.1. A) Experimental design of the study, B) Timeline of the experiment.

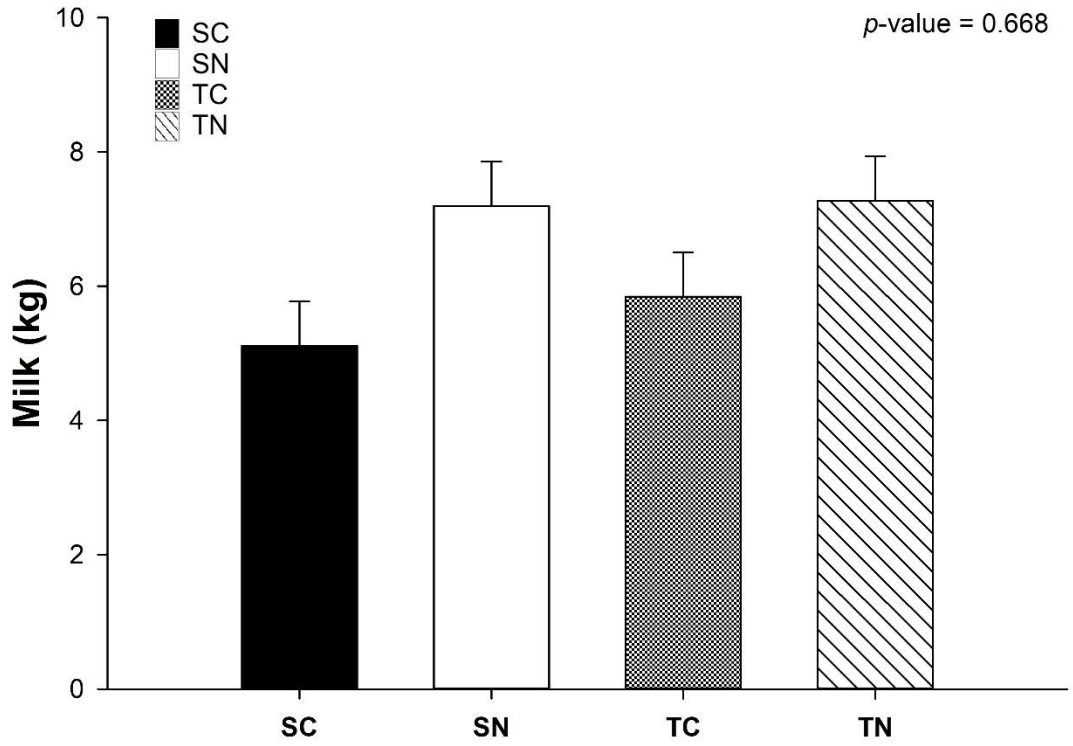


Figure 3.2. Effect of rumen-protected niacin supplementation on genetically tested Angus × Simmental dams exposed to endophyte-infected tall fescue during mid-gestation on milk production. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

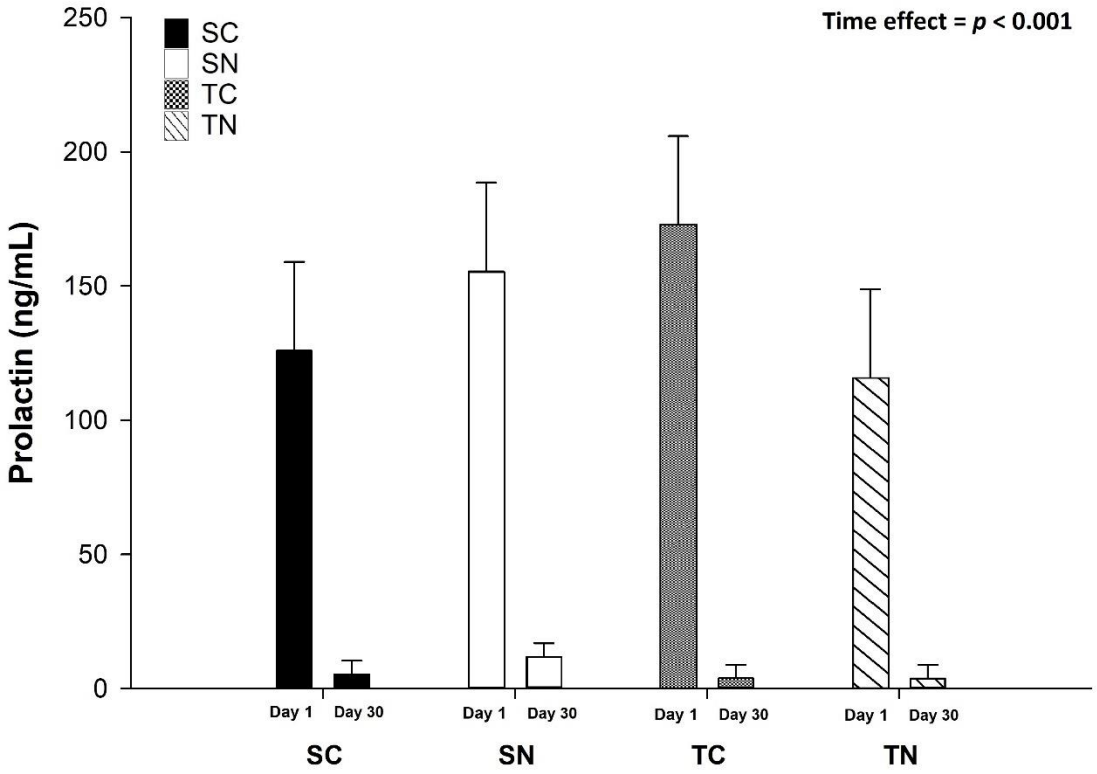


Figure 3.3. Effect of rumen-protected niacin supplementation on genetically tested Angus × Simmental dams exposed to endophyte-infected tall fescue during mid-gestation on circulating prolactin at Day 1 and Day 30. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent standard error means.

Chapter 4
Complete blood count analysis on beef cattle exposed to fescue toxicosis and rumen-protected niacin supplementation

The content of this chapter belongs to a manuscript published in *Animals*, 2021, *11*(4), 998 (Alfaro et al., 2021).

Introduction

In the Southeastern region of the United States, cow-calf operations, the main beef production systems of the region, are forage-based production. Tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort.) is a cool season perennial bunch grass with numerous positive physiological attributes, such as high nutritive value, herbage mass production, persistence, capability to be stockpiled, and pest resistance (Stuedemann and Hoveland, 1988; Kallenbach et al., 2003). Most tall fescue plants establish a mutualistic symbiotic relationship with the endophyte *Epichloë coenophiala*, which is responsible for producing ergot alkaloids as secondary metabolites. The location of the endophyte in the plant is tightly related to the survival strategy to colonize the plant and be propagated. The most important alkaloid produced is ergovaline, which enhances plant protection against biotic and abiotic stressors (Bacon and Siegel, 1988; Liebe and White, 2018; Dillard et al., 2019). Ergovaline concentration is greater in seed heads compared with vegetative organs such as leaves or steams (Rottinghaus et al., 1991). The use of fescue seeds in a research study has the advantage that animals under study can be provided with known concentrations of ergovaline in their dietary supplement, stimulating the occurrence of fescue toxicosis in a controlled situation. Therefore, animals consuming endophyte-infected tall fescue experience fescue toxicosis, which is characterized by a reduction in performance, elevated rectal temperature (RT) and respiration rate (RR), vasoconstriction, retention of winter hair coat, among others symptoms (Schmidt et al., 1982; Klotz et al., 2016;

Poole et al., 2019). Nevertheless, the utilization of endophyte-free tall fescue varieties, which lack a fungal endophyte, leads to superior animal performance outcomes; therefore, they have been an appropriate but expensive alternative. The consumption of endophyte-infected tall fescue by gestating dams also influences offspring's performance. Shoup et al., 2016 have reported a tendency for lower adjusted 205 d weaning weight for Angus × Simmental offspring that were exposed to endophyte-infected tall fescue during the last trimester of gestation. Hematological analyses, such as complete blood count (CBC), have been used extensively by researchers to assess the health status of cattle and to generate accurate disease diagnoses (Knowles et al., 2000). In a previous study, the genetic correlation and heritability of blood parameters, through CBC analysis, in crossbred beef cattle exposed to endophyte-infected and endophyte-free tall fescue was assessed (Chinchilla-Vargas et al., 2020). However, this study accomplished its objective to report the relationship between blood-based traits and phenotypic and genotypic heritability, but it did not show differences in blood parameters for cattle exposed to endophyte-infected and endophyte-free tall fescue. In a similar study, the effect of endophyte-infected tall fescue on a limited number of blood parameters, such as glutathione, oxidized glutathione, and total glutathione on beef cows was analyzed (Lakritz et al., 2002). Nevertheless, this study did not provide information about hematology disorders caused by fescue toxicosis through the utilization of CBC analysis. Therefore, it is possible to state that, to the best of our knowledge, no data reporting the effects of endophyte-infected tall fescue by means of a CBC analysis on beef cattle is currently available in the literature. The selection of animals based on their genetic resistance to fescue toxicosis has been used in commercial beef production systems as a strategy for reducing economic losses caused by this syndrome. A genetic test developed to determine the level of tolerance or susceptibility to fescue toxicosis (T-snip™, Ag. Botanica, Columbia, MO,

USA) is currently available to beef producers in the market. This commercial genetic test provides a tolerance index and the results are usually presented to producers in a six-point stars rating scale for most susceptible animals (zero stars) to most tolerant animals (five stars). The test showed promising results as a predictor of cow performance (Galliou et al., 2020). Finally, it has been broadly reported that one of the main effects of fescue toxicosis in cattle is vasoconstriction (Klotz et al., 2008; Klotz et al., 2016; Poole et al., 2018). Porter and Thompson (1992) have shown that in gestating ewes, vasoconstriction leads to a reduction in nutrient partitioning, causing lower performance outcomes in offspring. The inclusion of dietary niacin as a vasodilator agent on livestock diets could be utilized as an alternative for damping the negative effects of fescue toxicosis due to its vasoconstrictive effect in affected animals (Cheng et al., 2006; Rungruang et al., 2014). The objective of this study was to analyze changes in complete blood count (CBC) parameters, performance and health status in offspring' grouped based on maternal resistance to fescue toxicosis assessed with a commercially available genetic test and their supplementation with RPN for a period of 30 days after weaning.

Materials and methods

Animals and experimental design

All the procedures for this study were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Auburn University (IACUC Protocol #2019-3484). A genetic test for fescue toxicosis (T-snip™, Ag. Botanica, Columbia, MO) was used to select animals for this study. Hair samples were collected from 256 Angus × Simmental cows by pulling approximately 20–30 hairs from the animal's tail switch, ensuring that hair roots are present in the sample. This genetic test provides a tolerance index and results are expressed on a six-point star rating for most-susceptible animals (zero stars) or most-tolerant animals (five stars). The genetic test identified animals with low (0–1 stars, n = 34), medium (2–3 stars, n = 189), or high tolerance (4–5 stars, n = 33) to fescue toxicosis. A total of 28 pregnant cows, out of the 256 cows genetically tested, were selected for this study based on their genetic test results (14 susceptible and 14 tolerant). At mid-gestation, selected cows were randomly assigned to a control group (CTRL), which received only the base diet; or a rumen-protected niacin (RPN) group, fed with base diet and the addition of 6 g/hd/day of top-dressed ANEVIS™ RPN (QualiTech Inc., Chaska, MN) as a fixed rate for a 30-day period (from 1 July 2019 to 30 July 2019), while they received endophyte-infected tall fescue seeds. Therefore, 7 susceptible dams received a control diet (SC, n = 7), 7 susceptible dams received RPN top-dressed in the control diet (SN, n = 7), 7 tolerant dams received a control diet (TC, n = 7), and 7 tolerant dams received RPN in the control diet (TN, n = 7) during mid-gestation (~average 180 to 205 days pregnant). After a period of approximately 10 days, dams were successfully adapted to Calan gates (American Calan Inc., Northwood, NH) in order to ensure individual doses of fescue seeds and RPN. Base diet was *ad libitum* bermudagrass (*Cynodon dactylon*) hay in combination with a

nutritional supplement composed by 1.61 kg of tall fescue seeds: 1.61 kg of pellets composed by 46.5% ground corn, 46.5% soybean meal, 5% wheat middlings, and 2% soybean oil; and 0.1 kg of molasses per animal per day (Table 4.1). Base diet was formulated to meet nutrients animal requirements (NRC, 2016), and tall fescue seeds quantity offered was based on its ergovaline concentration. Ergovaline concentration results were obtain from the Veterinary Medical Diagnostic Laboratory at the University of Missouri (Columbia, MO). The two lots of tall fescue seeds utilized in this study had an ergovaline concentration of 7300 ppb and 2700 ppb, respectively. A total of 20 µg/kg BW/day was the daily dietary dose of ergovaline offered to the animals under study to produce characteristic signs of fescue toxicosis. This ergovaline concentration follows recommendations from previous studies (Spiers et al., 2004; Holtcamp et al., 2019).

Offspring calving season started on 11 September until 17 December 2019. After weaning (average 216 ± 25 days), offspring born to tolerant and susceptible dams received base diet with or without RPN and "KY 31" endophyte-infected tall fescue seeds (DLF Pickseed, Halsey, OR) for a period of 30 days (from 16 June 2020 to 16 July 2020). During the study, the average temperature was 26.1 ºC; whereas the average relative humidity was 77.3% (Auburn University Mesonet, Auburn, AL). The temperature-heat index (THI) was 70.57, and it was calculated as followed: "THI = ambient temperature + (0.55 - 0.55 × relative humidity / 100) × (ambient temperature - 58)", in which ambient temperature was expressed in Fahrenheit (Schlatter, 1987). Offspring steers (n = 19) and heifers (n = 9) with an average body weight (BW; 300 ± 44 kg) and age of 7–9 months-old were assigned to treatment groups based on their dam's genetic and nutritional treatments. Therefore, this study is composed by offspring that come from susceptible dams that received a control diet (SC, n = 7), offspring from susceptible

dams that received RPN top-dressed in the control diet (SN, n = 7), offspring from tolerant dams that received a control diet (TC, n = 7), and offspring from tolerant dams that received RPN in the control diet (TN, n = 7). Offspring used for this study belongs to the beef unit located at the Auburn University Black Belt Research and Extension Center (Marion Junction, AL), where they have permanent access to endophyte-infected tall fescue pastures. The offspring used in this study come from 9 different sires whose genetic information in terms of fescue toxicosis resistance was not evaluated. Although, sire used to breed each cow was a parameter considered at the time to select animals for this study. At weaning, offspring were relocated at the Beef Evaluation Center, Auburn University, Auburn, AL due to the accessibility to Calan gates system. Calan gates were used to ensure offspring individual RPN daily dosage which was top-dressed on the based diet mixed with endophyte-infected tall fescue seeds and molasses to increase palatability.

Animal performance

Individual BW, average daily gain, respiration rate, and rectal temperature from offspring heifers and steers were obtained on Day 1 (2 weeks after weaning), 7, 14, 21, and 30 of treatment from 0600 to 0800 h from offspring heifers and steers. Average daily gain was calculated considering the difference between two BW measurements within a known period of time. Rectal temperature data was measured using a digital rectal thermometer (Sharptemp V, Cotran Corporation, Portsmouth, RI), obtaining values in Fahrenheit for posterior conversion to Celsius using the formula $◦C = [(◦F − 32) × 5/9]$. Respiration rate was recorded as the number of breaths, determined by counting flank movements, per 20 s and multiplied by 3 to obtain breaths per minute. Hair shedding score was based on visual observation of the extent of winter hair, and it was reported on a 1 to 5 scale; being 1 when the animal had removed completely the winter coat

showing full shedding and a score of 5 indicating that the animal retained completely the winter coat. Hair shedding score was performed by the same trained person at Day 1 and Day 30.

Complete blood counting analysis

Complete blood count (CBC) analysis was used as a relatively easy technique for assessing the offspring immunological status (Leach et al., 2013). Ten mL of whole blood was obtained individually via venipuncture from the coccygeal vessels on Day 1 and Day 30. Whole blood samples were placed in BD Vacutainer® Plus blood collection tubes coated with a spray-dried K2 EDTA, which works as anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Right after collection, samples were gently inverted several times and immediately transported to the Clinical Pathology Laboratory at the College of Veterinary Medicine of Auburn University. Complete blood count analysis was performed automatically using an ADVIA 120 Hematology System apparatus (Siemens, Munich, Germany).

Statistical analysis

The response variables analyzed included BW, body temperature, respiratory rate, average daily gain, hair score, and blood parameters. The overarching linear mixed effects models used to describe all the response variables included the fixed effects of cow nutritional treatment (CTRL or RPN), genotype (tolerant or susceptible), sex, and interactions (two and third way). All response variables with the exception of ADG were analyzed assuming a repeated structure using calf as the subject. Both an unstructured and an unstructured order 1 variance–covariance structures were evaluated to accommodate for possible covariation across time points and heterogeneity of variances. The fits of both model specifications were compared using the Bayesian Information Criterion and, based on this assessment, the results from the unstructured order 1 specification are presented. The measurement protocols required the

inclusion of an additional explanatory variable to the previously described overarching model included additional explanatory variables. The models describing the profiles of BW, RT, RR, and blood parameters included BW at Day 1 as a covariate, and the time at measurement (Days 7, 14, 21, and 30) as main effect and interacting with the remaining model factors. The model describing the average daily gain excluded time because one observation was available per calf across the trial and excluded BW as a covariate because this measurement was used to compute the response variable. For hair score, the estimates and residual distribution from a generalized linear mixed effects model assuming a Poisson distribution and from a linear mixed effects model were consistent. In consideration of this finding, and to facilitate the interpretation of the estimates, results from the analysis of hair score using a linear mixed effects model are presented. BW, RT, RR, average daily gain, and hair score were analyzed in the observed scale. The blood parameters were transformed using a natural logarithm function to ensure that the distribution of the residuals of all variables followed a Gaussian distribution. The analysis of the mixed effect models was implemented using the MIXED procedure with the Kenward–Rogers adjustment of degrees of freedom (SAS/STAT software, Version 9.4, 2019, SAS Institute, Cary, NC). The least square means, pairwise contrasts, and associated standard errors were estimated for the model factors. A significant *p* value was declared at $p \leq 0.05$ and tendencies between $p > 0.05$ and < 0.1.

Results

Complete blood count analysis

Neutrophil to lymphocyte ratio, reticulocytes and basophils

There was a genetic treatment × nutritional treatment × time interaction for neutrophils to lymphocytes ratio (Figure 4.4). Offspring in the TN group had a greater neutrophils to lymphocytes ratio at Day 1 compared to the rest of the treatments ($P = 0.045$). In addition, there was a nutritional treatment × time interaction ($P = 0.031$) for basophils (Figure 4.4). Offspring in RPN group had a decrease in basophils concentration, while CTRL animals present an increment in basophils concentration between Day 1 and Day 30 ($P = 0.031$). Furthermore, there was a sex × time interaction ($P = 0.002$), and a nutritional treatment × time interaction for reticulocytes ($P = 0.027$). Control heifer offspring had an increment of reticulocytes percentage between Day 1 and Day 30 compared to RPN male offspring (Figure 4.4).

Hematocrit, hemoglobin, red blood cell distribution width, and white blood cells.

There was a nutritional treatment × sex interaction ($P = 0.026$) for hematocrit percentage and hemoglobin concentration (Figure 4.2). Concentration of hemoglobin and percentage of hematocrit was greater for CTRL male offspring and RPN female offspring as compared to CTRL female and RPN male offspring. Furthermore, there was a nutritional treatment × sex interaction for red blood cell distribution width (RDW, Figure 4.2). Heifer CTRL offspring had the greatest RDW dimensions ($P = 0.004$). Furthermore, there was a nutritional treatment × sex interaction ($P = 0.004$) for white blood cells (WBC) concentration (Figure 4.2). Control male and RPN female offspring had greater WBC concentration as compared to Control female and RPN male offspring ($P = 0.004$). A tendency for greater WBC concentration was observed in susceptible heifers and tolerant steers ($P = 0.091$)

Mean corpuscular hemoglobin, mean corpuscular volume, neutrophils and white blood cells

There was a genetic treatment × nutritional treatment interaction for mean corpuscular hemoglobin and mean corpuscular volume ($P = 0.013$ and $P = 0.031$, respectively) with greater concentrations for SC offspring and lower concentration for SN offspring (Figure 4.3). There was a genetic treatment × nutritional treatment interaction ($P = 0.043$) for neutrophil with greater concentration for TN offspring. Finally, there was a genetic treatment × nutritional treatment significant interaction ($P = 0.003$) for white blood cells with greater concentrations for SC and TN offspring (Figure 4.3).

Endophyte-infected tall fescue seeds effect on hematocrit, hemoglobin, red blood cells, and reticulocytes.

There was a time effect ($P < 0.01$) for hematocrit, hemoglobin, and red blood cells, with lower concentrations of these parameters at the end of the experiment (Figure 4.5). In contrast, reticulocytes percentage increased ($P = 0.002$) during the 30-day experimental period (Figure 4.5). These time effects could be attributed exclusively to the administration of endophyte-infected tall fescue seeds on the experimental diet.

Sex Effects on Red Blood Cell Distribution Width and Rumen-Protected Niacin Effect on Mean Corpuscular Hemoglobin and Mean Corpuscular Volume Discussion

Heifer offspring had greater red blood cells distribution width as compared to steers ($P < 0.01$). Furthermore, rumen-protected niacin supplementation decreased offspring mean corpuscular hemoglobin and mean corpuscular volume ($P = 0.02$ and $P = 0.032$, respectively) (Figure 4.6)

Animal performance

Figure 4.7 shows that administration of endophyte-infected tall fescue seeds increased rectal temperature in all the animals under study, especially between Day 7 and Day 14 on treatment ($P < 0.01$). Furthermore, respiratory rate also increased for all the animals under study ($P < 0.01$), especially between Day 21 and Day 30 under study (Figure 4.7). There was a genetic treatment × sex interaction for rectal temperature, with greater values for heifer offspring born to susceptible dams as compared to other treatments ($P = 0.04$). A significant nutritional treatment × sex interaction ($P = 0.02$) showed greater rectal temperatures for SC animals as compared to other treatments (Figure 4.8). Body weight only had a tendency for a significant difference between heifers and steers offspring ($P = 0.06$), steers being heavier than heifers offspring (Figure 4.8). Furthermore, these heavier males presented a tendency for greater respiratory rates ($P = 0.08$). There were not significant differences ($P > 0.05$) for average daily gain. Finally, hair shedding score decreased with time ($P < 0.01$) and, in general, it was lower ($P < 0.01$) for heifer offspring (Figure 4.9).

Discussion

Complete blood count

Red blood cells indices and niacin

The average amount of hemoglobin per red blood cell is represented as mean corpuscular hemoglobin (MCH). In contrast, mean corpuscular volume (MCV) is a measurement of the average size of the red blood cells. A lower level of MCH and MCV was expected in animals exposed to endophyte-infected tall fescue seeds. This is mainly due to copper concentration, needed to produce hemoglobin, which is usually present in reduced concentration in animals grazing endophyte-infected tall fescue (Stoszek et al., 1979; Coffey et al., 1992; Saker et al., 1998). Furthermore, decrease in copper levels reduces winter coats shedding (Saker et al., 2001) or causes anemia in more severe cases (Myint et al., 2018). During our study, in general, MCH and MCV were maintained below their lower threshold level (i.e., 14 pg for MCH and 40 fL for MCV, respectively), according to reference values for the bovine specie (Joerling and Doll, 2019). These results could lead us to think that offspring were slightly anemic due to toxic tall fescue seed supplementation, especially those susceptible receiving rumen-protected niacin (Figure 4.3 and Figure 4.6). Nicotinic acid is also known for its capability of forming chemical complexes with transition metals, such as iron, zinc, or manganese. The presence of nitrogen and oxygen atoms in niacin structure provides the necessary chelating site for metal ions attachment (Al-Saif and Refat, 2012). The chelating property of niacin has also a biological importance on blood parameters. Agte et al. (1997) reported an increase of blood hemoglobin levels due to the addition of dietary niacin on rats. In addition, the iron content in liver was greater for the group supplemented with niacin, which shows an improved iron bioavailability to the animal (Agte et al., 1997). Although, our results contradict these statements. Values of MCH typically mirror

mean corpuscular volume (MCV) results, small red blood cells have a lower MCH, and vice versa. In a previous study, it was observed that niacin supplementation increase hemoglobin levels in growing turkeys (Adebowale et al., 2018). Our results show that in a fescue toxicosis situation, in which animals are exposed to high concentrations of ergovaline, niacin supplementation did not exert these mentioned beneficial effects on hemoglobin concentration. Therefore, the administration of rumen-protected niacin did not have a significant effect on MCH or MCV in the animals under study. Finally, the percentage of packed red cells in blood was within the normal range for the specie (24–46%) (Roland et al., 2014; Joerling and Doll, 2019). Hematocrit percentage is an indicator of oxygen carrying capacity of the circulatory system of the animal (Craig, 1988) and dehydration (Costill and Saltin, 1974). Therefore, these results indicate that our offspring were not dehydrated even though the experiment was performed during the hot summer months.

White Blood Cells (WBC), Neutrophil:Lymphocyte Ratio, Neutrophils and Basophils

Beef steers grazing endophyte-infected tall fescue usually show reduced immunological response compared to steers grazing low-endophyte fescue (Saker et al., 1998). A previous study showed that fescue toxicosis lowers white blood cell count (Kishore, 2010). Our results had a similar response, showing that white blood cell number decreased in TC heifers and SN steers. The opposite response was observed for SC heifers and TN steers with greater WBC concentrations, showing signs of a negative reaction to ergovaline that leads to an activation of the innate immune system (Aristizábal and González, 2013). Nevertheless, further investigation will benefit the understanding of this response in animals under fescue toxicosis. The neutrophil:lymphocyte ratio is the number of neutrophils divided by the number of lymphocytes used as an indication of infection or inflammation (Forget et al., 2017). Under physiological

stress, the number of neutrophils increases, while the number of lymphocytes decreases (Davis et al., 2008). The lower neutrophil:lymphocyte ratio in TN offspring might be related to the increment in neutrophil's concentration (Figure 4.4). These results contradict previous research that demonstrates that nicotinic acid accelerates apoptosis in cultured neutrophils in a concentration-dependent manner (Kostylina et al., 2008). Therefore, more research needs to be done in order to understand the effects of niacin in neutrophil mobilization in vivo. Basophils are responsible for inflammatory reactions during immune response (Huntley, 1992). In our study, basophils concentration was above the upper threshold of 0.1 × 103/uL for CTRL offspring at Day 30 (Figure 4.4). In cattle, basophils release histamine as a consequence of a bacterial or viral infection (Motomura et al., 2014). Ergot alkaloids, including ergovaline, bind to amines (e.g., dopamine, histamine, and serotonin) and stop them from functioning, and as a result, can cause persistent vasoconstriction (Oliver, 2005). Therefore, we believe that the mentioned mechanism was taking place in CTRL offspring after 30 days of administration of endophyte-infected tall fescue seeds.

Reticulocytes and Red Blood Cells Distribution Width

The toxic effect of ergovaline present in endophyte-infected tall fescue seeds could be exerted on the hematopoietic system, resulting in alterations of red blood cell function. Glucose-6-phosphate dehydrogenase deficiency is typical under these situations and, the gene controlling this enzyme is located on the X-chromosome; thus, the defect is sex linked (Piomelli, 1981). A high reticulocyte count and red blood cell distribution width (RDW) in CTRL heifers exposed to endophyte-infected tall fescue seeds could mean that they are producing red blood cells larger than normal. An elevated RDW is a sign of iron deficiency anemia (d'Onofrio et al., 1995),

however, further research needs to be consider to explore the possibility of this condition under fescue toxicosis.

Animal performance

Body weight and average daily gain

It is widely known that the consumption of endophyte-infected tall fescue leads to substantially lower individual average daily gain when compared to animals consuming non-infected pastures (Stuedemann and Hoveland, 1988; Nihsen et al., 2004; Melchior et al., 2019; Mote et al., 2019). Interestingly, a meta-analysis conducted by Liebe & White (2018) reported a negative relationship between ergovaline concentration and average daily gain (Liebe and White, 2018). Furthermore, the consumption of diets based on endophyte-infected tall fescue usually leads to BW losses in gestating animals and their offspring during growth (Schmidt and Osborn, 1993; Greene et al., 2020). In utero exposure to ergot alkaloids causes a tendency to reduce Angus × Simmental offspring's adjusted 205 d BW (Shoup et al., 2016). Similarly, another study indicates that lambs born to ewes receiving toxic fescue during mid and late gestation had altered growth rate (Greene et al., 2020). Although, it was observed a decrease in individual feed intake, BW and average daily gain did not change due to any of the treatments applied. Nevertheless, it was recently reported that the commercially available genetic test used in this study may be considered as predictor of cow performance (Galliou et al., 2020). The offspring steers and heifers used in our study followed their previous maternal nutritional treatment; therefore, offspring presented in utero ergovaline and RPN exposure during mid to late gestation. There is no evidence reporting the effect of endophyte infected tall fescue and RPN on growing beef cattle BW and average daily gain. However, the addition of niacin to beef species is not well defined yet. According to a review publication, niacin supplementation levels up to 1 g/hd/day

lead to an average daily gain of 72 gBW/hd/day to 82 gBW/hd/day. Any variation of average daily gain may be related to energy and protein levels in the diet (Flachowsky, 1993).

Rectal temperature and respiration rate

Consumption of endophyte-infected tall fescue during summer months leads to hyperthermia as a fescue toxicosis symptom. The increase in respiration rate and rectal temperature are evidence of the occurrence of hyperthermia (Thompson and Stuedemann, 1993). The physiological effect of ergot alkaloids is to bind amine receptors in the peripheral blood vessels, leading to vasoconstriction and potential incapability to dissipate body heat (Rhodes et al., 1991; Aiken et al., 2007; Aiken et al., 2009). The respiration rate increment is a physical response to reduce the rising body temperature as a consequence of fescue toxicosis (Al-Haidary et al., 2001). Several studies have reported the effect of fescue toxicosis on respiration rate and rectal temperature (Browning and Leite-Browning, 1997; Browning, 2004; Mote et al., 2019). Rectal temperature may vary depending on the animal's sex. In a previous study, confined females have higher rectal temperature as compared to males (Williams et al., 2019). In our study, all the animals had greater rectal temperature as compared to a reference range for calves between 38.6–39.4 ◦C (Figure 4.7). Increments in body temperature due to fescue toxicosis were more pronounced in SC heifers. The difference in body temperature between heifers and steers could be attributed to different hormonal levels and temperament. To the best of our knowledge, no evidence of RPN supplementation in growing beef cattle consuming endophyte-infected tall fescue is currently available in the literature; however, RPN has been investigated in dairy cattle with conflicting performance results. Zimbelman et al. 2010 reported a significant decrease in rectal temperature in heat-stressed Holstein cows, whereas Di Constanzo et al. (1997) reported no difference for the same animal category under a similar environmental condition (Di

Costanzo et al., 1997; Zimbelman et al., 2010). The evidence of endophyte-infected tall fescue intake during summer months supports our results, in which we observed a greater rectal temperature and respiration rate throughout the study. Therefore, these parameters may indicate that animals consuming endophyte-infected tall fescue had a limited capacity to dissipate excessive body temperature and maintain homeostasis. In addition, TN offspring had the lowest rectal temperature as compared with the other treatments, suggesting that RPN may exert its vasodilator effect producing a greater blood flow, which might help to regulate body temperature.

Hair shedding score

Rough hair coat of animals exposed to endophyte-infected tall fescue is a result of winter hair coat retention or excessive hair coat growth during long-duration days in the summer (Aiken et al., 2011). The role of ergot alkaloids in prolactin metabolism could be a possible mechanism of disruption on the follicle cycle in cattle (Poole and Poole, 2019). Furthermore, ergot alkaloids are also used as a pharmacological inhibitor of pituitary hormones, such as prolactin (Tudzynski et al., 2001). It has been shown that prolactin plays a key role in hair growth in cattle (Littlejohn et al., 2014) and mice (Craven et al., 2006), usually delaying or inhibiting hair regrowth. Furthermore, prolactin levels remain low on animals consuming endophyte-infected tall fescue during increasing day length; therefore, they retain winter hair coat (Campbell et al., 2014). Although, studies performed in beef cattle consuming endophyte-infected tall fescue showed rougher hair coats as compared to animals grazing pasture without fungus (Hoveland et al., 1983; Coffey et al., 2001; Saker et al., 2001). Our study was performed in summer months; therefore, there was a natural winter hair loss during the trial for all treatments.

Gilbert et al. (1991) investigated the effect of sire genetics, sex, diet fed, and breed on hair coat characteristics of growing Angus bulls and heifers after traditional weaning. Interestingly, even though authors found that the density (number of hairs per cm^2) was greater for females than males; weight, length, and diameter was different in sires of different breeds (Gilbert and Bailey, 1991). We speculate that the visual estimation of hair coat at Day 1 resulted in higher values for males than females due to the greater weight, length, and diameter of hairs, not only by the appearance of presenting more hair coat, but also because they might slow down the process of shedding the winter coat.

Summary and conclusions

In our study, the genetic test utilized on dams that provide a tolerance index to fescue toxicosis, did not provide any clear result that could help to justify its implementation by beef producers to differentiate between tolerant or susceptible animals. Hematological parameters were characterized by differences between heifers, steers, and the administration of rumen-protected niacin in the supplemental diet. Results showed that susceptible control offspring presented signs of anemia denoted by low mean corpuscular hemoglobin and mean corpuscular volume after 30 days of exposure to endophyte-infected tall fescue seeds. High levels of white blood cells and basophils in combination with a low neutrophil to lymphocytes ratio were the signs of infection or inflammation detected in the complete blood count analysis, especially in tolerant niacin steers. Furthermore, offspring heifers control had a greater percentage of reticulocytes and red blood cells distribution width, denoting signs of anemia due to exposure to endophyte-infected tall fescue seeds. Offspring from dams exposed to endophyte-infected tall fescue seeds presented the typical symptoms of high rectal temperature and respiration rate and rough hair coats. Particularly, rectal temperature was greater for susceptible control heifers. Body weight, average daily gain, and health related parameters were not improved by rumen-protected niacin supplementation or the genetic test to detect fescue toxicosis resistance.

Tables and figures

Table 4.1. Chemical composition of diet fed to steers and heifers. Bermudagrass hay was fed *ad libitum*, whereas 1.46 kg of fescue seeds, 1.46 kg of pellets. and 0.07 kg of molasses were offered individually in daily basis. All ingredients are expressed in a DM basis.

Ingredients [1]	% DM	CP	NDF	ADF	TDN	Crude Fat
Fescue Seeds [2]	90.55	16.12	48.49	16.03	64.11	-
Pellets [2,3]	90.59	29.63	12.29	4.30	74.45	1.84
Molasses [2]	84.00	5.80	-	0.40	72.00	-
Bermudagrass hay	84.90	14.36	31.65	63.87	64.55	-

DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDN = total digestible nutrients

[1] All ingredients are expressed on a DM basis

[2] Fescue seeds, pellets, and molasses were fed as supplement on a 48.5:48.5:3 ratios.

[3] Pellets were composed by 46.5% ground corn, 46.5% soybean meal, 5% wheat middlings, and 2% soybean oil.

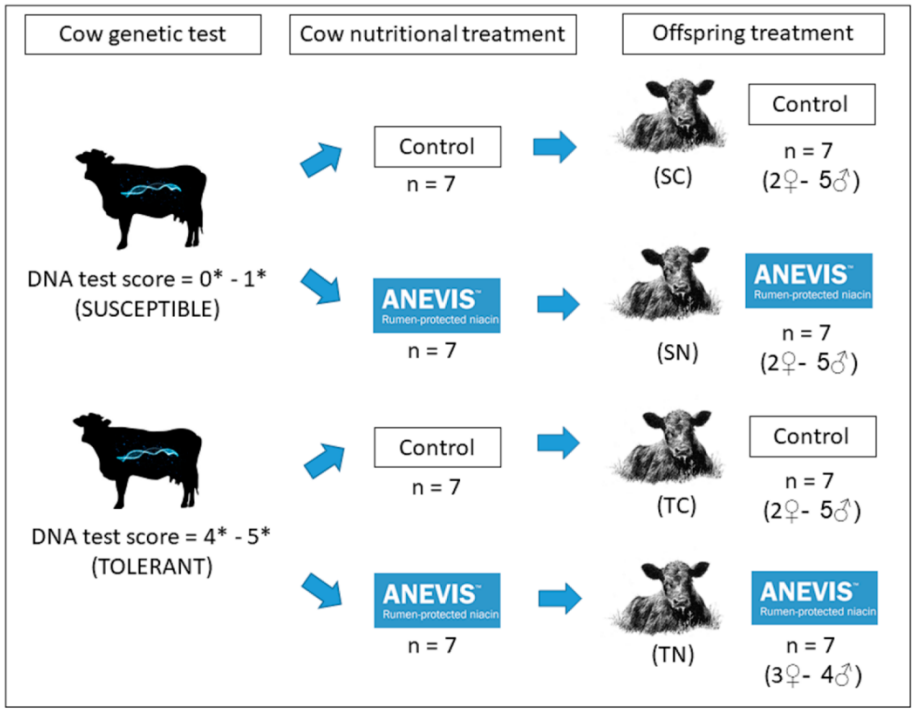


Figure 4.1. Experimental design of the study. Abbreviations: SC: susceptible control; SN: susceptible niacin; TC: tolerant control; TN: tolerant niacin.

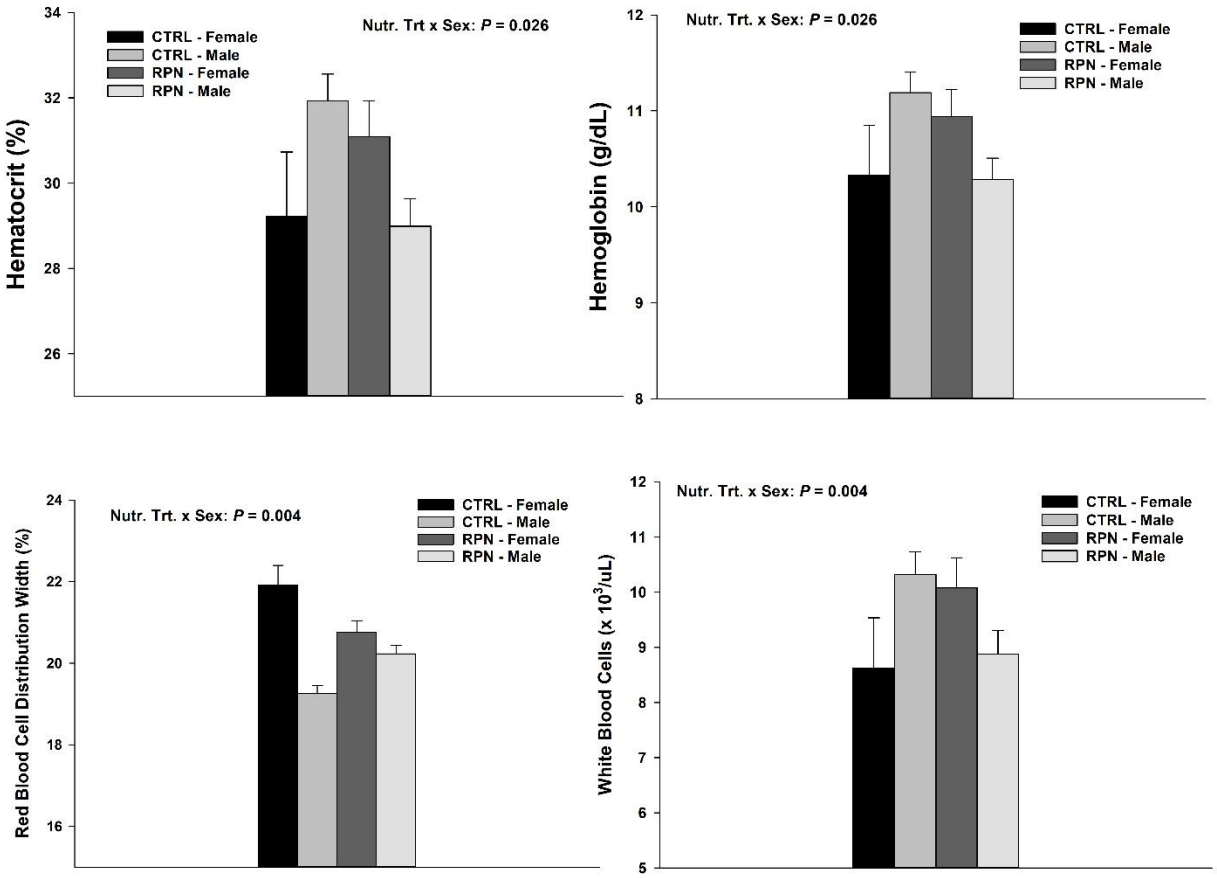


Figure 4.2. Significant nutritional treatment × sex interactions for hematocrit, hemoglobin, red blood cell distribution width and white blood cells of Angus × Simmental steers and heifers' offspring. Hematocrit is represented by the percentage of packed red cells in the blood, hemoglobin (g/dL), red blood cell distribution width (RDW, %), and white blood cells ($\times 10^3$/uL) results of Angus × Simmental steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN), or without rumen-protected niacin (CTRL), and endophyte-infected tall fescue seeds. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

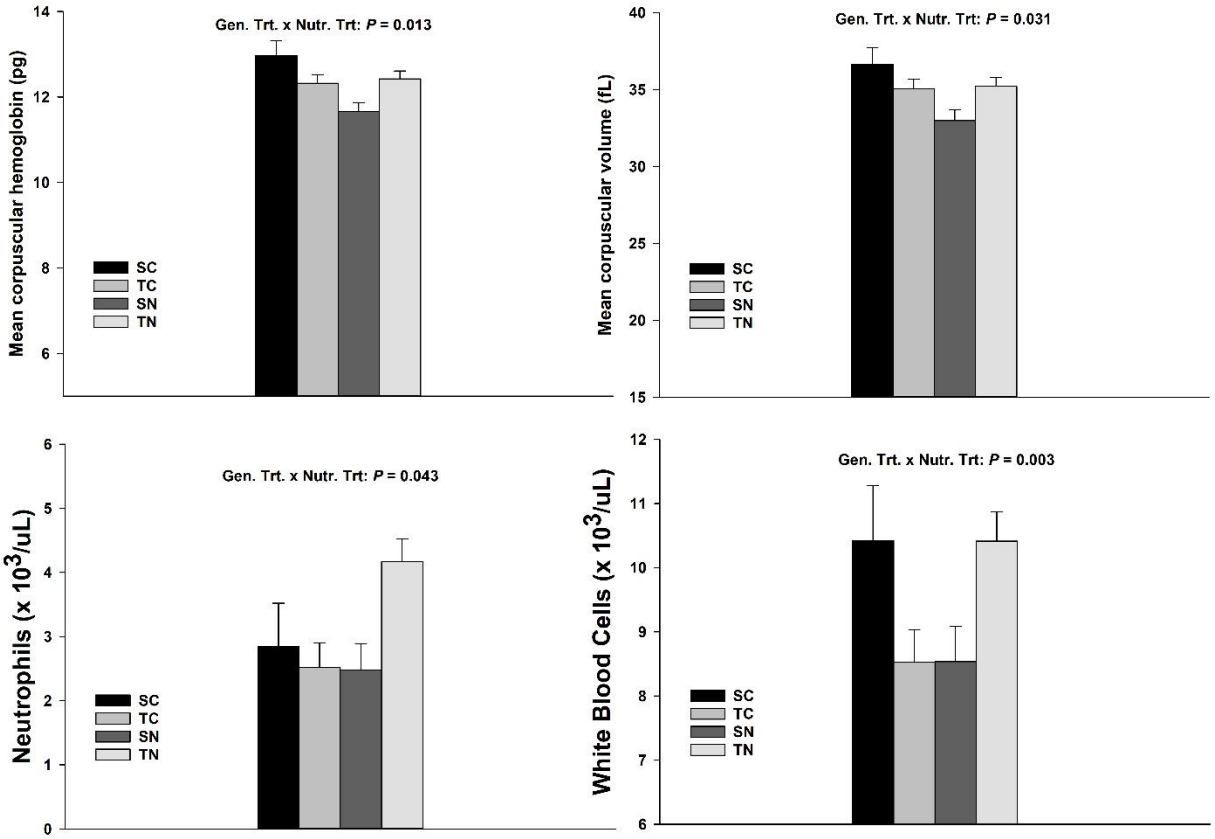


Figure 4.3. Significant genetic treatment and nutritional treatment interactions for mean corpuscular hemoglobin, mean corpuscular volume, neutrophils, and white blood cells of Angus × Simmental steers and heifers' offspring. Mean corpuscular hemoglobin (MCH, pg), mean corpuscular volume (fL), neutrophils (×103/uL), and white blood cells ($\times 10^3$/uL) concentrations for Angus × Simmental offspring steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN) or without rumen-protected niacin (CTRL), while receiving endophyte-infected tall fescue seeds. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

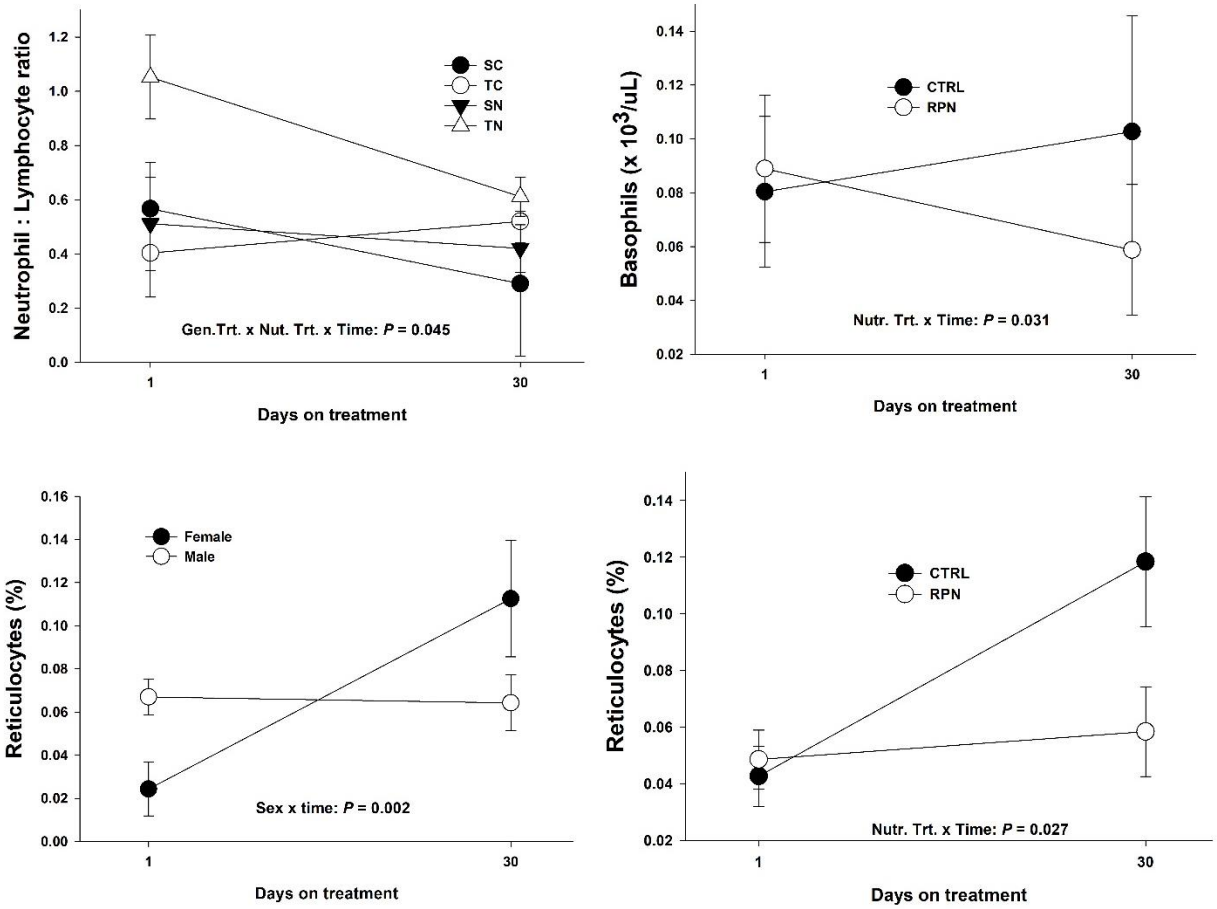


Figure 4.4. Significant interactions between genetic treatment, nutritional treatment and time for reticulocytes, basophils, and neutrophils to lymphocyte ratio for Angus × Simmental steers and heifers' offspring. Neutrophil to lymphocyte ratio, basophils ($\times 10^3$/uL) and percentage of reticulocytes results of Angus × Simmental steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN) or without rumen-protected niacin (CTRL), while receiving endophyte-infected tall fescue seeds. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

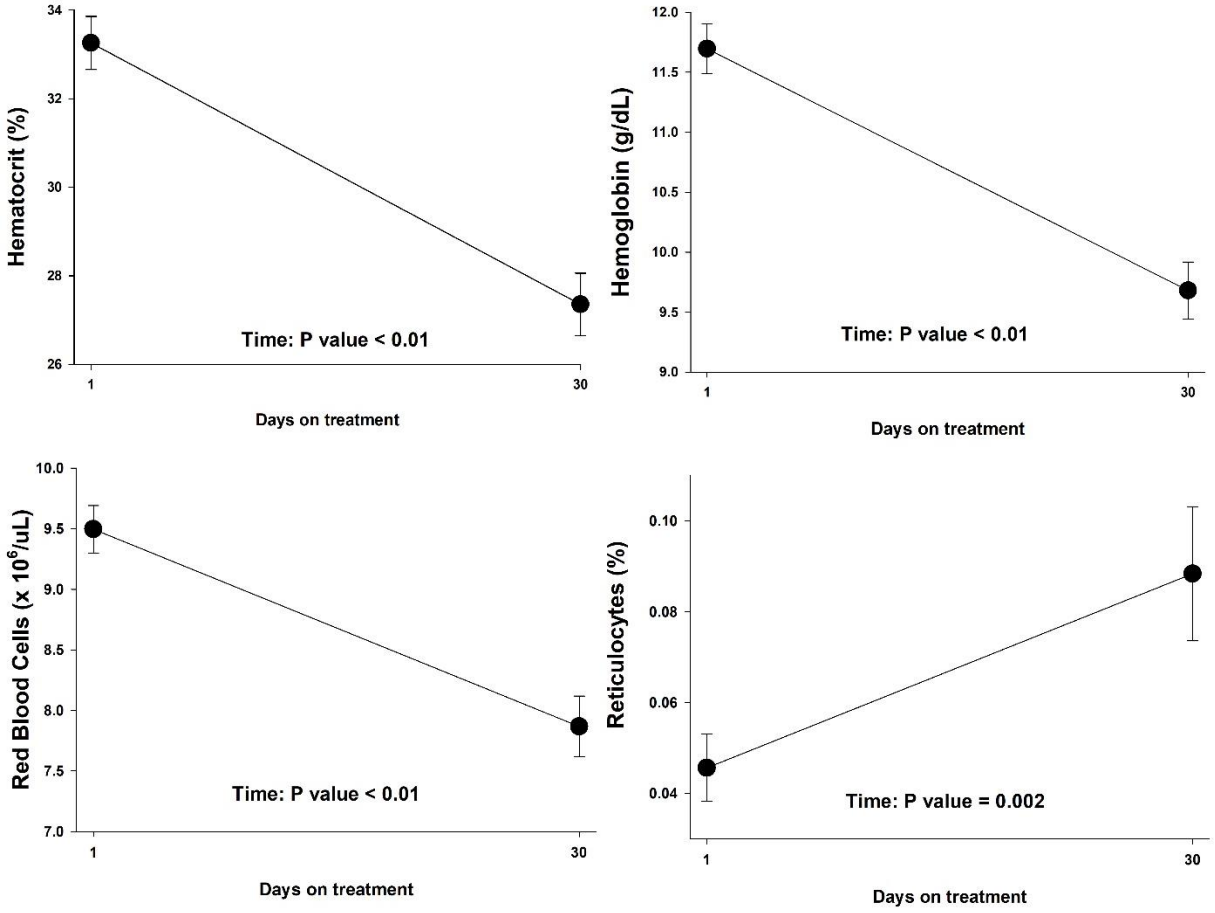


Figure 4.5. Time effect for hematocrit, hemoglobin, red blood cells and, reticulocytes during the 30-day treatment period for Angus × Simmental steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN) or without rumen-protected niacin (CTRL) and endophyte-infected tall fescue. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

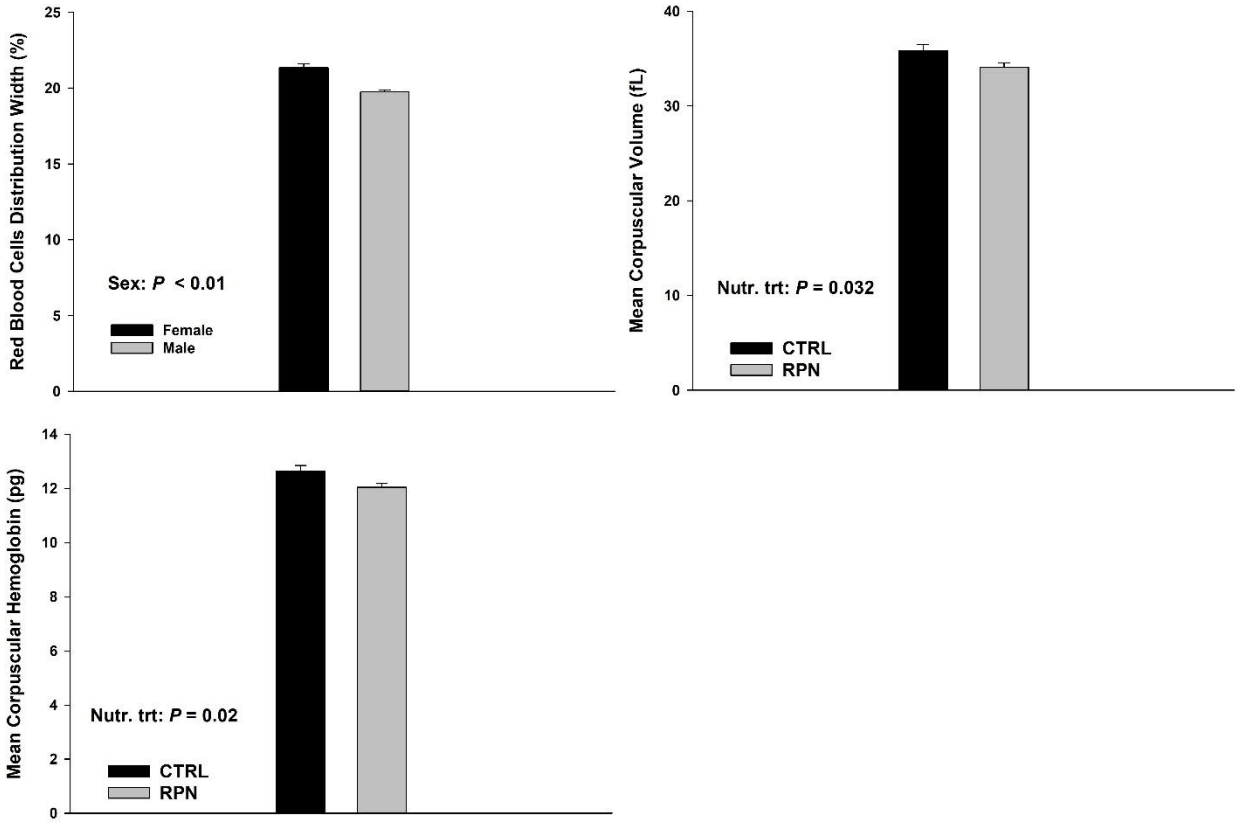


Figure 4.6. Sex effect for red blood cells distribution width and nutritional treatment effect for mean corpuscular hemoglobin and mean corpuscular volume during the 30-day treatment period for Angus × Simmental steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN) or without rumen-protected niacin (CTRL) and endophyte-infected tall fescue. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

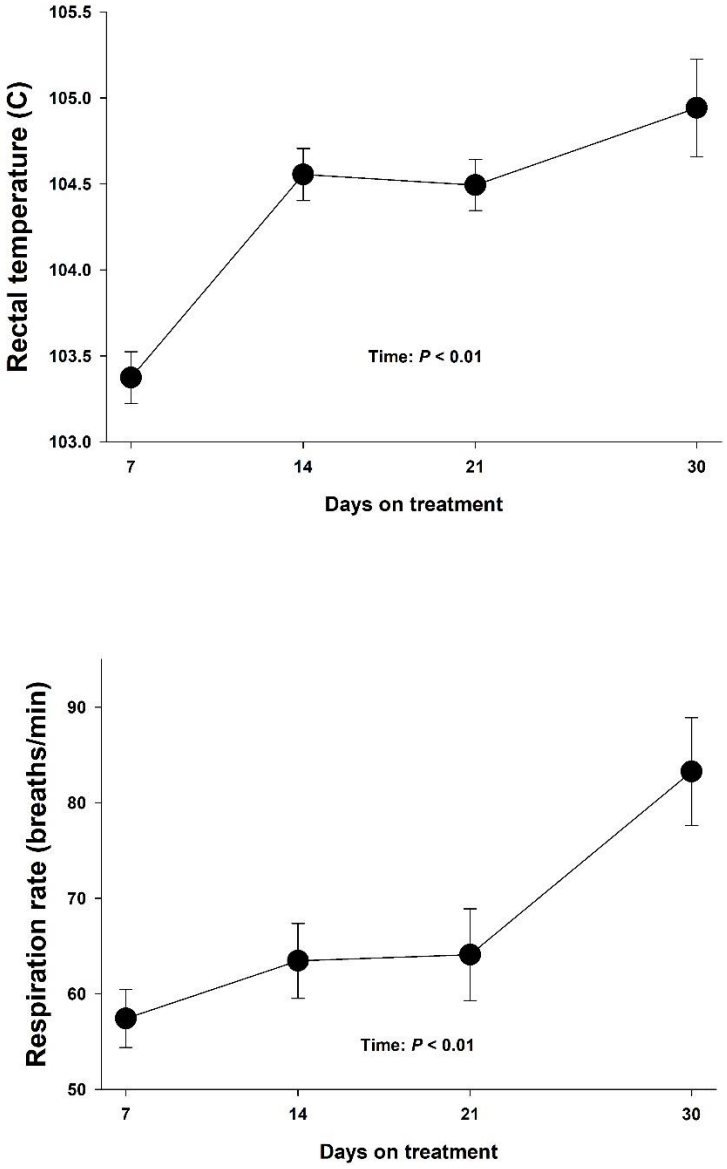


Figure 4.7. Time effect for rectal temperature and respiration rate during the 30-day treatment period for Angus × Simmental steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN) or without rumen-protected niacin (CTRL) and endophyte-infected tall fescue. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

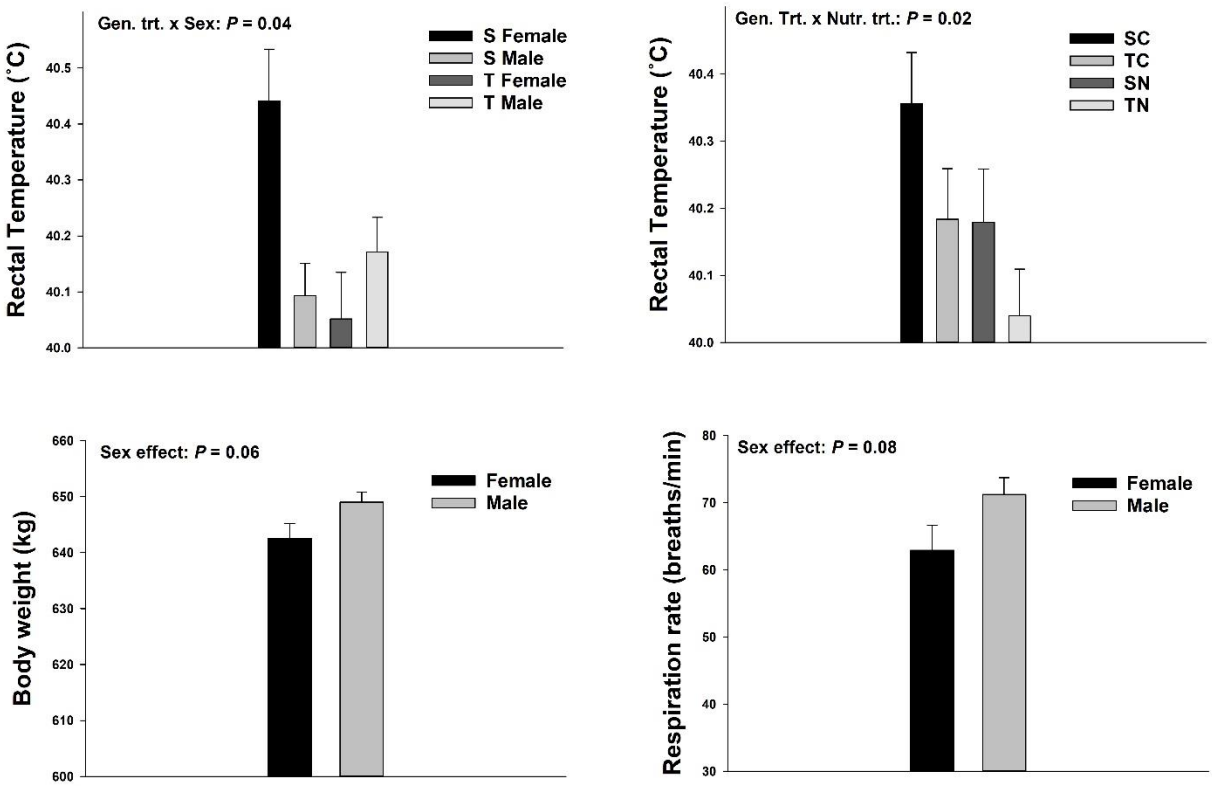


Figure 4.8. Significant interactions for rectal temperature and sex effect for body weight and respiration rate during the 30-day treatment period for Angus × Simmental steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN) or without rumen-protected niacin (CTRL) and endophyte-infected tall fescue. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1.

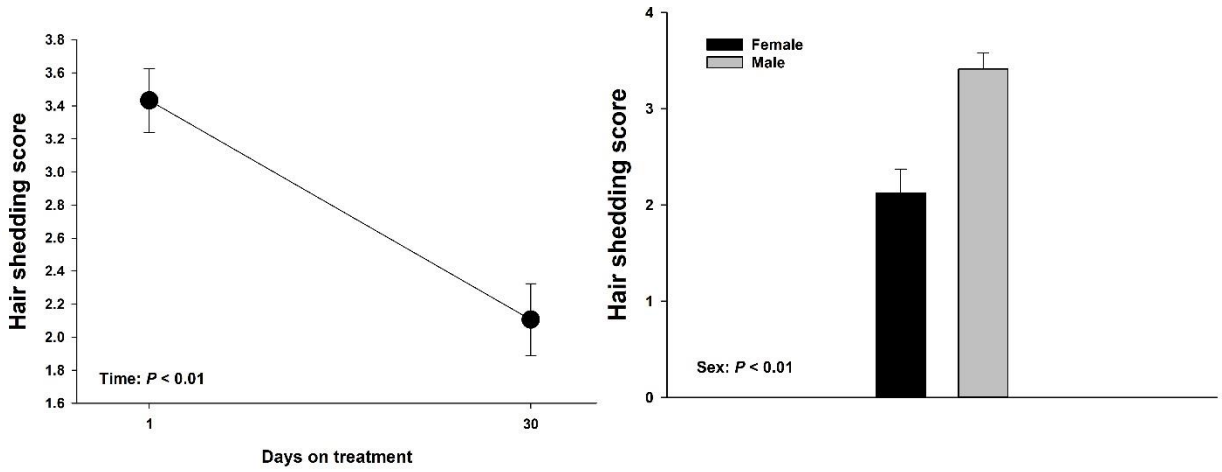


Figure 4.9. Time effect and sex effect for hair shedding score for Angus × Simmental steers (male) and heifers (female) exposed to diets containing rumen-protected niacin (RPN) or without rumen-protected niacin (CTRL) and endophyte-infected tall fescue during the 30-day treatment period. Statistically significant differences were declared at $p \leq 0.05$ and tendencies at $p > 0.05$ and < 0.1. Error bars represent SEM.

Chapter 5
Comparative analysis of the liver transcriptome of beef cattle under fescue toxicosis using RNA-seq

Introduction

Tall fescue (*Schedonorus arundinaceus* (Schreb.) Dumort.) is the predominant cool-season forage in the southeastern region of the United States due to its excellent productive characteristics. However, the superlative aptitudes of tall fescue rely on the symbiotic relationship with an endophyte called *Epichloë coenophiala* (Chai et al., 2020). Ergot alkaloids are produced as secondary metabolites by the fungus. Furthermore, the fungus strategically relocates in the plant based on its reproduction capacity. Since the unique reproduction method of the fungus is asexual, the concentration of the endophyte differs at different locations of the plant. Thus, leaves present low ergot alkaloids concentration, whereas the crown usually has a medium concentration, and the seed head is the portion of the plant in which the highest level of ergot alkaloids are present (Rottinghaus et al., 1991). The asexual transmission of the endophyte through seeds is an essential strategy for ensuring the survivability of the fungus. Interestingly, endophyte-infected (E+) varieties could present ergot alkaloids for a period of up to 1 year. Consequently, to avoid high levels of endophyte contamination, producers may utilize older seeds for planting; however, the vigor, germination, and survivability could be substantially lower when stored at ambient temperature and humidity (Welty et al., 1987).

Tall fescue plants might have come to the United States as a contaminant with other grasses from Europe. The most popular variety of tall fescue is known as “KY31” (K31) because it was found in a pasture located Menifee County, in eastern Kentucky (Stuedemann and Hoveland, 1988). Low cost and optimal forage characteristics of K31 are the reasons behind the preference of producers in selecting this variety. However, in order to avoid fescue toxicosis

occurrence, a variety lacking toxic endophyte was developed, commonly called “endophyte-free tall fescue”. Contrary to K31, the absence of the symbiotic relationship between the endophyte-free (E-) varieties and the fungus results in lower forage characteristics and production. A comparative study reported that beef cows grazing E+ tall fescue had accelerated body weight loss, reduced milk production, and calved offspring with lower BW compared with cows grazing E- tall fescue (Peters et al., 1992). Similarly, beef steers grazing E+ have greater rectal and skin temperature, lower average daily gain, and lower prolactin levels compared to animals grazing E- tall fescue (Johnson et al., 2012). In addition, the cost of E- tall fescue is also higher compared with E+, causing a difficulty in the adoption by producers, especially if the infestation rate is not high (Zhuang et al., 2005).

Previous reports indicate that consumption of ergot alkaloids causes changes in the transcriptome of the liver, the main detoxifying organ in mammals (Bhusari et al., 2006; Liao et al., 2015). Thus, the main objective of our study was to analyze changes in the liver transcriptome in beef cattle consuming E+ vs. E- tall fescue seeds.

Materials and methods

Animals and experimental design

All the procedures for this study were conducted following a protocol approved by the Institutional Animal Care and Use Committee of Auburn University (IACUC Protocol #2019-3484).

A group of 9 Angus × Simmental, weaned steers (n = 6) and heifers (n = 3) with average body weight (BW; 331 ± 36 kg) and age of 7-9 months old were allocated in two groups based on dietary treatment: 1) Endophyte-infected tall fescue (E+; n = 6), and 2) Endophyte-free tall fescue (E-; n = 3). There was a steer:heifer ratio of 2:1 in all treatments (e.g., E+ = 4 steers and 2 heifers; E- = 2 steers and 1 heifer). Calves were born to genetically tested dams for fescue toxicosis tolerance. Animals in the E+ were born to tolerant and susceptible dams and received E+ tall fescue seeds based on results obtained by a genetic test (T-Snip™ Ag. Botanica, Columbia, MO). In contrast, those in E- were born to medium-tolerant dams and received E- tall fescue seeds. All dams grazed tall fescue pastures during early to mid-gestation at the Black Belt Research Center (32º28'16.32"N 87º13'54.12"W, Marion Junction, Alabama) belonging to Auburn University. Susceptible and tolerant dams experienced a 30 days trial in which they received endophyte-infected tall fescue seeds during mid-gestation, and they returned to a tall fescue and dallisgrass consociation. During mid to late gestation, and lactation period all dams were in tall fescue/dallisgrass consociation pastures.

At weaning, steers and heifers were relocated at the Beef Evaluation Center, Auburn University, Auburn, AL due to the accessibility to Calan gates system (American Calan Inc., Northwood, NH). After a training period of approximately ten days, steers and heifers were successfully adapted to Calan gates, utilized for ensuring the correct amount of seeds per head

per day. During the study, the average temperature was 26.1 ºC; whereas the average relative humidity was 77.3% (Auburn University Mesonet, Auburn, AL). The temperature-heat index (THI) was 70.57, and it was calculated as followed: "THI = ambient temperature + (0.55 - 0.55 × relative humidity / 100) × (ambient temperature - 58)", in which ambient temperature was expressed in Fahrenheit (Schlatter, 1987). The diet offered was *ad libitum* bermudagrass (*Cynodon dactylon*) hay combined with a nutritional supplement composed of 1.61 kg of E+ or E- tall fescue seeds, and 1.61 kg of pellets composed of 46.5% ground corn, 46.5% soybean meal, 5% wheat middlings, and 2% soybean oil; and 0.1 kg of molasses per animal per day (Table 5.1). The diet was formulated to meet animal nutrient requirements (NRC, 2016). In the E+ group, tall fescue seeds were offered based on ergovaline concentration. Ergot alkaloids concentration was measured at the Veterinary Medical Diagnostic Laboratory at the University of Missouri (Columbia, MO). There were two lots of tall fescue seeds used in this study, with an ergovaline concentration of 7300 ppb and 2700 ppb, respectively. A total of 20 µg/kg BW/day was the daily dietary individual dose of ergovaline to E+ steers and heifers for ensuring signs of fescue toxicosis. This pharmacological ergovaline concentration follows the recommendation from previous studies (Spiers et al., 2004; Holtcamp et al., 2019).

RNA extraction and library construction

The total RNA of cattle liver samples was extracted using the ZYMO Quick DNA/RNA Miniprep Plus Kit (Zymo Research, CA). For homogenization, the tissue samples with DNA/RNA Shield were mechanically homogenized by Qiagen TissueRuptor II (Qiagen, MD). The RNA concentrations were measured by Qubit fluorometer 3.0 (Thermo Fisher Scientific, MA) with Qubit RNA BR Assay Kit. RNA sequencing libraries were constructed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs, MA)

and NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, MA), with a 1500 ng total RNA input. The concentrations and the size distribution of the libraries were checked by LabChip GX Touch HT machine using the HT DNA NGS 3K Assay (Perkin Elmer, MA). The fragment size of final libraries ranged from 343 to 409 bp. The libraries were individually barcoded and pooled. The libraries were sequenced on an Illumina NovaSeq 6000 sequencing machine to generate 150-bp paired-end reads.

Quality control and preprocess of the RNA-seq data

A total number of 794,813,112 paired-end reads were generated for the nine transcriptomes.

The reads quality was checked by FastQC v11.5 (Andrews, 2010). Illumina adapter sequences and low-quality bases were removed using Trimmomatic v0.36 (Bolger et al., 2014). And the second round of QC was also conducted by FastQC (Andrews, 2010). After trimming, the high-quality reads were mapped to the cattle reference genome by Tophat-2.1.1 and Bowtie2 (Trapnell et al., 2009; Langmead and Salzberg, 2012). There was a total of 3,500,984,881 reads mapped to the reference genome, and the average mapping percentage is 87.45%.

Counts generation and normalization

Gene reads counts were performed in three software packages, Cufflinks-2.2.1, Bedtools -2.30.0, and HT-seq (Trapnell et al., 2009; Quinlan and Hall, 2010; Anders et al., 2015). The counts agreed well for most gene models. When they disagreed, a manual check for each gene model was performed in IGV (Integrative Genomics Viewer) to determine the correct counts (Thorvaldsdóttir et al., 2013). EdgeR and DESeq2 packages in R were used to calculate the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) value and detect significant differentially expressed genes (DEGs) (Robinson et al., 2010; Melchior et al., 2019),

at a FDR (False Discovery Rate) cutoff of 0.05. The log fold change (LogFC) was in a range between -11.5 and 2.9. The total amount of DEG detected when applying cutoffs values comparing liver samples from E+ to E- animals were 326 genes upregulated and 503 downregulated. When comparing animals based on genetic treatment, non-DEG were detected. In addition, a volcano plot was designed to show the DEG in animals receiving E+ using VolcaNoseR tool (Goedhart and Luijsterburg, 2020; Figure 5.2).

KEGG and PANEV analyses

The cutoff criteria for selecting KEGG pathways for discussions was to select those with an Impact value > 890, which accounts for the top 50% impact values observed. The categories 'Cellular processes', 'Environmental information processing', and 'Organismal system' were included for discussion based on the cutoff value criteria. PANEV (Pathway Network Visualizer) v.1.0 is an R (RStudio, Boston, MA) package which utilizes KEGG database in order to retrieve information about each KEGG pathway. This method helps to visualize the interconnection among key genes and KEGG pathways that were significatively impacted by the treatment applied. PANEV analysis was performed as described by Palombo et al. (2020).

Results

We utilized The Dynamic Impact Approach (DIA) analysis for estimating the impact and flux of all the manually-curated pathways associated with the KEGG database (Bionaz et al., 2012). We defined the term 'impact' as the change in the expression of the genes belonging to the pathway due to the administration of E+ tall fescue seeds; and 'flux' as the report of the average direction in the expression as downregulation, upregulation, or no change. The entire dataset, including Entrez gene IDs, FDR, Fold Change (FC), and *p*-values of each seed type group (E+ and E- seeds) were uploaded into DIA, and the overall cut-off was applied on FDR and *p*-value ≤ 0.05 as threshold (Table 5.2). The most representative KEGG categories ('Metabolism', 'Genetic information processing', 'Environmental information processing', 'Cellular processes', and 'Organismal system') were impacted by the consumption of E+ tall fescue showing, in general, an inhibition (or down-regulation).

More specifically, the KEGG subcategories affected in "Cellular processes" KEGG category were "Cell Growth and Death", "Cellular community – eukaryotes", and "Transport and catabolism". First, "Cell Growth and Death" KEGG subcategory presented the downregulation of 'Cellular senescence' pathway. Second, "Cellular community – eukaryotes" KEGG subcategory showed two downregulated pathways: 'Focal adhesion', and 'Signaling pathways regulating pluripotency of stem cells'. Finally, "Transport and Catabolism" KEGG subcategory presented the downregulation of 'Autophagy – animal'.

In addition, "Environmental information processing" KEGG category had two KEGG subcategories affected: "Signal transduction" and "Signaling molecules and interaction". On the one side, "Signaling transduction" KEGG subcategory presented the downregulation of the following pathways: 'Phospholipase D signaling pathway', 'Apelin signaling pathway', 'ErbB

signaling pathway', 'VEGF signaling pathway', 'cAMP signaling pathway', 'AMPK signaling pathway', 'Hippo signaling pathway', 'FoxO signaling pathway', and 'PI3K-Akt signaling pathway'. On the other side, 'ECM-receptor' pathway, which belongs to "Signaling molecules and interaction" KEGG subcategory, was downregulated.

The KEGG subcategories affected belonging to "Organismal System" KEGG category were "Aging", "Circulatory System", "Digestive system", "Endocrine system", "Immune system", and "Nervous system". First, the pathways downregulated in "Aging" KEGG subcategory were 'Longevity regulating pathway', and 'Longevity regulating pathway - multiple species'. Second, "Circulatory system" KEGG subcategory showed the downregulation of 'Adrenergic signaling in cardiomyocytes'. Third, "Digestive system" KEGG subcategory presented the downregulation of 'Protein digestion and absorption'. Forth, 'Regulation of lipolysis in adipocytes', 'Relaxin signaling pathway', 'Insulin signaling pathway', 'Estrogen signaling pathway', 'Thyroid hormone signaling pathway', 'Growth hormone synthesis, secretion and action', and 'Parathyroid hormone synthesis, secretion and action' were the downregulated KEGG pathways belonging to "Endocrine system" KEGG subcategory. Fifth, "Immune system" KEGG subcategory showed the downregulation of the following KEGG pathways: 'B cell receptor signaling pathway', 'Fc epsilon RI signaling pathway', 'Fc gamma R-mediated phagocytosis', 'Chemokine signaling pathway', 'Toll-like receptor signaling pathway', 'Platelet activation', and 'RIG-I-like receptor signaling pathway'. Finally, 'Cholinergic synapse', 'Dopaminergic synapse', 'Neurotrophin signaling pathway' were the most impacted and down-regulated KEGG pathways in "Nervous system" KEGG subcategory.

Finally, RNA-seq validation performed using RT-qPCR showed consistent results with the overall downregulation caused by E+ intake in KEGG pathways (data not shown).

Discussion

KEGG pathways

Cellular processes

The liver is a unique organ responsible for numerous metabolic, vascular, detoxifying, secretory, and excretory functions. Its uniqueness relies on the capability of being regenerated through hepatocytes proliferation after the exposure to injury related to a toxin (Michalopoulos, 2007). During the E+ tall fescue, the liver of different mammalian species experiences a reduction in weight per unit of kg as a result of the numerous detoxification processes, as shown in rats (Chestnut et al., 1992; Settivari et al., 2006) or beef cattle (Brown et al., 2009). More specifically, the underlying mechanisms causing liver size reduction could be linked to numerous detoxification processes. Among them, AKT Serine/Threonine Kinase 2 (*AKT2*), a regulator of several endocrine pathways, is a key factor involved in KEGG pathways affected by ergot alkaloids consumption, such as 'Cellular senescence', 'Focal adhesion', 'Signaling pathways regulating pluripotency of stem cells', and 'Autophagy' (Table 5.3; Figure 5.3). Senescence is a mechanism characterized by permanent cell cycle arrest, usually occurring due to a normal aging process or exposure to stressors agents (Pazolli and Stewart, 2008; Huda et al., 2019). A previous study indicates that the inhibition of *TGFβ*, one of the most important regulators of cellular senescence, enhance liver regeneration after a mild to severe injury by reducing hepatic senescence (Bird et al., 2018). In addition, liver cells are linked extracellularly thanks to focal adhesions, composed by a large number of integrin transmembrane and cytoplasmic proteins that are connected to the extracellular matrix and the actin cytoskeleton (Wolfenson et al., 2009). The activation of both *TGFβ* and *AKT2* stimulates focal adhesion and cellular proliferation (Wang and Basson, 2011; Chen et al., 2020). Therefore, cell-extracellular structure and connection may

be impaired by the downregulation of *AKT2*. This result is consistent with the downregulation of 'ECM-receptor interaction' KEGG pathway in our study. 'ECM-receptor interaction' KEGG pathway indicates the activity of the extracellular matrix and its receptors formed mainly by epithelial cells (i.e., hepatocytes) and stromal cells (Kim et al., 2011). The most important function of ECM is providing both physical support and receptor signaling. Integrin, one of the ECM proteins, is normally able to activate cellular growth, in a synergistic manner with other membrane receptors signaling pathways. For example, integrin interacts with insulin receptors, VEGF receptors, TGF-β receptors, among others (Goel and Mercurio, 2012; Williams et al., 2015; Dewidar et al., 2019). Remarkably, our results indicate that the consumption of E+ tall fescue led to a downregulation of 'Insulin signaling pathway', 'VEGF signaling pathway', and 'Growth hormone synthesis, secretion, and action'. Therefore, we suggest that there might be a reduction in liver size that could be affected by the disruption in growth-related signaling pathways.

Autophagy is a normal and necessary process occurring in the liver that enhances the intake of lipids, carbohydrates, and amino acids to hepatic cells, maintaining cellular homeostasis (Chun and Kim, 2018). In eukaryotes, three primary autophagy forms occur: microautophagy, chaperone-mediated autophagy, and macroautophagy. Microautophagy consists in the degradation of small portion of cytoplasm near lysosome and endosome that are engulfed and degraded. In chaperone-mediated autophagy, transport proteins enhance the translocation of targeted compounds through the lysosome membrane into the lysosome lumen. Finally, macroautophagy comprises the formation of autophagosome, an organelle capable of engulfing a portion of cytoplasm or different organelles for subsequent fusion with lysosome for degradation (Majeski and Fred Dice, 2004; Mizushima et al., 2008). Macroautophagy (herein mentioned as

autophagy, as referred in KEGG) is a catabolic process that is also associated with cellular senescence. More specifically, injured hepatic cells due to toxicosis incur in a detoxification process, including autophagy. Damaged proteins, if not removed through autophagy, lead to hepatotoxicity (Schneider and Cuervo, 2014). Likewise, autophagy may play a homeostatic role by suppressing cellular senescence under stressful conditions. One of the major examples is the occurrence of autophagic senescence due to high oxidative stress impairing homeostatic regulation of senescence (Tai et al., 2016). Inhibition of autophagy could lead to cell death (Czaja et al., 2013). Currently, contradicting results are available in the literature regarding ergot alkaloids metabolism and liver detoxification process. A previous study pointed out that ergot alkaloid consumption upregulates genes related to apoptosis in heat-stressed rats (Settivari et al., 2009). However, autophagy plays an important role in lipid metabolism since it is required for lipid droplets breakdown in hepatic cells. Consistent with our results, a previous experiment revealed that the knockdown of Autophagy gene related-5 (*Atg5),* one of the genes involved in 'Autophagy' KEGG pathway, inhibited autophagy in rat hepatocytes. In addition, authors reported an increase triglyceride and lipid droplets content in both *in vitro* and *in vivo* (Singh et al., 2009). Finally, autophagy is highly regulated by insulin and glucagon (Mizushima and Klionsky, 2007; Ezaki et al., 2011). A study conducted using steers as the animal model indicates that consumption of 2 - 3.06 mg of ergot alkaloids in a daily basis for a period of 14 days increased insulin levels, suggesting a possible insulin resistance (Eisemann et al., 2014). Therefore, since animals in our study were receiving E+ tall fescue for 30 days, impairments in insulin concentration could have occurred, leading to the downregulation of the related pathways 'Autophagy', 'Regulation of lipolysis in adipocyte' and 'Insulin signaling pathway' in the 'Organismal System' KEGG category.

Environmental information processing

Phospholipase D (*PLD*) is an enzyme involved in the secretion of phosphatidic acid, a lipidic second messenger, by catalyzing the hydrolysis of the phosphodiester bond of glycerophospholipids. Furthermore, PLD acts on cellular processes such as cell proliferation and cell survival (McDermott et al., 2004). Phospholipase D products, such as phosphatidic acid, regulate autophagy in liver cells. For example, mice lacking phospholipase 1 (*Pld1*) present lipid homeostasis imbalance due to autophagy impairment, which leads to lipid accumulation in the liver (Hur et al., 2016). Similarly, mice lacking *Pld1* and *Pld2* presented higher circulating lipid levels and insulin resistance (Viera et al., 2016). Furthermore, it is widely known that thyroid hormones bind nuclear receptors and regulate gene expression (Yen, 2001; Shibusawa et al., 2003; Cheng et al., 2010). However, a previous study found that thyroxine (T_4) activates phospholipase D in a non-genomic manner in liver cells (Kavok et al., 2001). Interestingly, our results show that the consumption of E+ tall fescue led to a downregulation of 'Autophagy', 'Thyroid hormone signaling pathway', and 'Regulation of lipolysis in adipocytes' (Table 5.4; Figure 5.4). Therefore, these mentioned pathways may be causing lipid accumulation as a result of ergovaline detoxification (Browning et al., 2000; Ferguson, 2020).

Vascular endothelial growth factor (VEGF) is universally known as one of the most critical contributors to cell proliferation and angiogenesis (Mustonen and Alitalo, 1995; Ferrara and Davis-Smyth, 1997). Our results indicate that hepatic cells experienced a reduction in cell proliferation occurrence due to the lower expression of 'VEGF signaling pathway' in the E+ group compared to the E- group. Additionally, both 'Apelin signaling pathway', which is associated to cell proliferation, and 'PI3K-Akt signaling pathway', a major regulator of cell cycle and apoptosis, were also downregulated. The lower expression of these pathways was congruent

with the downregulation of ‘VEGF signaling pathway’. The liver is a highly vascularized organ, and the vasoconstrictive effect of fescue toxicosis might also play a role in angiogenesis in hepatic cells. In mice peritoneal tissue, dopamine inhibits angiogenesis by inducing endocytosis of VEGF receptor 2, which is critical for VEGF binding (Basu et al., 2001). The downregulation of ‘VEGF signaling pathway’ could also be explained by the fact that ergot alkaloids present a similar chemical structure to amines and are able to mimic them (Klotz, 2015). Consequently, ergot alkaloids bind dopamine receptors causing numerous metabolic imbalances, including antiangiogenic effects (Šoškić et al., 1986; Larson et al., 1995; Delgado-Rosas et al., 2011). Relaxin is part of the insulin superfamily of peptide hormones, and it was originally known to be present in pregnant individuals due to its secretion by the corpus luteum. However, current research identified other biological functions in both males and females. Relaxin proteins differ among species, being relaxin-3 and insulin-like peptide 3 conserved in mammal species (Bathgate et al., 2013). One of the physiological roles of relaxin is the stimulation of vasodilation, as shown in humans (Dschietzig et al., 2009), and rats (Debrah et al., 2005). Therefore, the downregulation of ‘Relaxin signaling pathway’ could be related to the vasoconstrictive effect of ergot alkaloids. More specifically, ergot alkaloids are adrenergic agonist (Rhodes et al., 1991; Aiken et al., 2007; Aiken et al., 2009), in which α-adrenergic is a potent vasoconstrictor agent (Oliver, 2005). However, more evidence is needed to clarify any antagonist relation between the vasodilatory effect of relaxin and the vasoconstrictive effect of ergot alkaloids in cattle consuming E+ diets.

The downregulation of *AKT2* is also associated with the downregulation of HRas Proto-Oncogene, GTPase (*HRAS*), a gene that codifies for “rat sarcoma virus” (RAS) proteins. The RAS family protein is involved in basic cellular functions such as cell proliferation and apoptosis

(Wittinghofer and Pai, 1991). Our PANEV visualization results for 'Environmental Information Processing' shows that the consumption of E+ tall fescue impacted 'PI3K-Akt signaling pathway', 'FoxO signaling pathway', 'VEGF signaling pathway', 'Apelin signaling pathway', and 'Phospholipase D signaling pathway' all connected with *HRAS*, supporting the hypothesis of the occurrence of cell proliferation inhibition.

Organismal systems

The production and release of insulin, the main anabolic hormone that promotes energy storage, occurs by the action of β pancreatic cells. It is tightly related to animals' food intake and energy balance, controlling glucose homeostasis (Loh et al., 2017). Furthermore, the consumption of E+ tall fescue leads to a reduction in dry-matter intake (DMI) (Hemken et al., 1981). Accordingly, in our study, animals consuming E- tall fescue presented greater DMI compared with animals receiving endophyte-infected tall fescue. Therefore, we believe that the downregulation of 'Insulin signaling pathway' could be partly explained by reducing DMI on steers and heifers consuming E+ diets (Table 5.5; Figure 5.5).

The inhibition of nutrient-signaling pathways (i.e., 'Insulin signaling pathway') could affect a variety of other pathways. For example, mammals experiencing a decrease in insulin levels are usually associated with a shorter cell half-life (Rincon et al., 2004). Impaired sensitivity to insulin and reduced in glucose disposal rate, named insulin resistance, could also be related to the developing of aging process. Furthermore, dietary restriction inactivates insulin/insulin-like growth factor signaling (IIS), AMPK signaling pathway, mTOR pathway, and PI3K-Akt signaling pathway in mammals (Santos et al., 2016). Interestingly, 'Longevity regulating pathway' is negatively affected by the mentioned pathways. Our study indicates that cattle consuming E+ presented a downregulation in 'Longevity regulating pathway', and it could

be caused by the inactivation of 'AMPK', 'PI3K-Akt', and 'Insulin' signaling pathways. Therefore, the reduction in DMI because of ergot alkaloids consumption may reduce the lifespan of hepatic cell.

Lipolysis, the process of triglycerides breakdown, occurs in white adipocytes cells which are in cross-talk with hepatic cells. In addition, lipolysis could be affected by the consumption of E+ tall fescue. The KEGG pathway 'Regulation of lipolysis in adipocytes' was inactivated in animals exposed to ergot alkaloids in our study. In accordance with our results, Oliver (1997) suggested a possible reduction of lipolysis due to an adrenergic receptor activity in the liver (Oliver, 1997). In human adipocytes, α-adrenergic agonists, namely methoxamine, phenyl epinephrine, and clonidine, inhibit lipolysis *in vitro* by interaction with α_2-adrenergic receptors (Wright and Simpson, 1981). It has been shown that ergotamine, an ergot alkaloid also present in E+ fescue (Guerre, 2015), is an α_2-adrenergic agonist in rats (Roquebert and Grenié, 1986). Therefore, we suggest that the animals receiving toxic seed could have lower lipolysis activity in white adipocytes in the liver due to the agonistic action of ergot alkaloids on adrenergic receptors.

Even though estrogen is widely associated with sexual physiology, previous studies have revealed physiological effects on liver health. For example, estrogen receptors α and β can enhance or inhibit gene transcription of the immune system, such as interleukin-6 and -1 (Palmisano et al., 2017). The main sites of estrogen secretion are gonads (i.e., ovaries in females and testis in males). Although male cattle used in the present experiment were castrated previous to the beginning of the study, estrogen could have been synthetized in other tissues, such as the brain, adrenal glands, or adipose tissue (Barakat et al., 2016). In addition, a recent study pointed out that ergot alkaloids in E+ diets could impair follicular development and estrogen secretion

(Poole and Poole, 2019). However, more research is needed to understand the effects of ergot alkaloids in growing, non-cycling heifers and steers on estrogen production.

Thyroxine (T_4) triiodothyronine (T_3) can bind thyroid receptors in all cells, including hepatic cells. Thyroid receptors, in conjunction with retinoic acid, retinoid X, vitamin D, and peroxisome proliferator receptors, belongs to the nuclear superfamily group of receptors (Evans, 1988). The liver and kidney present type 1 deiodinase, which is the enzyme responsible to the conversion of T_4 to T_3, and accounts for up to 40% of extrathyroidal production of T_3 (Sanders et al., 1997). Furthermore, the major inactivator of T_4 and T_3, namely type 3 deiodinase system, is also found in the liver (Bianco et al., 2002). Consequently, thyroid hormones (e.g., T_4 and T_3) are essential regulators of liver metabolism. For example, thyroid hormones regulate lipid homeostasis by increasing the expression of LDL receptors and apolipoprotein A1 (Malik and Hodgson, 2002). Conflicting evidence in literature reports the effects of E+ consumption in thyroid hormone metabolism on the liver. Some studies showed that ergot alkaloids do not affect circulating thyroid hormone levels on Holstein calves (Hurley et al., 1980), ewes (Elsasser and Bolt, 1987), and horses (Breuhaus, 2003). On the other hand, Holstein cows and heifers receiving ergotamine, an ergot alkaloid present in E+ tall fescue (Guerre, 2015), had reduced circulating levels of T_3, glucagon, insulin, and cortisol. During a previous study, cows and heifers were exposed to an ergotamine challenge, in which they received an intravenous dose of ergotamine tartrate of 19-20 µg/kg BW (Browning et al., 2000). Circulating T_3 and T_4 were not measured in the present study. Therefore, a possible explanation for the downregulation of 'Thyroid hormone signaling pathway' in liver tissue could be related to the downregulation of dopamine-related pathways. As previously shown, dopaminergic system regulates the synthesis and release of numerous hormones, including thyroid hormones (Harkitis et al., 2015).

Dopamine is an important neurotransmitter, and its normal activity could be impaired by the presence of ergot alkaloids. A previous study reported that dopamine receptors interact with ergot alkaloids in an antagonistic manner in dopamine receptor 2 (*DRD2*) and agonist fashion in dopamine 3 (*DRD3*) in the caudate nucleus (Šoškić et al., 1986). This finding was also confirmed by Li et al. (2017), who showed a downregulation of *DRD2* in pituitary glands of steers consuming high-endophyte infected tall fescue compared to animals exposed to low-endophyte infected tall fescue (Li et al., 2017). Another effect of the antagonistic mechanism of ergot alkaloids on dopaminergic receptors is observed in the synthesis of parathyroid hormone. Calcium and phosphorus homeostasis regulation takes place by the action of parathyroid hormone, and its synthesis is tightly related to the presence of dopamine and cAMP. For example, a previous study using bovine parathyroid cells showed that dopamine increases parathyroid hormone and cAMP accumulation (Attie et al., 1980). Interestingly, ergot alkaloids can bind dopamine receptors and inhibits cAMP production (Larson et al., 1995), suggesting the antagonistic effect on parathyroid hormone formation. In addition to dopamine, the synthesis of parathyroid hormone occurs by the stimulation of epinephrine and norepinephrine (Blum et al., 1980). Similarly, the inhibitory effects of ergot alkaloids on the synthesis of epinephrine and norepinephrine is widely accepted since it has been reported for more than 90 years (Rothlin, 1929; Panaccione, 2011). Based on this evidence, it is possible to suggest that the 'dopaminergic synapse', and 'parathyroid hormone synthesis, secretion and action' KEGG pathways are both negatively affected by ergot alkaloids compared with animals receiving an E- diet. Presumably, ergot alkaloids played a role in inhibiting the dopamine receptors, which affected parathyroid hormone pathway. The effects of the downregulation of parathyroid hormone pathway in calcium and phosphorus metabolism in growing beef cattle remain to be investigated.

The liver is a complex organ that is innervated with afferent and efferent neurons, which are involved in numerous physiological processes of the liver. More specifically, the autonomic nervous system belongs to the peripheral nervous system, present in visceral organs including the liver (Jensen et al., 2013). The exposure to ergot alkaloids affected the neuronal system not only due to the inhibition of 'dopaminergic synapse', but also 'cholinergic synapse' and 'neurotrophin signaling pathway'. Cholinergic synapse mode of action relies on the conversion of an electric signal into a chemical signal as acetylcholine; whereas neurotrophin signaling consists in a set of growth factors that are involved in the development and maintenance of neurons (Roux and Barker, 2002). Interestingly, cholinergic signaling is also involved in liver metabolism by regulating inflammation processes (Metz and Pavlov, 2018).

B cells, also called B lymphocytes, are part of the adaptive immune system, and their activation is negatively affected by stressful conditions, such as E+ diets consumption (Davis et al., 2008). It has been shown that the consumption of E+ diets decreases white cell counts compared with E- diets in rodents (Dew et al., 1990). The reduction in lymphocyte concentration was also validated in beef cattle consuming E+ by Jackson et al. (2015), who reported a decline of 18% in Angus crossbred steers grazing high endophyte-infected tall fescue compared with low endophyte-tall fescue (Jackson et al., 2015). B cells present receptors in the membrane that allow sensing surrounding environmental signals. Once antigens are recognized by B cells receptors, signaling cascades are activated leading to immune responses (Hasler and Zouali, 2001). Remarkably, toll-like receptors contribute to the activation of B cells (Browne, 2012). In accordance with the previous evidence, our results indicate that the most affected pathway was 'B cell receptor signaling pathway', emphasizing the relevance of ergot alkaloids on the adaptive immune system. Consistently, 'Toll-like receptor signaling pathway' was inhibited by the

consumption of E+ tall fescue. In addition to B cell receptors, fragment crystallizable (Fc) receptors (e.g., Fc epsilon and Fc gamma receptors) also belong to the adaptive immune system. For example, Fc gamma plays a vital role in adaptive immunity since it is associated with the structure of numerous immunoreceptors and signal transduction. The activation of Fc gamma occurs by the action of IgG. Likewise, Fc gamma is involved in the activation signaling of natural killer cells, which are specialized cells from the adaptive immune system (Hamdan et al., 2020). However, in the presence of cytotoxins or pathogens, Fc gamma may exert phagocytosis, through the 'Fc gamma R-mediated phagocytosis' pathway (Kang and Jung, 2019). In our study, this KEGG pathway was downregulated in E+ group. Similarly, Fc epsilon RI signaling pathway represents the action of Fc epsilon receptor and its interaction with IgE. The activation of Fc epsilon RI signaling pathway helps with inflammation processes. It has been previously shown that the β subunit of the Fc epsilon RI could be in close interaction with Fc gamma III receptor in mast cells, natural killer cells and macrophages (Kurosaki et al., 1992). Another receptor pathway downregulated by the intake of E+ seeds is retinoic acid-inducible gene I (RIG-I)-like receptors. The main function of these receptors is to detect and restrict the invasion of both RNA and DNA viruses. Moreover, RIG-I-like factors are involved in promoting B cell differentiation (Loo and Gale, 2011). The downregulation of 'RIG-I-like receptor signaling pathway' could partly explain the inhibition of 'B cell receptor signaling pathway' in animals receiving to E+ tall fescue seeds.

A group of important of chemical signaling during inflammatory processes are called chemokines, which are formed by peptide chains and interact with G couple receptors. During inflammation, pro-inflammatory chemokines are responsible for enhancing the recruitment of leukocytes. In fact, cells with chemokine receptors in their structure (i.e., monocytes and

neutrophils) are attracted and led to the infection site occurring in the bloodstream. In addition, macrophages also are able to release chemokines (Graves and Jiang, 1995). The effects and mechanisms of impact of ergot alkaloids on chemokines are not fully elucidated in literature. Although, Poole et al. (2019) reported greater circulating of CCL2, CCL4, and MIG chemokines concentration on Angus steers receiving seeds with 185 µg ergovaline/kg of BW compared with steers exposed to E- tall fescue seeds (Poole et al., 2019). The difference in the results with our study could rely on the concentration of ergovaline (185 vs 20 µg ergovaline/kg of BW) and the tissue sampled (blood vs. liver).

Platelets, also called thrombocytes, are widely known as the cells involved in clotting. More specifically, platelets play an essential role in liver regeneration by providing a scaffold for the coagulation process (Hugenholtz et al., 2009). Interestingly, an ergot derivative compound called nicergoline, known for its pharmacological function as an α1-adrenoreceptor antagonist, detain and inhibit platelet aggregation (Alvarez-Guerra et al., 1999). Therefore, it is possible to suggest that the similar chemical structures of ergot alkaloids present in E+ tall fescue may play an inhibitory role in 'platelet activation' KEGG pathway.

The overall downregulation on immune system-related KEGG pathways could be partly explained by the inhibitory effect that ergot alkaloids have on cAMP. It has been previously reported that cAMP triggers the induction of antibody production by B cells (Gilbert and Hoffmann, 1985). In addition, immunosuppression exerted by fescue toxicosis could occur in cattle due to a lower concentration of serum prolactin (Borba et al., 2018). A previous study indicated that the typical lower circulating prolactin could play a role in immunomodulation. Consequently, authors suggested that animals exposed to E+ diets could have reduced antibody production compared to animals receiving an E- diet (Dawe et al., 1997). Even though our study

did not report circulating prolactin levels, it is well established in the literature that the consumption of E+ causes a reduction in this hormone.

Summary and conclusions

RNA-sequencing technique has been widely utilized in current omics studies to broadly understand the genetic response to nutritional disturbances. For example, consumption of E+ tall fescue is a very common nutritional disturbance among cattle producers in the southeastern region of the US that leads to significant economic losses. Our study analyzed the effects of E+ tall fescue consumption on hepatic transcriptome of Angus × Simmental steers and heifers, which is a widely used cross-breed in the southeastern region of the US. The most impacted pathways in animals consuming E+ tall fescue seeds experienced an overall downregulation compared with those exposed to E- tall fescue seeds. The downregulation of pathways associated with 'Cellular processes' KEGG category indicate that hepatic cells may experience reduced senescence; however, the size of cells could also be smaller due to the lower catabolic activity, expressed by the downregulation of autophagic pathways.

Furthermore, animals consuming ergot alkaloids had a downregulation of signal transduction pathways involved in 'Environmental information processing'. These pathways are associated with numerous endocrinal-related and immune-related pathways. For example, the downregulation of 'Phospholipase D signaling pathway', which was the most affected pathway in 'Environmental information processing, could have a negative effect on the regulation of lipolysis and autophagy. Similarly, ergot alkaloids bind dopamine receptors inhibiting the production of cAMP. Consequently, there was a downregulation of 'cAMP signaling pathway' in animals exposed to E+ diet compared to those receiving E- tall fescue seeds.

Lastly, ergot alkaloids impaired endocrine pathways involved in the "Organismal Systems" KEGG category. For example, the lower DMI intake in animals exposed to E+ could negatively affect 'Insulin signaling pathway'. Furthermore, the detrimental impact of ergot

alkaloids on cAMP also negatively affects the production of B cell. In our study, the most impacted pathway was ‘B cell receptor signaling pathway’ and, together with other immune system-related pathways, may indicate a possible immunosuppression in animals in E+ group.

In conclusion, the overall downregulation of KEGG pathways on animals exposed to E+ compared with those receiving E- might suggests that ergot alkaloids played a substantial role in disturbing normal liver metabolism. Previous evidence reports the difficulty of finding a unique solution to dampen fescue toxicosis. Thus, our findings contribute to the extensive data already published in the literature and could scaffold novel research. Based on our results, future research should be aimed at improving biological functions represented by the negatively impacted pathways, such as B cells production, insulin secretion, and thyroid hormone production.

Tables and figures

Table 5.1. Chemical composition of diet fed to steers and heifers. Bermudagrass hay was fed *ad libitum*, whereas 1.46 kg of tall fescue seeds, 1.46 kg of pellets. and 0.07 kg of molasses were offered individually in daily basis.

Ingredients [1]	% DM	CP	NDF	ADF	TDN	Crude Fat
Fescue Seeds [2]	90.55	16.12	48.49	16.03	64.11	-
Pellets [2,3]	90.59	29.63	12.29	4.30	74.45	1.84
Molasses [2]	84.00	5.80	-	0.40	72.00	-
Bermudagrass hay	84.90	14.36	31.65	63.87	64.55	-

DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDN = total digestible nutrients

[1] All ingredients are expressed on a DM basis

[2] Fescue seeds, pellets, and molasses were fed as supplement on a 48.5:48.5:3 ratios.

[3] Pellets were composed by 46.5% ground corn, 46.5% soybean meal, 5% wheat middlings, and 2% soybean oil.

Table 5.2. Summary of flux and impact uncovered results by the Dynamic Impact Approach (DIA) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways databases analysis of the bovine liver transcriptome of growing steers and heifers exposed to E- and E+ seeds.

KEGG category	KEGG subcategory	Impact	Flux
Global and overview maps	Metabolic pathways		
	Carbon metabolism		
	Biosynthesis of amino acids		
Metabolism			
	Energy Metabolism		
	Lipid Metabolism		
	Nucleotide Metabolism		
	Amino Acid Metabolism		
	Metabolism of Other Amino Acids		
	Glycan Biosynthesis and Metabolism		
Genetic Information Processing			
	Transcription		
	Translation		
	Folding, Sorting and Degradation		
	Replication and Repair		
Environmental Information Processing			
	Signal Transduction		
	Signaling Molecules and Interaction		
Cellular Processes			
	Transport and Catabolism		
	Cell Growth and Death		
	Cellular community - eukaryotes		
	Cell Motility		
Organismal Systems			
	Immune System		
	Endocrine System		
	Circulatory System		
	Digestive System		
	Excretory System		
	Nervous System		
	Sensory System		
	Development		
	Aging		
	Environmental Adaptation		

Flux represents the direction of each category and the corresponding subcategory: green color represents inhibition, whereas red color shows activation. Blue lines show the impact of each category and the corresponding subcategory (P value ≤ 0.05; FDR ≤ 0.05).

Table 5.3. Results of flux and impact uncovered by the Dynamic Impact Approach (DIA) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) 'Cellular processes' pathway database analysis of the bovine liver transcriptome of growing steers and heifers exposed to E- and E+ seeds.

KEGG category	KEGG subcategory	KEGG pathway	Impact	Flux
Cellular Processes	Cell Growth and Death	Cellular senescence		
	Cellular community - eukaryotes	Focal adhesion		
	Cellular community - eukaryotes	Signaling pathways regulating pluripotency of stem cells		
	Transport and Catabolism	Autophagy - animal		

Most downregulated	
Most upregulated	

Flux represents the direction of each subcategory belonging to 'Cellular processes' KEGG category: green color represents inhibition, whereas red color shows activation. Blue lines show the impact of each category and the corresponding subcategory (*P* value $\leq$ 0.05; FDR $\leq$ 0.05).

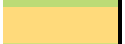
Table 5.4. Results of flux and impact uncovered by the Dynamic Impact Approach (DIA) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) 'Environmental information processing' pathway database analysis of the bovine liver transcriptome of growing steers and heifers exposed to E- and E+ seeds.

KEGG category	KEGG subcategory	KEGG pathway	Impact	Flux
Environmental Information Processing	Signal Transduction	Phospholipase D signaling pathway		
	Signal Transduction	Apelin signaling pathway		
	Signal Transduction	ErbB signaling pathway		
	Signal Transduction	VEGF signaling pathway		
	Signal Transduction	cAMP signaling pathway		
	Signal Transduction	AMPK signaling pathway		
	Signal Transduction	Hippo signaling pathway		
	Signal Transduction	FoxO signaling pathway		
	Signal Transduction	PI3K-Akt signaling pathway		
	Signaling Molecules and Interaction	ECM-receptor interaction		

Most downregulated	
Most upregulated	

Flux represents the direction of each subcategory belonging to 'Environmental information processing' KEGG category: green color represents inhibition, whereas red color shows activation. Blue lines show the impact of each category and the corresponding subcategory (*P* value $\leq$ 0.05; FDR $\leq$ 0.05).

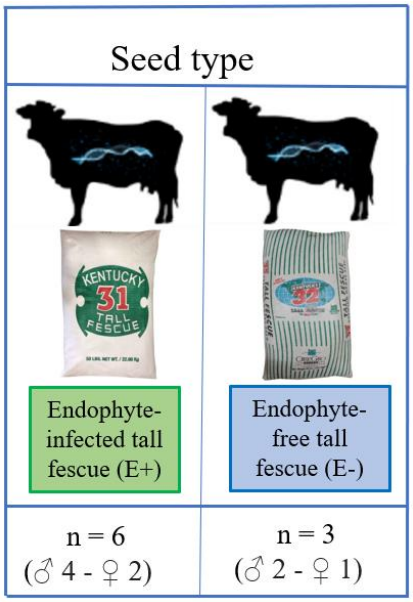
Table 5.5. Results of flux and impact uncovered by the Dynamic Impact Approach (DIA) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) 'Organismal Systems' pathway database analysis of the bovine liver transcriptome of growing steers and heifers exposed to E- and E+ seeds.

KEGG category	KEGG subcategory	KEGG pathway	Impact	Flux
Organismal Systems	Aging	Longevity regulating pathway		
	Aging	Longevity regulating pathway - multiple species		
	Circulatory System	Adrenergic signaling in cardiomyocytes		
	Digestive System	Protein digestion and absorption		
	Endocrine System	Regulation of lipolysis in adipocytes		
	Endocrine System	Relaxin signaling pathway		
	Endocrine System	Insulin signaling pathway		
	Endocrine System	Estrogen signaling pathway		
	Endocrine System	Thyroid hormone signaling pathway		
	Endocrine System	Growth hormone synthesis, secretion and action		
	Endocrine System	Parathyroid hormone synthesis, secretion and action		
	Immune System	B cell receptor signaling pathway		
	Immune System	Fc epsilon RI signaling pathway		
	Immune System	Fc gamma R-mediated phagocytosis		
	Immune System	Chemokine signaling pathway		
	Immune System	Toll-like receptor signaling pathway		
	Immune System	Platelet activation		
	Immune System	RIG-I-like receptor signaling pathway		
	Nervous System	Cholinergic synapse		
	Nervous System	Dopaminergic synapse		
	Nervous System	Neurotrophin signaling pathway		

Most downregulated	
Most upregulated	

Flux represents the direction of each subcategory belonging to 'Organismal systems' KEGG category: green color represents inhibition, whereas red color shows activation. Blue lines show the impact of each category and the corresponding subcategory (*P* value $\leq$ 0.05; FDR $\leq$ 0.05).

A)



B)

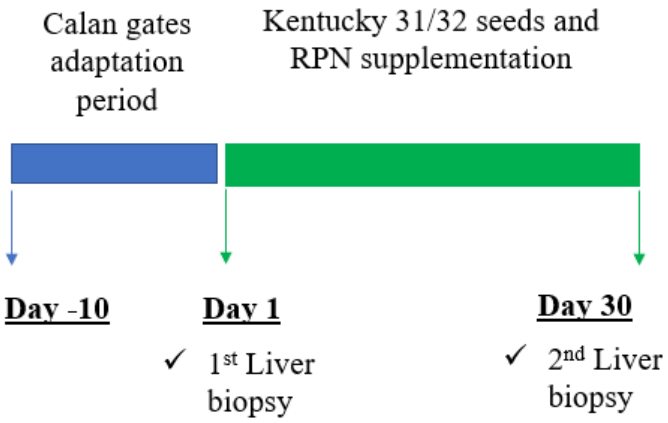


Figure 5.1. A) Experimental design of the study, B) Experiment timeline.

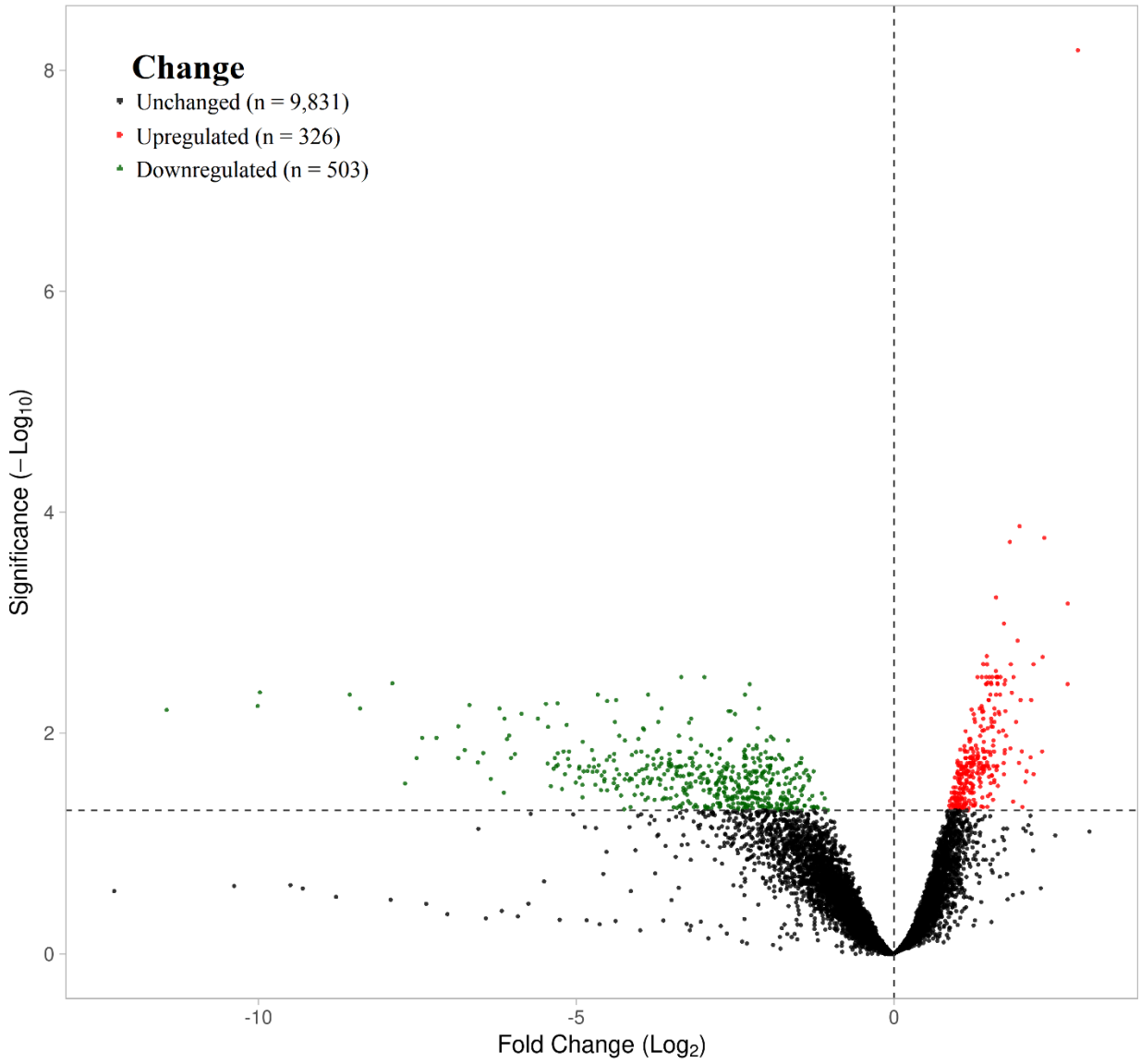
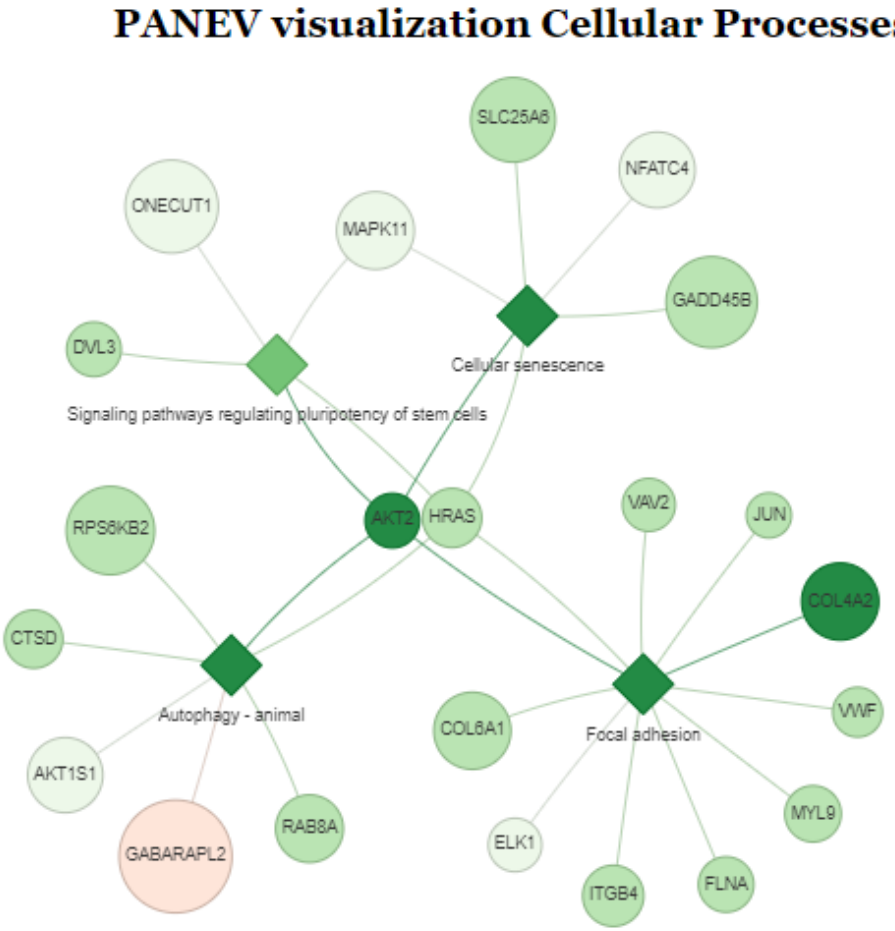


Figure 5.2. Vulcano plot of differentially expressed genes in animals receiving E+ tall fescue diets.



Most downregulated	
Most upregulated	

Figure 5.3. PANEV visualization of 'Cellular Processes' KEGG pathway KEGG category. Circles, rhombuses, and lines in green color represent downregulation of the specific pathway or gene. Size of circles strictly depends on gene transcript name length.

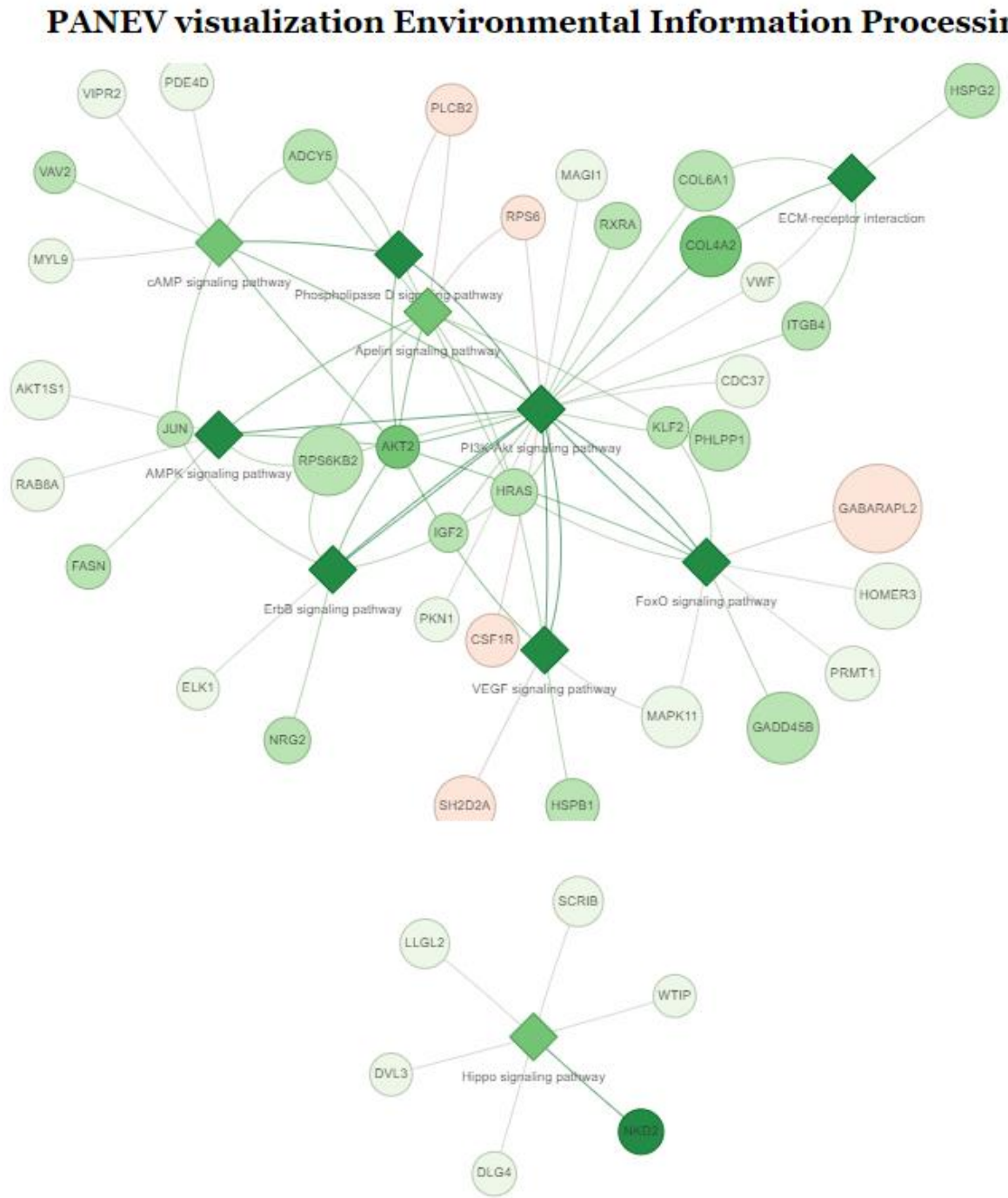
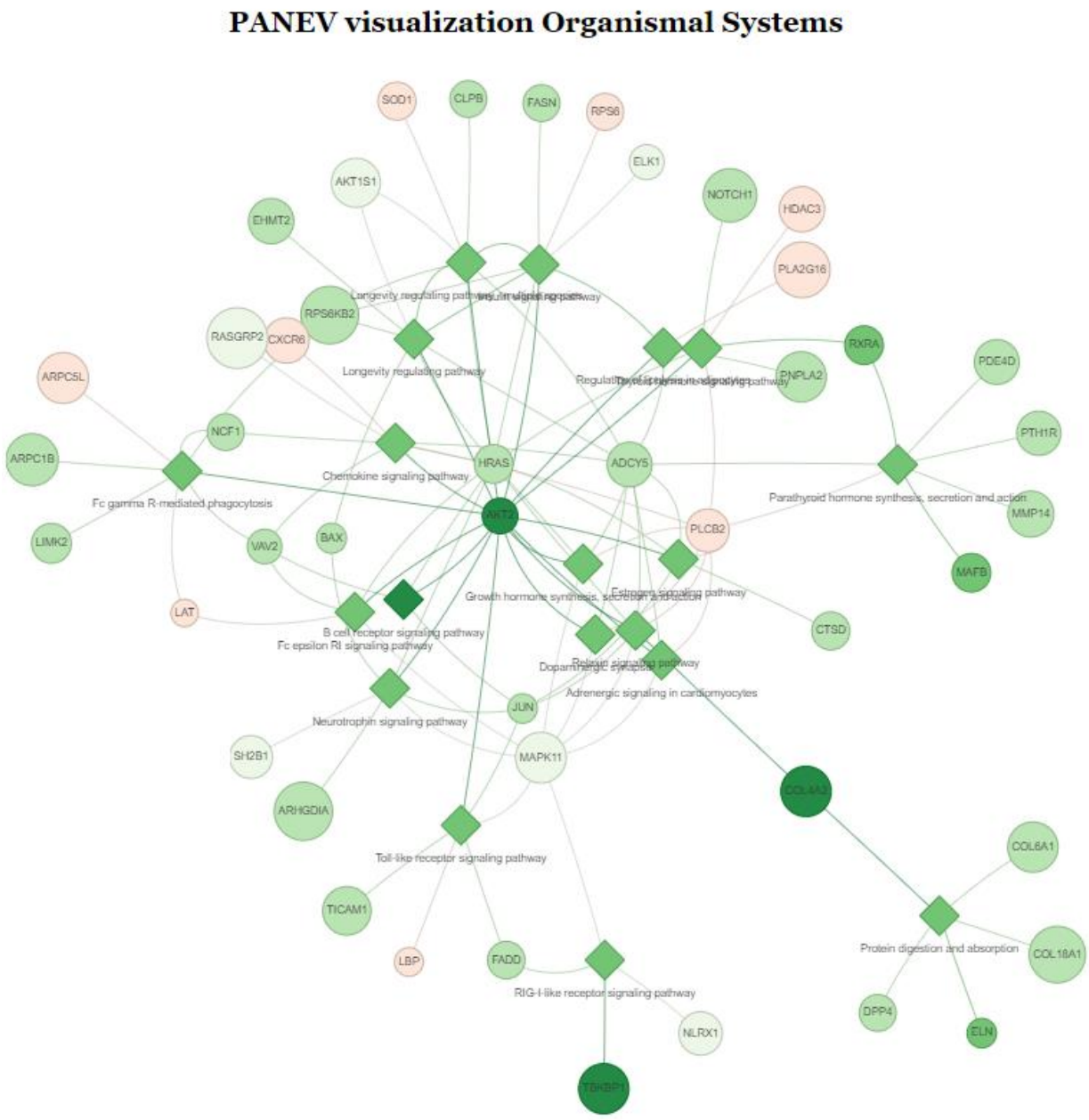


Figure 5.4. PANEV visualization of 'Environmental Information Processing' KEGG category. Circles, rhombuses, and lines in green color represent downregulation of the specific pathway or gene. Size of circles strictly depends on gene transcript name length.



Most downregulated	
Most upregulated	

Figure 5.5. PANEV visualization of 'Organismal systems'. Circles, rhombuses, and lines in green color represent downregulation of the specific pathway or gene. Size of circles strictly depends on gene transcript name length.

Chapter 6
Summary, conclusions, and future directions

Gestation could be identified as the most critical period on beef dams because it directly impacts overall maternal health and performance and postnatal growth and development of calves.

The experiment presented in Chapter 2 showed that maternal supplementation with RPM supplementation on PRIM dams altered the expression of adipose, oxidative stress, and DNA methylation-related genes in skeletal muscle in offspring. These are novel results that could improve carcass quality due to a potential accumulation of intramuscular fat. In addition, a greater hypertrophy may occur in PRIM-RPM due to greater expression of *SOD2* and *NOS3*. Further investigation is needed to fully understand the mechanism behind the different expressions of the named pathways in PRIM vs. MULT calves and their impact on postnatal growth and development.

Chapters 3, 4, and 5 focused on strategies for dampening fescue toxicosis on beef cattle. The occurrence of fescue toxicosis due to E+ tall fescue consumption has become one of the most limiting problems to overcome in cow-calf operation systems in the southeast region of the US over the last century. Based on the numerous strategies investigated for mitigating this deleterious symptomatology to date, there is no unique solution productively and economically viable to overcome fescue toxicosis. Still, a large amount of approaches must be implemented in different areas of production, such as nutrition, reproduction, and animal health.

In Chapter 3, gestating beef dams were genotyped for fescue toxicosis tolerance and were fed E+ tall fescue seeds and RPN for a period of 30 days. The tolerant niacin group experienced an accelerated reduction in BW compared with the rest of the treatments. However, this

reduction in BW was not translated into a decrease in offspring's birth nor weaning weight. All dams experienced a marked reduction in circulating prolactin, proving the occurrence of fescue toxicosis in all treatments. Furthermore, there was a consistent reduction in blood metabolites that serve as an indicator of liver health, which is the major detoxifying organ of mammals' bodies. Further research is needed to understand if genotyping for fescue tolerance and supplementation of RPN during mid-gestation can improve the reproductive performance of beef dams by .

Chapters 4 and 5 were performed using the offspring of dams receiving E+ tall fescue seeds. Therefore, it could be possible to consider both chapters are a continuation of Chapter 3. In Chapter 4, after 30 days of exposure to E+ tall fescue seeds, susceptible control animals showed signs of anemia due to the low mean corpuscular hemoglobin and mean corpuscular volume. Furthermore, tolerant niacin steers presented high levels of white blood cells and basophils and low neutrophils to lymphocytes ratio, suggesting a possible inflammation infection. Offspring born to dams exposed to E+ diets presented high rectal temperature, respiration rates, and retention of the winter coat. Our results indicate that there was no improvement in average daily gain or health-related parameters by the addition of RPN nor the utilization of genetic test for fescue toxicosis tolerance. Future research needs to be done to corroborate the impact of anemia due to exposure of ergovaline either in the womb or postnatally on feedlot performance. In addition, investigation should be aimed at identifying any possible lasting effect of the supplementation of RPN on growing steers and heifers.

Finally in Chapter 5, RNA-sequencing of liver tissue was analyzed from randomly selected steers and heifers born that received E+ tall fescue seeds supplementation for 30 days and were born to dams exposed to E+. The novelty of this study was that the major pathways in

the liver were downregulated due to the toxicosis effect of ergot alkaloids. Namely, pathways associated with 'Cellular processes' KEGG category showed a reduction of senescence. Pathways related to 'Environmental information processing' were also downregulated, suggesting a possible impairment in lipolysis and autophagy processes. In addition, animals exposed to ergot alkaloids had a downregulation in the pathways from the 'Organismal Systems' KEGG category, in which the lower expression of 'B cell receptor signaling pathway' be suggestive for potential immunosuppression. Future investigations could be oriented toward the development of therapeutic techniques that improve B cell metabolism as a possible strategy to dampen the negative effects of tall fescue toxicosis.

Overall, our results provide insightful evidence of the effects of nutritional changes on gene expression. More specifically, the main goal of showing the lasting effect of maternal nutrition on postnatal life was fulfilled, showing numerous expected results. The utilization of novel molecular biology techniques (e.g., epigenetics) could be helpful to confirm the mechanism behind these nutrigenetic and nutrigenomics changes. Consequently, identifying molecular markers for developing new, novel nutritional strategies will help to improve beef cattle production.

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