EFFECTS OF LATE GESTATIONAL COPPER, ZINC, AND MANGANESE SOURCE AND INCLUSION IN BEEF CATTLE

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By
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EFFECTS OF LATE GESTATIONAL COPPER, ZINC, AND MANGANESE SOURCE AND INCLUSION IN BEEF CATTLE

presented by Emma Stephenson,

a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

To my family and friends,

for constantly being my biggest supporters and always believing in me when I fail to believe in myself.

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EFFECTS OF LATE GESTATIONAL COPPER, ZINC, AND MANGANESE SOURCE AND INCLUSION IN BEEF CATTLE

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ABSTRACT

A study was conducted to determine the effects of source and inclusion of Cu, Zn, and Mn during late gestation on cow and calf mineral status, fetal growth, and offspring immune function and pre-weaning performance. Cows received 1 of 4 treatment during late gestation: no additional Cu, Zn, and Mn (CON), inorganic trace minerals to supply 133% of recommendations (ITM), chelated trace minerals to supply 133% of recommendations (CTM), or both inorganic and chelated trace minerals to supply 100% of recommendations (RR). Cows fed CTM had greater post-calving liver Cu and colostrum yield and lactose. Gestational trace mineral treatment did not affect fetal growth; however, the inclusion of chelated Cu, Zn, and Mn in the maternal diet resulted in greater neonatal calf liver Cu. Calves born to cows fed RR tended to have less liver Mn than all other treatments. Calf plasma Zn was maintained from 0 to 48 h of age in ITM and CTM calves but decreased during that period in CON and RR. Calf serum immunoglobulins at 48 h of age were not affected by treatment. Also at 48 h of age, an ex vivo whole blood stimulation assay using Toll-like receptor (TLR) agonists to determine immune responsiveness was utilized for a subset of calves. The inclusion of chelated trace mineral in the diet during late gestation resulted in greater mRNA expression of pro-inflammatory cytokines interleukin 1β and 8, as well as inducible nitric oxide synthase, in calf whole blood when exposed to TLR agonists. In conclusion, gestational trace mineral supply did not alter fetal growth or passive transfer, but these data indicate

that late gestational supply of chelated trace minerals improves cow and calf Cu status, neonatal calf Zn metabolism, and calf innate immune responsiveness to bacterial pathogens.

REVIEW OF LITERATURE

INTRODUCTION

Of beef calves born alive, 5.5% die prior to weaning (APHIS, 2017). A majority of that death loss occurs within the first 3 wk of life (APHIS, 2010) which emphasizes how challenging the neonatal period is for calves. They are expected to stand, find the udder, and successfully suckle shortly after birth. This is a major transitionary time as well because the calf has to switch from a parenteral (placental) to an enteral source (colostrum and milk) of nutrients and breathe and thermoregulate on its own to maintain homeostasis. Proper development in utero is crucial to neonatal survival because it ensures organ systems are best prepared for this difficult time.

Essential trace minerals Co, Cu, Fe, I, Mn, Se, and Zn are components of or cofactors to many enzyme systems therefore they are important for many biological functions (McDowell, 1992). These trace minerals are vital for fetal development because they are important for bone formation, glucose utilization, lipid metabolism, antioxidant capacity, and DNA synthesis and transport (Hostetler et al., 2003). Most of these processes are also important for maternal adaptation to pregnancy. However, beef cows are typically fed forage-based diets that are highly variable in trace minerals and their antagonists, which often leads to deficiency (McDowell, 1992).

The maternal environment is strongly influenced by nutrition and greatly affects fetal and neonatal development which can have long-term effects on the offspring (Fowden et al., 2006; Greenwood and Cafe, 2007; Funston et al., 2010). In ruminants, the dam is completely responsible for the supply of trace minerals to the fetus and neonate (Hidiroglou and Knipfel, 1981). Severe trace mineral deficiencies during gestation can

result in extreme abnormalities such as skeletal and connective tissue malformations or enzootic ataxia (Hidiroglou, 1980; Hostetler et al., 2003) that prevent the calf from standing and finding the udder. In some cases, trace mineral deficiencies can cause fetal death (Hurley, 1981), but the effects of marginal trace mineral deficiencies during gestation on the offspring are complex in ruminants. Thus, the objective of this literature review is to investigate the role of trace minerals in biological processes required for fetal and neonatal development in ruminants in order to better understand the effects of marginal trace mineral deficiencies during gestation on the calf.

TRACE MINERAL EFFECTS ON THE PREGNANT DAM

Maternal Adaptation to Pregnancy

Pregnancy elicits many physiological changes in the dam to provide adequate nutrients and a healthy intrauterine environment for the fetus to appropriately develop, and to prepare the dam for parturition and lactation. There are metabolic, cardiovascular, respiratory, and renal changes to ensure adequate nutrients and oxygen are delivered to the fetus and metabolic waste products can be removed (Thornburg et al., 2006). For example, cardiac output, or the amount of blood volume pumped by the heart each minute, increases throughout gestation, which is mainly driven by an increase in blood volume (Rosenfeld, 1977). The thoracic cavity expands to allow for more lung capacity, and gas exchange in the alveoli increases during gestation (Thornburg et al., 2006). These adaptations accommodate the increase in metabolic demands of the dam and fetus, as well as an increase in uterine blood flow (Rosenfeld, 1977).

Glomerular filtration rate and effective renal plasma flow increase during early

and mid-gestation and are sustained until term or several weeks prior to term, respectively (Thornburg et al., 2006), to sustain greater waste disposal. These dramatic changes disrupt normal metabolism because they require greater energy and nutrient expenditure, trace minerals included. These requirements are either similar or greater after parturition, depending on milk yield, to support lactation. Therefore, it is important to maintain energy and nutrient stores during gestation. This is a challenging time for the dam, and perturbations with these changes can affect both the dam and fetus.

Placental Transfer

There are limited data available on trace mineral transfer from bovine dams to fetuses, and placental transfer of Co and I in general. Ruminants have epitheliochorial placental barriers which make human and rodent placental transport data difficult to compare with as they have hemochorial placental barriers (Stulc, 1997). Pigs have epitheliochorial barriers, thus swine placenta data are a better representation of bovine placental transport. In a study where gilts were orally dosed or intravenously injected with radiolabeled Mn, transfer from gilt to fetus was primarily affected by fetal mass (Gamble et al., 1971), which could indicate placental transfer is driven fetal demand. Matte and Audet (2019) calculated the ratio of pre-suckling neonate serum mineral concentration to prepartum maternal serum mineral for Cu, Fe, Se, and Zn as an indicator of prenatal transfer in pigs. Ratios for Cu, Fe, and Se indicated that placental transfer was limited (< 1), whereas the ratio for Zn indicated that placental transfer was active (> 1). The serum trace mineral concentrations in the neonatal pigs could be overstated because samples were collected in the first 20 min of life when the neonate could be mobilizing

endogenous trace mineral stores at that time (Aggett, 1998). This could artificially inflate the prenatal transfer ratios. However, the rate of trace mineral store mobilization in neonates is ambiguous. In ewes, source of Se provided affected placental transfer such that ewes provided Se-enriched wheat grain had more efficient placental transfer of Se than ewes fed Na selenite (Taylor et al., 2009). They hypothesized this was due to Se-enriched wheat containing selenomethionine instead of Se, which could be transported across the placenta using amino acid transporters instead of Se-selective transporters. These data indicate that trace mineral transfer is regulated and could be driven by demand of the fetus or affected by trace mineral source.

Mechanisms for placental transfer of Cu, Se, and Fe, have been studied in species that have hemochorial placentas. It is currently hypothesized that circulating Cu binds to Cu transporter protein 1, a high affinity carrier present on the cell surface, that transports Cu into a placental cell where it binds to chaperone proteins. The chaperone proteins transfer Cu to ATPases that either transport the Cu across the basolateral membrane or, when excess Cu is present, transport Cu back into maternal circulation (McArdle et al., 2008). Placental transfer of Se is through anion exchange with S (Boyd and Shennan, 1986; Shennan, 1988). Placental transport of Fe is hypothesized to start with transferrin, the primary Fe carrier in the blood, binding to transferrin receptors on the cell surface which initiates pinocytosis. Then the endosomal pH decreases which releases the Fe ions for transport across the basolateral membrane (McArdle et al., 2008). Although these data are from species that have hemochorial placentas, it could provide insight into placental transfer of these minerals in species that have epitheliochorial placentas.

Maternal Metabolic Status

Despite the important role of trace minerals in energy and protein metabolism (Spears, 1999), there are few data available regarding effects of trace mineral supply to pregnant ruminants on dam metabolic status. Both Fe and Cu are important components of the electron transport chain. Manganese is important for glucose utilization. Iodine and Se are important in the thyroid hormone axis and Co is important for B12 synthesis, both of which are crucial for basic metabolic function. Zinc is important for various enzymes involved in energy metabolism (McDowell, 1992).

Dairy cows receiving inorganic trace mineral boluses the last 30 d of gestation had greater circulating β-hydroxybutyrate and γ-glutamyltransferase concentrations at d 15 and 30 post-calving compared with cows receiving organic trace mineral boluses (Osorio et al., 2016). Circulating glucose and urea N were not affected by gestational trace mineral supply. This suggests that inorganic trace mineral supplemented cows had impaired energy pathway signaling and were more susceptible to subclinical ketosis than organic trace mineral cows. Supplementing dairy cows with different concentrations of Co during late gestation and early lactation did not alter circulating NEFA concentrations (Kincaid and Socha, 2007). In ewes fed adequate or supranutritional Se from mid-gestation to parturition, Se in the diet did not affect circulating urea N or NEFA (Lekatz et al., 2010). In these 3 studies, trace mineral requirements were met or exceeded in all treatments. These studies demonstrate that trace mineral supply can affect postpartum dam metabolic status but suggest that trace mineral supply may have minimal effects on prepartum dam metabolic status. Further investigation is needed to determine the effects of trace mineral supply on prepartum dam metabolic status which affects the maternal

environment and consequently fetal development.

Maternal Antioxidant Status

Many of the processes involved in maternal adaptation to pregnancy induce production of reactive oxygen species (**ROS**) to stimulate tissue differentiation or growth (Al-Gubory et al., 2010). Still, an imbalance of ROS and antioxidant capacity can lead to oxidative stress, which can result in intrauterine growth restriction or, in extreme cases, fetal death (Gupta et al., 2007). Enzymes responsible for mitigating oxidative stress are dependent on trace minerals. Superoxide dismutase (Yasodhara et al.) is dependent on Cu, Zn, or Mn, catalase is dependent on Fe, and glutathione peroxidase (**GPX**) is dependent on Se (McDowell, 1992). Accordingly, beef cows supplemented Se during late gestations had greater whole blood GPX activity compared with beef cows not supplemented Se (Hidiroglou et al., 1987; Rowntree et al., 2004). These data indicate that Se-supplemented cows could mitigate oxidative stress events better than non-supplemented cows.

Among transition dairy cows supplemented different sources of trace minerals, cows provided the less bioavailable source had greater total antioxidant capacity at d 14 pre-calving (Osorio et al., 2016) or prepartum (Yasui et al., 2014) compared with cows that were provided an organic source. In both of these studies, ROS and thiobarbituric acid reactive substances (**TBARS**), respectively, were not affected by trace mineral supplementation during gestation. This could indicate that greater antioxidant capacity was required to neutralize ROS in cows fed the less bioavailable trace mineral because the antioxidant enzymes responsible were inefficient compared with cows fed the more

bioavailable sources. These data demonstrate the importance of trace minerals in maintaining the balance between production of ROS and oxidative stress.

Maternal Endocrine Status

There are few data available on the effects of dietary trace minerals supplied to the ruminant dam on circulating hormones. The majority of the data available address dietary Se and thyroid hormone production in sheep. Thyroid hormones, triiodothyronine (**T_3**) and thyroxine (**T_4**), are important during gestation because they regulate metabolic activity of the dam and are important for fetal nervous system development (Kim, 2008; Moog et al., 2017). Supplementing ewes adequate Se or supranutritional Se during mid- and late gestation did not affect circulating thyroid hormones (Lekatz et al., 2010). Yet, supranutritional Se supplementation of ewes on an adequate plane of nutrition resulted in greater circulating progesterone in mid-gestation compared with adequate Se supplementation (Lekatz et al., 2010). Beef cows fed 60 ppm Na selenite or selenized yeast starting 90 d prepartum and continuing through the subsequent pregnancy had greater plasma T_3 concentration during that time than cows fed 20 or 120 ppm Na selenite (Awadeh et al., 1998). Awadeh and others (1998) also reported plasma T_3:T_4 was greater in cows fed 20 ppm Na selenite compared with all other treatments. These changes in circulating T_3 could affect fetal nervous system development and nutrients supplied to the fetus by affecting dam metabolism.

During pregnancy, estrogen is important for normal fetal growth and development because it promotes placental vasculature, biosynthesis of progesterone, the onset of parturition, and fetal maturation (Albrecht and Pepe, 2010). In humans, Zn deficiency can

interfere with normal estrogen function by impairing estrogen receptors which are Zn-finger proteins (Caulfield et al., 1998). Lemley and others (2014) reported ewes fed supranutritional Se in mid- and late gestation had greater circulating estrogen concentrations during the first 24 h postpartum compared with ewes that received adequate dietary Se, but circulating estrogen was not affected prepartum. These data indicate function or concentration of circulating estrogen could be responsive to changes in dietary Zn and Se, which could impact fetal growth and development.

Colostrogenesis

The transfer of maternal circulating immunoglobulins (**Ig**) into mammary secretion during colostrogenesis, is markedly upregulated in the last few weeks of gestation and ends at parturition (Barrington et al., 2001). This process is primarily controlled by changes in estrogen and progesterone as gestation progresses (Guy et al., 1994; Stark et al., 2015) but can also be affected by timing of lactogenesis (Barrington et al., 2001).

When provided organic trace minerals during late gestation, dairy cows had greater colostral IgG concentration (Kincaid and Socha, 2004) and beef cows had greater colostral IgM concentration (Price et al., 2017) than cows provided inorganic trace mineral. However, late gestational trace mineral supply did not affect colostral Ig in other studies using beef cows (Muehlenbein et al., 2001; Wallace et al., 2017), dairy cows (Kincaid and Socha, 2007; Karkoodi et al., 2012; Jacometo et al., 2015), or sheep (Boland et al., 2005; Boland et al., 2008; Swanson et al., 2008). These data suggest late gestational trace mineral supply may have minimal effects on Ig transfer from circulation

to mammary secretions.

TRACE MINERAL EFFECTS ON FETAL AND NEONATAL DEVELOPMENT

Critical Windows in Fetal and Neonatal Development

During the fetal and neonatal periods there are several developmental windows (Fowden et al., 2006; Symonds et al., 2006). In general, organogenesis occurs in early and mid-gestation, exponential fetal growth occurs in late gestation, and tissue maturation occurs in the perinatal period (Fowden et al., 2006). During each critical window, nutrient supply and hormone concentrations can greatly affect development, which can have long-term effects on the offspring (Fowden et al., 2006; Fowden and Forhead, 2009).

Fetal accretion of trace minerals increases with gestational age (Hansard et al., 1968; Ferrell et al., 1982; Grace et al., 1986) and endogenous stores are mobilized after birth to aid in normal growth and development (Aggett, 1998). This demonstrates the importance of trace minerals during the fetal and neonatal period, as stores are accumulated during gestation and mobilized postnatally. Effects of trace mineral deficiencies during these times will be discussed in the following sections.

Endocrine Status

Thyroid hormones are crucial for normal fetal growth and development, particularly through the endocrine axis responsible for prenatal tissue maturation (Fowden and Forhead, 2009). Thyroidectomized lamb fetuses had prolonged gestation and poor survival past 24 h of age which was likely due to less tissue maturation

(Hopkins and Thorburn, 1972). This emphasizes the importance of proper thyroid hormone synthesis to neonatal survival. Iodine is a major component of both thyroid hormones; T_3 contains 3 I molecules and T_4 contains 4 I molecules. Both T_3 and T_4 are synthesized and released from the thyroid gland, however, T_3 is metabolically active. The enzyme responsible for converting T_4 to T_3 is type 1 deiodinase, which is Se-dependent (Allan et al., 1999). Both I and Se are required for normal thyroid function, which is important for fetal development and consequently, neonatal survival.

Lambs born to ewes provided 20 mg I twice weekly in the last month of gestation had greater T_4 concentrations, greater birth weight and growth rate, as well as greater survival percentage compared with lambs born to ewes that did not receive I supplement (Knights et al., 1979). Ewes in this study had either ad libitum or restricted access to the basal diet which demonstrates that production measures improved in offspring born to dams supplemented I regardless of plane of nutrition and with only minor changes in circulating thyroid hormones. Boland and others (2008) fed ewes an I adequate or supranutritional I diet (26.6 mg I daily) during the last 3 wk of gestation and reported similar lamb birth weights and serum T_4 concentrations at 1 h of age, but lower serum T_3 concentrations in lambs born to ewes fed supranutritional I. They hypothesized that lower serum T_3 in lambs born to ewes provided supranutritional I could be due to greater conversion of T_4 to reverse T_3, which is biologically inactive. However, the mode of action that favors the conversion of T_4 to reverse T_3 has yet to be elucidated. This study did not include lamb morbidity, mortality, or growth data; therefore, it is unclear how low serum T_3 at birth affected production traits. These highlight the importance of providing adequate I to dams during gestation to ensure there is proper thyroid hormone

metabolism in the offspring.

Lambs born to ewes fed a Se-deficient basal diet and supplemented Na selenite, selenized yeast, or no additional Se starting in the last 2/3 of gestation had similar birth weights, and pre-suckling serum T_4 and T_3:T_4, but lambs born to Se supplemented ewes tended to have greater serum T_3 concentrations (Rock et al., 2001). Supranutritional Se supplementation to dams has resulted in inconsistent circulating thyroid hormone data in offspring. Beef calves born to cows supplemented supranutritional selenized yeast during late gestation had greater plasma T_3 concentrations compared with calves born to Na selenite supplemented cows; however, birth weights and plasma T_4 or T_3:T_4 were not different (Awadeh et al., 1998). Conversely, Rowntree and others (2004) reported providing daily drench of supranutritional Na selenite to beef cows throughout gestation did not alter neonatal calf thyroid hormone concentrations or calf birth BW. These data demonstrate that providing adequate Se to the dam during gestation is also important for normal offspring thyroid hormone function, and that alterations to calf endocrine status does not always result in altered calf birth BW.

Insulin-like growth factors (**IGF**) are important for tissue differentiation and cell proliferation (Zapf and Froesch, 1986), which are both important for fetal development. In transformed fibroblast and muscle cell lines, Zn decreased IGF binding affinity to IGF binding protein (Sackett and McCusker, 1998) and increased binding affinity to IGF receptors (McCusker et al., 1998). In rats, Zn deficiency resulted in less serum IGF-1 concentrations (MacDonald, 2000); therefore, adequate Zn is needed to ensure IGF that is free to bind to receptors on the cell surface is present in order to promote growth and differentiation.

The effects of Zn deficiency and circulating IGF on fetal development are described by a study performed by Hanna and others (2010). Rat dams were fed a Zn-adequate diet, Zn-deficient diet, or a pair-fed diet that provided adequate Zn but matched the overall intake of the Zn-deficient rats. These diets were fed during gestation and tissues of dam and offspring were collected in late gestation. Fetal BW and size were lowest in the Zn-deficient treatment, greatest in the Zn-adequate treatment, and intermediate in the pair-fed fetuses were intermediate. Relative mRNA expression of IGF-1 in fetal liver was greater in pair-fed and Zn-adequate fetuses compared with Zn-deficient fetuses, and relative mRNA expression of both IGF-1 and IGF-2 binding proteins was greater in pair-fed fetuses compared with the other treatments. These data show that maternal Zn-deficiency can reduce fetal IGF gene expression in liver which likely contributes to impaired fetal growth and development of Zn-deficiency during gestation.

Organogenesis and Development

Nervous system. Proper nervous system development in utero is vital for the neonatal ruminant as they need to be mobile shortly following birth. There are several trace minerals that are important for fetal nervous system development, including Cu, Fe, Zn, and I.

Enzootic ataxia (i.e. Swayback) is a well-known consequence of maternal Cu deficiency during gestation, especially in sheep. Affected offspring are uncoordinated, weak in the hind limbs, and sometimes unable to stand or walk (Innes, 1939). Inadequate Cu provided to the dam results in less cytochrome oxidase activity in brain of the

offspring (Mills and Williams, 1962). Cytochrome oxidase is a Cu-dependent enzyme and is responsible for myelination of motor neurons (Howell and Davison, 1959; Wainio et al., 1959). Myelin increases the speed of signal transmission and protects the axon itself (Hyung et al., 2015). This decrease in myelination in Cu-deficient offspring leads to increased degeneration of spinal cord fibers (Smith et al., 1977) which develops into enzootic ataxia postnatally (Lewis et al., 1967).

In rats, Zn deficiency during gestation can have detrimental effects on brain development. Developmental retardation, resulting from widespread apoptosis, in early neural tissues has been reported in Zn-deficient rat fetuses (Rogers et al., 1995). It has been hypothesized that Zn deficiency weakens the cytoskeleton network which leads to poor neural development (Ashworth and Antipatis, 2001). Additionally, it could be hypothesized that Zn deficiency weakens the oxidative defense system of the fetus which could also lead to greater cell death. Unfortunately, there are limited data available regarding gestational Zn deficiency and offspring nervous system development in ruminants.

Thyroid hormones regulate the metabolic pattern of the brain (Ashworth and Antipatis, 2001); therefore, alterations to thyroid hormones can affect nervous system development. Maternal I deficiency can result in offspring brain damage and mental retardation, which is likely driven by impaired thyroid hormone synthesis (Delange, 2000). In a study conducted by Bastian and others (2011), rat pups born to Cu-deficient dams had impaired sensorimotor function at 3 mo of age, even after pups were fed a Cu-adequate diet postnatally. Pups born to Cu-deficient dams also had lower serum T_3 and Fe concentrations, which can be expected as Cu is required for Fe absorption (McDowell,

1992). Decreased circulating thyroid hormone in Cu-deficient pups could be due to greater oxidative stress in the thyroid gland because superoxide dismutase and catalase (Cu- and Fe-dependent, respectively) activity are impaired. Adequate trace minerals, specifically Cu, Fe, and I, are required for normal thyroid gland function in the fetus and are essential for proper nervous system development.

Iron in essential for hematopoiesis and oxidative defense which can also affect fetal brain development. Again in rats, maternal Fe deficiency resulted in greater angiogenesis in fetal rat brain which was likely due to compensatory mechanisms driven by cellular hypoxia caused by anemia (Bastian et al., 2015). Increased angiogenesis in the brain typically results in degradation of extracellular matrix and basal membranes (Baburamani et al., 2012), which compromises the blood-brain barrier integrity. This could explain why anemia resulting from maternal Fe deficiency has also led to behavioral deficits in rat pups (Felt and Lozoff, 1996). Pups born to dams that were fed a diet marginally deficient in Fe during gestation and lactation had greater oxidative stress in brain tissue and impaired motor development, indicated by lower grip strength (Kwik-Uribe et al., 1999). Although it is difficult to determine if the insult to nervous system development was prenatal or postnatal in this study, it interesting that maternal Fe deficiency did not affect hematocrits (Kwik-Uribe et al., 1999). Thus, anemia likely did not play a role in impaired motor development. With or without anemia, maternal Fe deficiency appears to be detrimental to fetal nervous system development.

Immune system. Neonatal ruminants are born immunologically immature which makes them more susceptible to infections (Osburn, 1981). There are components of the immune system that develop in utero thus, insults to fetal immune system development

could further constrain calf immune function postnatally. Innate immune cells (i.e. neutrophils and macrophages) and complement activity are present in late gestational fetuses and approach adult concentrations and activity, but are functionally depressed near term and in the neonate due to increased cortisol concentrations (Barrington and Parish, 2001). In the fetus and neonate, adaptive immune cells are present but do not reach adult cattle concentrations or functionality (Chase et al., 2008). Neonates are also agammaglobulinemic due to lack of placental transfer of Ig, therefore they rely upon colostrum consumption and transcytosis in the small intestine to absorb Ig to obtain passive immunity (Barrington and Parish, 2001). This highlights that neonatal calves are immunologically disadvantaged, thus improvements or diminutions in fetal and neonatal immune system development can have major effects on calf survival.

Trace minerals Cu, Fe, Mn, Se, and Zn are important for immune system development and function. Oxidants are used by immune cells to destroy pathogens. The involvement of trace minerals in antioxidant enzymes (superoxide dismutase, GPX, and catalase) is crucial for protecting the immune cell from oxidant damage as well (Maggini et al., 2007). The Se-dependent enzyme, thioredoxin reductase, is important for redox regulation of enzymes, transcription factors, and receptors in the inflammatory pathway (Maggini et al., 2007). The Fe-dependent enzyme, protein kinase C, is important for cell proliferation, which is essential to rapid cell turnover in the immune system, and Fe-dependent myeloperoxidase is important for pathogen destruction in neutrophils (Maggini et al., 2007).

Copper deficiency has resulted in neutropenia and anemia, fewer myeloid precursors in bone marrow, and lower ability of leukocytes and macrophages to destroy

pathogens. However, the exact mechanism driving these consequences of Cu deficiency are unclear (Stafford et al., 2013). Zinc's involvement in immunity is complex. It is important for membrane integrity; therefore it is important in maintaining the physical barrier that prevents pathogens from entering the body. Zinc is also important in cytokine signaling, through metalloenzymes and transcription factors that initiate an inflammatory response (Maares and Haase, 2016). Inflammation response is reflective of the innate immunity, but it also communicates with adaptive immunity to signal there are pathogens present and aids in maturation of adaptive immune cells (Murphy et al., 2008). Although Zn has been shown to increase microbicidal activity of macrophages, it also affects multiple anti-inflammatory processes in the immune system which are hypothesized to regulate the immune response (Jarosz et al., 2017). Adequate trace mineral supply during gestation and lactation is crucial for proper immune system development and function.

Keen and others (1989) performed a pivotal study demonstrating the effects of marginal gestational Zn deficiency on immune function of offspring by feeding Rhesus monkeys an adequate or marginally deficient Zn diet during pregnancy and the 1st month of lactation. They found neonates born to mothers fed a marginally Zn-deficient diet had similar tissue Zn concentrations compared with control infants but had reduced peripheral lymphocyte responsiveness to mitogens. This showed that immune function was affected by marginal Zn deficiency during gestation and early lactation. Neonatal calves born to dairy cows receiving organic trace mineral (Co, Cu, Mn, and Zn) boluses the last 30 d of gestation had less indicators of oxidative stress, and inflammatory mRNA and miRNA were downregulated compared with calves born to cows receiving inorganic trace mineral (Jacometo et al., 2015). Because these calves were all managed similarly after

birth, they were all subjected to a similar pathogen load in the neonatal period. It could be hypothesized that lower oxidative stress indicated there was less ROS production from immune cells because transcription of inflammatory mRNA was reduced, which was interpreted by the authors as the calves born to organic trace mineral cows having a more efficient immune system. Wallace and others (2017) reported feeding beef cows alfalfa hay or Se-enriched alfalfa hay the last 3rd of gestation did not affect colostrum or neonatal calf serum *E. coli* antibody concentrations. All cows in this study received supranutritional dietary Se, therefore results may have differed if some calves were developed in a Se-deficient environment. Although these studies give insight into how gestational trace mineral supply affects offspring peripheral (circulating) immune system development, they do not include morbidity or mortality data.

Formigoni and others (2011) fed dairy cows either inorganic or organic forms of Cu, Mn, and Zn the last 60 d of gestation and reported calf mortality rates at calving were greater in inorganic trace mineral supplemented cows than organic trace mineral supplemented cows. They hypothesized this was due to greater IgG in organic trace mineral cow colostrum; however, their definition of 'mortality at calving' was unclear. If that time point indicated the act of parturition, then differences in calf mortality are likely due to problems such as dystocia or poor calf vigor. If it is indicating the neonatal period, then it could be due to greater colostrum IgG available to organic trace mineral calves. Calf mortality after calving (defined as: from birth to weaning) was not affected by gestational trace mineral treatment; age at weaning was also unclear. In a 2-yr study, ewes fed a restricted, adequate, or high nutritional plane diet were supplemented either adequate Se or supranutritional Se throughout gestation and lamb morbidity and mortality

data were collected (Hammer et al., 2011). There were no differences in lamb mortality in either year, but in yr 1, lamb morbidity at d 57 of age was greater in lambs born to restricted dams fed adequate Se compared with supranutritional Se. Conversely, lambs born to ewes on high plane of nutrition and supplemented adequate Se had less morbidity at d 57 of age than supranutritional Se lambs. In yr 2, lambs born to ewes fed an adequate plane of nutrition diet and supplemented adequate Se had lower morbidity than lambs born to ewes provided supranutritional Se. All lambs in this study received artificial colostrum to achieve similar IgG consumption. Neonatal lamb serum Ig differences were analogous to morbidity data in yr 1 where serum Ig was greater in lambs born to ewes on a high plane of nutrition with adequate Se than lambs born to ewes receiving supranutritional Se. These data demonstrate that gestational trace mineral supply can affect offspring survival and immune function pre-weaning, and calf Ig absorption could be affected by gestational trace mineral supply.

Blood concentrations of Ig in neonatal ruminants indicate passive immune transfer and is important for survival. Lambs born to ewes fed high trace mineral diet (Co, I, Mn, Se, and Zn) the last 7 wk of gestation had less IgG absorption at 24 h of age compared with lambs born to ewes fed adequate trace minerals during gestation (Boland et al., 2005). Boland and others (2008) also determined IgG absorption in lambs born to ewes fed a high Co, high I, or adequate trace mineral diet the last 3 wk of gestation, or a high I diet the last week of gestation and reported lambs born to ewes fed a high I diet the last 1 or 3 wk of gestation had less IgG absorption at 24 h of age than Co-supplemented lambs and control lambs. In this study, serum T_3 concentrations were greater in control lambs compared with lambs born to ewes fed a high I diet for 3 wk. Similar results were

reported by McGovern and others (2016) where lambs born to ewes supplemented a high I diet had less IgG absorption and lower circulating T_3 and ileal expression of thyroid hormone receptor β compared with control lambs. They hypothesized that disruption in offspring IgG absorption when the dam is fed excess I is mediated by the altered thyroid hormone axis in the offspring but the exact mechanism is still unclear.

Neonatal Ig absorption data are also inconsistent and unclear when other trace minerals in the maternal diet are being studied. Providing organic sources of Co, Cu, Mn, and Zn to beef cows in the last 3rd of gestation resulted in greater calf serum IgA than calves born to cows provided inorganic trace minerals (Price et al., 2017). In this study colostral IgA concentration was not affected by gestational trace mineral treatment but colostral IgM concentrations were greater in organic trace mineral-supplemented dams compared with inorganic-supplemented dams. Although there were inconsistencies between calf serum and colostrum IgA and IgM data, the IgG data were consistent and it is the main Ig isoform. In yr 2 of a study where ewes were fed at different planes of nutrition and supplemented adequate or high Se, serum IgG at 24 h of age was not affected by maternal Se supplementation (Hammer et al., 2011). Calves born to beef cows fed alfalfa hay or Se-enriched alfalfa hay did not have different serum IgG concentrations at 48 h of age, despite colostrum from Se-enriched hay fed cows having greater colostrum IgG concentrations compared with alfalfa hay fed cows (Wallace et al., 2017). This could indicate that calves from cows fed Se-enriched hay had less IgG absorption compared with calves from hay fed cows, but colostrum consumption was not measured in this study. Calves born to beef cows supplemented 120 ppm Na selenite had greater serum IgG than calves born to cows fed 20 ppm Na selenite, and colostral IgG

was greater in cows fed 60 and 120 ppm Na selenite than 20 ppm Na selenite (Awadeh et al., 1998). Adequate Se during gestation is important for normal thyroid hormone production along with I. Hence, it could be hypothesized that differences in Ig absorption when different sources and concentrations of Se were supplied to the fetus could be mediated by the thyroid hormone axis.

Bone and connective tissue. Development of fetal bone and connective tissue is crucial to neonatal ruminants because they are expected to be mobile shortly after birth. Trace minerals Cu, Zn, and Mn are important for several enzymes involved in the formation of these tissues. Thus, inadequate trace mineral supply can lead to malformations which can decrease neonatal survival.

There are 2 Mn-dependent enzymes needed for mucopolysaccharide formation. They are important components of the organic bone matrix (Leach and Muenster, 1962). Both lambs and calves born to Mn-deficient dams had reductions in cartilage uronic acid and hexosamines (Rojas et al., 1965; Hidiroglou et al., 1979). These are the main subunits of proteoglycans, which are important for bone organization and structure (Lamoureux et al., 2007), indicating that offspring had disturbed development of the bone matrix. In a more recent study, Hansen and others (2006) observed calves born to heifers fed a Mn-deficient diet throughout gestation weighed less at birth and had greater incidence of dwarfism, weakness at birth, and superior brachygnathism compared with calves born to heifers fed a Mn-adequate diet. These findings were further supported by Hidiroglou and others (1990) who reported a relationship between calf serum Mn and incidences of congenital joint laxity and dwarfism.

Neonatal ruminant dwarfism has also been observed as the result of Zn deficiency

(Groppel and Hennig, 1971). This could be due to Zn-dependent enzymes, alkaline phosphatase and carbonic anhydrase II, being crucial for bone formation, and matrix metalloproteinases being important for organization and adjustment of connective tissues (Sloup et al., 2017). Similar to Hansen et al. (2006), maternal Zn deficiency in rats resulted in greater incidence of fetal bone malformation including under-developed ribs, under-developed tibia and fibula, and malformed spine (Catalanotto et al., 1979).

Lysyl oxidase is a Cu-dependent enzyme required for collagen and elastin cross-linking (McDowell, 1992). Although connective tissues are important for bone and joints, impaired connective tissue formation due to Cu deficiency can also affect vital organ development. Rabbit pups born to dams fed a marginally Cu-deficient diet had less lung lysyl oxidase activity compared with pups born to dams fed a Cu-adequate diet, which resulted in incomplete collagen maturation in the lungs (Abdel-Mageed et al., 1994). Hunsaker and others (1984), reported elastin clumps of irregular size and shape in aortas of rat pups born to dams fed a Cu-deficient diet which led to aortal abnormalities; however, pups had no other signs of Cu deficiency.

SUMMARY AND CONCLUSION

Gestational trace mineral supply is important for proper fetal bone and connective tissue, endocrine, nervous, and immune system development and function in the offspring. Trace mineral deficiencies in the dam during the gestation and the perinatal period are detrimental to these systems which can lead to poor calf survival or growth. Further investigation into effects of marginal trace mineral deficiency during gestation on ruminant dam and offspring is necessary to understand the role of trace minerals in

developmental programming, and therefore long-term effects on the offspring.

CHAPTER 2

EFFECTS OF COPPER, ZINC, AND MANGANESE SOURCE AND INCLUSION DURING LATE GESTATION ON BEEF COW PERFORMANCE, MINERAL STATUS, AND COLOSTRUM AND MILK YIELD AND QUALITY

ABSTRACT

To determine the effects of source and inclusion of Cu, Zn, and Mn during late gestation on beef cow performance, mineral status, oxidative stress, and colostrum and milk yield and quality, multiparous, fall-calving, Sim-Angus cows (n = 48; 649 ± 80 kg BW; 5.3 ± 0.5 BCS; 4.3 ± 1.2 yr of age) were individually-fed tall fescue-based hay (12.2% CP, 61.5% NDF) and supplemented to meet or exceed all nutrient recommendations except Cu, Zn, and Mn. From 91.2 ± 6.2 d pre-calving to 11.0 ± 3.2 d post-calving, cows received: no additional Cu, Zn, or Mn (CON); Cu, Zn, and Mn sulfates supplying 133% NASEM recommendations (ITM); Cu, Zn, and Mn metal methionine hydroxy analogue chelates (MMHAC, MINTREX, Novus International) supplying 133% recommendations (CTM); or Cu, Zn, and Mn sulfates and MMHAC supplying 100% recommendations (reduce and replace, RR). Data were analyzed with treatment and breeding group as fixed effects, and animal as experimental unit. Sampling time was a repeated effect for circulating metabolites and oxidative stress markers. Cow BW, BCS, and gestational and 1 h post-calving metabolites were not affected ($P \geq 0.13$) by gestational trace mineral treatment. Post-calving cow liver Cu was greater ($P \leq 0.07$) in cows fed CTM compared with all other treatments. Treatment did not affect ($P \geq 0.28$) post-calving liver Zn or Mn. Cows fed CTM had greater ($P \leq 0.05$) pre-suckling

colostrum volume, as well as lactose concentration and content, compared with ITM and RR cows. Treatment affected ($P = 0.09$) colostrum triglyceride concentration, where RR cows had greater ($P \leq 0.04$) triglycerides concentration than CTM and CON. Colostrum weight and all other components were not affected ($P \geq 0.10$) by treatment. Milk Zn content at d 60 of lactation was greater ($P \leq 0.04$) in CTM cows than ITM and RR; however, milk Zn concentrations at d 60 were not affected ($P = 0.18$) by treatment. Treatment did not affect ($P \geq 0.15$) 4-h milk yields or all other milk components at d 35 or 60 of lactation. Treatment affected ($P = 0.10$) serum thiobarbituric acid reactive substances (TBARS) during gestation where CON cows had greater ($P = 0.01$) circulating TBARS than CTM. Lactating cow serum TBARS were affected ($P = 0.09$) by treatment x day interaction, where RR cows had greater ($P = 0.04$) serum TBARS at d 35 of lactation than CON, and CTM cows had greater ($P \leq 0.09$) serum TBARS than all other treatments at d 60 of lactation. Data suggest inclusion of chelated Cu, Zn, and Mn in late gestational beef cow diet improves Cu status and results in greater colostrum yield and lactose.

INTRODUCTION

Nutritional demands of the cow increase during the last third of gestation due to exponential fetal growth and during lactation due to demands for milk production (NASEM, 2016). Both of these physiological states disrupt trace mineral homeostasis of the dam in order to partition trace minerals from the diet or storage (primarily liver) to the placenta (Mills and Davies, 1979) or the mammary gland (Annenkov, 1982). Because essential trace minerals Cu, Zn, and Mn are constituents of metalloenzymes and cofactors in many enzyme systems (McDowell, 1992), they are important in almost all normal

biochemical processes (Spears, 1999). In reproducing females, these 3 trace minerals are particularly important for the antioxidant defense system (McDowell, 1992; Spears and Weiss, 2008), which works to combat greater oxidative stress resulting from the increased metabolic demand during late gestation and lactation (Sordillo, 2005). Cows are also completely responsible for providing Cu, Zn, and Mn to the fetus and postnatal calf (Hidiroglou and Knipfel, 1981); these are necessary for immune, endocrine, reproductive, skeletal, and nervous system development (Hostetler et al., 2003).

Beef cows are typically fed forage-based diets that are highly variable in trace minerals and their antagonists which often leads to deficiency (McDowell, 1992). Therefore, beef cows can be provided free choice trace mineral supplements that vary in source. In ruminants, organic sources of Cu, Zn, and Mn (chelated with amino acids or amino acid analogues) are generally more bioavailable than inorganic salts (sulfates and oxides; Spears, 2003). This is due to greater stability in the rumen which reduces mineral antagonism and delivers the mineral to the absorptive cells of the small intestine (Nuzback et al., 2019). Overall, we hypothesized that supplementing beef cows a chelated source of Cu, Zn, and Mn in late gestation would improve cow mineral status and colostrum quality, which would improve fetal development and calf mineral status. Our specific objective was to determine the effects of Cu, Zn, and Mn source and inclusion during late gestation on beef cow performance, mineral status, oxidative stress, and colostrum and milk yield and quality.

MATERIALS AND METHODS

All animal procedures were approved by the University of Missouri Animal Care

and Use Committee (Protocol #9045) and took place at University of Missouri Beef Teaching and Research Farm (Columbia, MO).

Animal Management and Diets

Treatment diets and housing. Forty-eight crossbred beef cows (Sim-Angus, average initial BW = 649.3 ± 79.6 [SD throughout] kg, average initial BCS = 5.30 ± 0.46, age at calving range: 3 to 7 yr [average = 4.25 ± 1.20 yr]) bred by AI (n = 36, due date: September 17, 2017) or natural service (n = 12) were fed tall fescue-based hay and a pelleted supplement to meet or exceed all nutrient recommendations (NASEM, 2016) except Cu, Zn, and Mn. Cows were allocated by BW, BCS, age, and breeding group (AI or natural service) to 1 of 4 gestational trace mineral treatments from 91.2 ± 6.2 d pre-calving to 11.0 ± 3.2 d post-calving: 1) basal diet with no additional Cu, Zn, or Mn (control; **CON**); 2) basal diet with Cu, Zn, and Mn sulfates to supply 133% NASEM (2016) recommendations (inorganic trace minerals; **ITM**); 3) basal diet with Cu, Zn, and Mn metal methionine hydroxy analogue chelates (**MMHAC**; MINTREX, Novus International, St. Charles, MO) to supply 133% NASEM recommendations (chelated trace minerals, **CTM**); or 4) basal diet with Cu, Zn, and Mn sulfates and MMHAC to supply 100% NASEM recommendations (reduce and replace strategy; **RR**).

Three different hays (Table 2.1) were fed throughout the treatment period, with all treatments receiving the same hay on any given day. All hays were cut and baled in the late spring or early summer from the University of Missouri Beef Teaching and Research Farm. Treatments were delivered in a pelleted supplement based in soybean hulls or soybean hulls and dried distillers grains with solubles (**DDGS**), depending on nutrient

composition of the hay fed at the time. Supplements were formulated for each treatment based on the initial core samples of the corresponding hay to meet or exceed NE_m, CP, Ca, Na, vitamin A, vitamin D, vitamin E, I, Se, and Co recommendations (NASEM, 2016). Inclusion of Cu, Zn, and Mn in the supplements were also formulated using initial hay core samples and individual treatment goals. Sulfate-based Cu, Zn, and Mn included in ITM and RR supplements were Cu sulfate pentahydrate, Zn sulfate monohydrate, and Mn sulfate monohydrate. Methionine hydroxy analogue (MFP, Novus International) was included in CON, ITM, and RR supplements to provide similar amounts of methionine hydroxy analogue that cows fed CTM supplements received from MMHAC Cu, Zn, and Mn.

All treatment supplements corresponding with hays 1 and 3 were composed of DDGS, soyhulls, choice white grease, calcium carbonate (Fre-Flo, ILC RESOURCES, Urbandale, IA), salt (Top-Flo, Cargill, Minneapolis, MN), vitamin ADE (ADE NutraMix, Nutra Blend, LLC, Neosho, MO), calcium iodate, sodium selenite, and cobalt sulfate. Treatment supplements corresponding with hay 2 had similar composition to supplements fed with hays 1 and 3 but did not include DDGS due to hay CP concentration. Nutrient composition of treatment supplements corresponding with hays 1, 2, and 3 were 21.0, 8.5, and 25.1% CP (DM basis), respectively, and 1.74, 1.16, and 1.93 Mcal NE_m/kg (DM basis; calculated based upon ingredient inclusion using NASEM, 2016 values), respectively.

Cows were housed in partially-covered 3.7 x 15.8 m pens (n = 4/pen) with concrete floors bedded with sawdust. Each pen had 4 electronic feeding gates (American Calan, Northwood, NH) in order to individually feed each cow. Cows were acclimated to

electronic gate feeding system for ≥ 15 d prior to study initiation. Daily grab samples of hay and supplement were collected at 2 wk intervals and analyzed for nutrient composition. Supplement was weighed for each individual cow and fed every morning (0730) in a feed pan to prevent wastage. Approximately equal amounts of hay were weighed and fed every morning (0800) and evening (1800). Amount of hay offered to each cow was initially calculated assuming cows would consume 1.2% BW of NDF, and then was adjusted based on actual intakes for each cow to have approximately 10% refusal rate. Two-day BW were collected every 28 d, and amount of hay fed was adjusted accordingly. Supplement was fed to be 11.5% of total DMI and was adjusted weekly based on hay offered the previous week. Hay refusals were weighed back weekly and sampled for DM analysis. On the rare occasion there were supplement refusals, the refusal was collected in a plastic bag, stored at 4 °C, and fed to the same cow with the next day's supplement.

Prior to calving (17.3 ± 7.0 d pre-calving) cows were moved to 18 x 61 m dry lot calving pens (Duncan and Meyer, 2018) by treatment. In the calving pens, ad libitum hay (hay 3 in Table 2.1) was provided, treatment supplements were pen-fed at 1800, and cows had free access to water. Three stadium lights allowed for continuous monitoring of cows throughout the night. The amount of supplement fed to each pen was calculated by estimating hay DMI assuming cows could consume 1.2% BW of NDF, using total pen BW (hay DMI = 1.20% BW / % hay NDF [DM]). Supplement was fed to be 11.5% of total pen DMI, similar to when cows were individually-fed. Supplement grab samples were collected daily, and hay core samples were collected from each bale upon delivery to the calving pens.

Nutrient intake calculations. While being individually-fed in the Calan gates, daily hay DMI was calculated by multiplying daily hay offered by hay subsample % DM to determine hay DM offered. The weekly refusal amount was multiplied by each refusal subsample % DM, and divided by 7 d to estimate daily refusal DM. Daily refusal DM was subtracted from daily hay DM offered to determine daily hay DMI. Supplement DMI was calculated by multiplying supplement DM offered by supplement subsample % DM.

After cows were moved to the calving pens, hay DMI was estimated as 1.2% BW in hay NDF. Supplement DMI was estimated for each cow using the same equations used to calculate amount of supplement to feed to each pen. Daily hay and supplement DMI were then multiplied by nutrient densities of corresponding subsamples to determine daily nutrient intakes and dietary trace mineral provided.

Dry matter intakes, dietary trace mineral concentrations provided, and target dietary trace mineral concentrations for each treatment are presented in Table 2.2. Actual dietary trace minerals provided varied from target concentrations for ITM, CTM, and RR because composition of hay fed differed from the initial hay core sample composition used to formulate treatment supplements. This was likely due to high variability of trace minerals in forages (McDowell, 1992). Cows fed CON had dietary Cu and Zn concentrations below the NASEM recommendations (2016) throughout the treatment period and had dietary Mn concentrations below recommendations from d 29 to 56 of study.

Post-treatment management. After treatment termination, all cow-calf pairs were housed in an additional calving pen for ≥1 wk for monitoring, provided ad libitum hay, and allowed access to a pressed, molasses-based supplement that provided vitamins and

inorganic minerals (minimum Ca [6.0%], maximum Ca [6.5%], minimum NaCl [3.0%], maximum NaCl [3.5%], and minimum amounts of the following: P [6.0%], Mg [3.0%], K [3.0%], Cu [1,100 mg/kg], I [50 mg/kg], Mn [600 mg/kd], Se [9 mg/kg], Zn [9 mg/kg], vitamin A [441,000 IU/kg], vitamin D3 [99,225 IU/kg], and vitamin E [287 IU/kg]; MLS #12 Minera-lix, Midcontinent Livestock Supplements, Moberly, MO). Pairs were then moved to and rotated through 3 tall fescue-based pastures, provided ad libitum access to water and the same vitamin and mineral source, and monitored until weaning at 195.3 ± 8.3 d post-calving.

Gestational Data Collection and Sampling

Jugular blood samples and 2-d BW were collected before morning feeding at 2 and 9 days prior to study initiation for AI and NS cows, respectively. At d 28 and 56 of study, jugular blood samples and 2-d BW were collected before morning feeding as well. Jugular blood samples and 1-d BW were collected to prevent cow stress and induction of parturition in late gestation prior to moving to the calving pens (17.3 ± 7.0 d pre-calving). This time point was termed "pre-calving." Three trained technicians recorded BCS (9-point scale: 1 = emaciated, 9 = obese) for each cow at study initiation, d 28 and 56 of study. Blood samples were collected into 4 tubes (2 Vacutainer serum collection tubes containing no additives [10 mL draw; Becton Dickinson, Franklin Lakes, NJ], 1 Monoject plasma collection tube containing 0.10 mL of 15% K_3EDTA [10 mL draw; Covidien, Mansfield, MA], and 1 Vacutainer plasma collection tube containing 15 mg of sodium fluoride and 12 mg of potassium oxalate [6 mL draw; Becton Dickinson, Franklin Lakes, NJ] for glucose determination). Blood tubes were inverted, placed on ice (serum

tubes were allowed to clot before placing on ice), and centrifuged for 30 min at 1,500 x *g* at 4°C within 10 h of collection.

Initial liver biopsies were performed at study initiation sampling using a protocol modified from Davies and Jebbett (1981). Organic matter was removed from the surgical area, the hair was clipped, and the area was scrubbed in non-overlapping, concentric circles with iodopovidine followed by 70% alcohol. The scrubbing cycle was repeated ≥ 3 times until the surgical area was clean of dirt and hair. Five milliliters lidocaine (20 mg/mL) was administered subcutaneously at the surgical site and an approximately 1 cm incision was made with a #10 scalpel blade in the intercostal space between the 11th and 12th rib approximately 20 cm ventral to the transverse spinal processes on the right side of the cow. A trocar and cannula were inserted through the peritoneal tissue then the trocar was removed, and the cannula was advanced into the liver with a twisting motion by angling the cannula towards the left elbow joint. The cannula was retracted, and the liver sample was placed in a plastic 2 mL microcentrifuge tube, then was flash frozen on dry ice and stored at -80°C for mineral analysis at a later date. Chromic gut suture was used to place 1 suture to close the skin at the incision site, then the incision and surrounding area were sprayed with 5% permethrin (Prozap Screw Worm Aerosol, Neogen Coorporation, Pleasantville, IA). Incision sites were monitored daily for ≥ 1 wk to ensure they healed properly.

Post-calving Data Collection and Sampling

While in the calving pens, cows were closely monitored by trained personnel to detect when cows were in stage II of parturition by walking through pens at least once

every hour except 0200 and 0300 during heavy calving. Once stage II was detected, the cow was continuously monitored to record time of birth. After the calf stood but before it suckled, both cow and calf were removed from the pen and the cow was directed into the chute. Cow blood samples were collected from the jugular vein (76.3 ± 77.5 min post-calving) into the same 4 tubes types as gestational blood sampling and were processed as previously described.

One rear quarter of the mammary gland (the quarter that was most full with no signs of tissue damage) was hand-milked completely prior to suckling (57.5 ± 17.5 min post-calving). Colostrum volume and weight were recorded, and subsamples were collected and stored at -20°C until composition analysis.

Liver biopsies were performed on cows at 11.0 ± 3.2 d post-calving as described previously. After biopsies, pairs were moved to an extra calving pen for post-biopsy monitoring, and treatments were terminated.

4-h Milk Yields and Pre-weaning Data Collection

Cow milk yields and samples were collected at d 35 (34.6 ± 1.8 d post-calving) and d 60 (60.7 ± 3.7 d post-calving) of lactation. Milk yields were measured in the evening for each cow (average: 2002 ± 1.2 h, range: 1734 to 2303 h) in order to be able to accurately extrapolate 4-h yields to daily yields. Due to time constraints, 10 to 12 pairs could be milked in an evening. Pairs to be milked that evening were selected to keep day of lactation similar among treatments, sorted from the rest of the herd, and brought to a working facility with pens. Calves were separated from dams and housed in pens with at least a 1 pen separation from the cows. Cow 1-d BW were collected, and BCS were

recorded by 2 trained technicians. Jugular blood samples were collected into serum tubes (previously described) prior to oxytocin administration. Next 1.0 mL of oxytocin was administered into the jugular vein, and debris was wiped from teats. Cows were completely milked using a single-cow portable milking machine, followed by hand-milking to ensure complete milk removal. Cows were then housed in pens with access to water but no feed for 4 h.

The same milking protocol was performed 4 h (4.0 ± 0.1 h for both d 35 and 60 milk yields) after initial milking to determine 4-h milk yields. Milk weight and volume for each cow were recorded and subsamples were collected and stored at -20°C until composition analysis. The portable milking machine container and pitchers were rinsed thoroughly with water between each milking. Several cows were found to have a nonfunctional teat, but those cows were included in the data set because their yields were not outliers. Jugular blood samples were processed as previously described.

Cow 2-d BW were collected and BCS were recorded by 2 trained technicians when calves were weaned at 195.3 ± 8.3 d post-calving.

Tissue and Feed Mineral Analysis

All mineral analyses were performed at Novus International Inc. Hay, supplement, colostrum, and milk samples were analyzed for Cu, Zn, and Mn concentration by inductively coupled plasma (**ICP**) optical emission spectrometry with a cyclonic spray chamber and high solids gemcone nebulizer (PerkinElmer, Waltham, MA). Liver samples were analyzed for Cu, Zn, and Mn concentration by ICP mass spectrometry (Agilent 7500, Agilent Technologies, Santa Clara, CA). Samples were

digested using HNO_3 acid and heat (260°C). After acid digestion, an internal standard (0.2 ml of 250 ppm yttrium solution) was added, and all samples were brought up to a common volume using deionized water and mixed thoroughly. After cooling to room temperature, samples were centrifuged at 3,650 rpm for 15 min and filtered through a 0.2 µm nylon filter. A calibration curve was generated for each mineral. Samples were injected, normalized to the internal standard, and analyzed in triplicate. Analysis was acceptable if the tested value and theoretical value of the internal standard had a relative percent difference < 10%.

Circulating Metabolite Analysis

Gestational and 1 h post-calving cow serum was thawed at 4°C, then analyzed for urea N using a commercially available urea N kit (Urea Nitrogen Procedure Number 0580; Stanbio Laboratory, Boerne, TX) based on the diacetylmonoxime method, and for NEFA using a modified procedure of the NEFA C kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) based on the acyl-CoA synthetase-acyl-CoA oxidase method. Gestational and 1 h post-calving cow plasma was thawed at 4°C, then analyzed for glucose concentration using the Infinity glucose hexokinase commercially available kit (Fisher Diagnostics, Middletown, VA) based on the glucose-6-phosphate dehydrogenase method. For each assay, samples were analyzed in duplicate in 96-well polystyrene plates (Corning Inc., Corning, NY) read on a microplate reader (Biotek Synergy HT, Biotek Instruments Inc., Winooski, VT) at 520, 550, and 340 nm, for urea N, NEFA, and glucose, respectively. Pooled gestational cow serum was used as an internal control for urea N and NEFA assays, and pooled gestational cow plasma was used as an internal

control for glucose assays. The intraassay and interassay CV for urea N were 3.40% and 4.99%, respectively. The intraassay and interassay CV for NEFA were 2.97% and 5.22%, respectively. The intraassay and interassay CV for glucose were 3.29% and 1.07%, respectively.

Serum Oxidative Stress Markers

At Novus International Inc., gestational, 1 h post-calving, and lactational cow serum was thawed at 4°C and analyzed for thiobarbituric acid reactive substrates (**TBARS**) concentration using a commercially available TBARS assay kit (Cayman Chemical, Ann Arbor, MI) based on the reaction of malondialdehyde (a naturally occurring product of lipid peroxidation) and thiobarbituric acid to form malondialdehyde-thiobarbituric acid adduct in high temperature (90 to 100°C) and acidic conditions. Serum samples were analyzed for glutathione peroxidase (**GPX**), reduced glutathione (**GSH**), and oxidized glutathione (**GSSG**) concentration using enzyme linked immunosorbent assay (GSH-Px ELISA kit, GSH ELISA kit, and GSSG ELISA kit, MyBioSource, Inc., San Diego, CA). For GPX and GSSG analysis, samples were diluted 2x and 3x, respectively. Samples were analyzed in duplicate using a microplate spectrophotometer (Epoch 2, BioTek, Winooski, VA) at 532 nm for TBARS assay and 450 nm for GPX, GSH, and GSSG assay. Intraassay and interassay CV for TBARS, GPX, GSH, and GSSG were < 3.0% and < 2.7%, respectively.

Serum protein concentrations were used to report GPX, GSH, and GSSG concentrations relative to serum protein in order to ensure differences in these oxidative stress marker concentrations were not confounded by dilution of serum. Gestational, 1 h

post-calving, and lactational cow serum was thawed at 4°C and analyzed for protein concentration using commercially available Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, Rockford, IL) based on Bradford coomassie-binding, colorimetric method. Samples were analyzed in duplicate in 96-well polystyrene plates (Corning Inc.) on a microplate reader at 595 nm. Intraassay and interassay CV were 5.23% and 2.85%, respectively.

Hay and Supplement Nutrient Analysis and Intakes

All feed and refusal samples were first dried in a 55°C oven for a minimum of 48 h. Dried feed samples were ground through a 2 mm screen using a Thomas Wiley Mill (Thomas Scientific, Swedensboro, NJ), then ground through a 1 mm screen using a Cyclotec sample mill (Model 1093, Cyclotec Tecator, Höganäs, Sweden). Neutral detergent fiber and ADF were separately analyzed using an ANKOM Fiber Analyzer (Model 200, ANKOM Technology Corp., Fairport, NY). Nitrogen content of forages was determined by thermoconductivity using an Elementar Nitrogen Analyzer (Vario Macro Cube, Elementar Americas, Mt. Laurel, NJ) and used to calculate CP (N x 6.25). To determine total DM, samples were dried in a 105°C oven for 24 hours, then put into a 500°C oven for at least 4 h to determine ash content.

Colostrum and Milk Quality

Colostrum and milk were thawed at 4°C, and then analyzed for protein using a commercially available Coomassie (Bradford) protein assay kit (Thermo Scientific), for urea N using a commercially available QuantiChrom urea assay kit (Bioassay Systems,

Hayward, CA) based on an improved Jung method (Zawada et al., 2009), for lactose using a commercially available EnzyChrom lactose assay kit (Bioassay Systems) in which lactose is cleaved and galactose is produced to form a colored product, and for triglycerides using a commercially available Infinity Triglycerides kit (Thermo Scientific) based on the glycerol-3-phosphate oxidase method and modifications from Fossati and Prencipe (1982) and McGowan and others (1983).

Colostrum samples were diluted with a dilution factor of 1:100, 1:5, 1:150, and 1:75 for protein, urea N, lactose, and triglycerides, respectively. Milk samples were diluted with a dilution factor of 1:100, 1:10, 1:150, and 1:75 for protein, urea N, lactose, and triglycerides, respectively. Prior to dilution for triglycerides analysis, milk samples were sonicated using an ultrasonic processor (Sonics VC 505, power 500 W, Sonics and Materials Inc, Newtown CT) for 1 min at 40% intensity and diluted ≤ 1 h after sonication.

All samples were analyzed in duplicate in 96-well polystyrene plates (Corning Inc.) on a microplate reader (Biotek Synergy HT) at 595, 430, and 570 nm for protein, urea N, and lactose assays, respectively. For colostrum and milk triglycerides, plates were read at 500 and 660 nm and final results were calculated by subtracting the 660 nm reading from the 500 nm reading.

Pooled colostrum and milk samples were used as internal controls for colostrum and milk assays, respectively. The intraassay and interassay CV for colostral protein were 4.08% and 8.35%, respectively and for milk protein were 3.17% and 3.19%, respectively. The intraassay and interassay CV for colostral urea N were 2.37% and 2.61%, respectively and for milk urea N were 2.87% and 8.31%, respectively. The intraassay and interassay CV for colostral lactose were 2.80% and 6.70%, respectively and for milk

lactose were 2.22% and 3.78%, respectively. The intraassay CV for colostral triglycerides was 4.11%, and the intraassay and interassay CV for milk TG were 3.37% and 6.36%, respectively.

Colostrum samples were thawed at 4°C then analyzed for immunoglobulins (**Ig**) G, A, and M using an enzyme linked immunosorbent assay (Bovine IgG ELISA Quantitation Set, Bovine IgA ELISA Quantitation Set, and Bovine IgM ELISA Quantitation Set, Bethyl Laboratories, Inc., Montgomery, TX). Bovine reference serum was used to make a 7-point standard curve ranging from 7.8 to 500 ng/mL for IgG and 15.675 to 1000 ng/mL for IgA and IgM assays to generate a 4-parameter curve fit. Pooled colostrum was used as an internal control and placed on the top half and bottom half of the plate in order to control for plate location differences, likely driven by time spent on the plate. Samples were diluted with a dilution factor of 1:1,000,000 for IgG, and 1:30,000 for IgA and IgM, then analyzed in duplicate in 96-well polystyrene plates (Corning Inc.) on a microplate reader at 450 nm. Standard, sample, or internal control CV < 15% were considered acceptable. If a standard had a CV > 15% due to 1 value in the triplicate being an outlier, then that value was masked. Standard curves were considered acceptable if $R^2 > 0.990$. The intraassay and interassay CV for IgG were 3.57% and 2.38%, respectively, for IgA were 6.96% and 11.62%, respectively, and for IgM were 3.94% and 4.51% respectively.

Nutrient content of milk and colostrum and Ig content of colostrum were determined by multiplying the nutrient or Ig concentration by the weight or volume of sample collected. For colostrum samples, yield was from 1 rear quarter of initial colostrum, and for milk samples, yield was from all quarters over 4 h.

Statistical Analysis

Three cows were removed from the study due to late gestational abortion, and 1 cow was removed from the study because she was later bred than all other natural service cows, resulting in 44 cows that calved. After post-calving liver biopsies, 1 cow was removed due to temperament resulting in 43 cows that were followed through weaning. One cow was not included in 4-h milk yields due to poor udder conformation but was included for all other pre-weaning sampling. Final sample numbers are provided in Table 2.3. Measures with fewer sample numbers are due to cows calving overnight which allowed for the calf to suckle or cow temperament, that prevented personnel from safely collecting data.

Data were analyzed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) with treatment and breeding group as fixed effects and cow as experimental unit. Initial cow liver mineral concentration was used a covariate for post-calving cow liver mineral concentration. For circulating metabolites and oxidative stress markers over time, treatment, sampling day, and their interaction were also considered fixed effects. Day was considered a repeated effect for these measures, using the best-fit covariate structure (chosen from compound symmetry, heterogeneous compound symmetry, autoregressive, and heterogenous autoregressive). Circulating metabolites at 1 h post-calving were not included as part of gestation due to inconsistencies in timing compared with study initiation. Main effects of sampling time will not be discussed due to differences in hay fed throughout the treatment period and because this was not part of the objective. Means were separated if the treatment or treatment x day $P \leq 0.10$. Means

were considered different if $P \leq 0.05$ and were considered tendencies if $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Performance and Metabolic Status

Cow BW and BCS at study initiation were not different ($P \geq 0.87$) among treatments (Table 2.4). Throughout late gestation, Cu, Zn, and Mn source and inclusion did not affect ($P \geq 0.47$) cow BW or BCS. This was expected because, per study design, energy and protein intakes were not different ($P \geq 0.54$) among treatments during late gestation (data not shown). In similar studies, gestational BW and BCS were not altered by source and concentration of Cu supplementation (Muehlenbein et al., 2001) or Cu, Zn, Mn, and Co supplementation (Ahola et al., 2004) in late gestational beef cows where the basal diet was marginally deficient in the trace mineral(s) being studied. Beef cow pre-calving BW and BCS were not affected by supranutritional Cu, Zn, Mn, and Co supplementation in late gestation (Marques et al., 2016). Cows offered free choice mineral that contained Zn and Mn methionine in the last third of gestation had greater BW gain than cows offered no supplemental Zn and Mn or Zn and Mn oxides (Spears and Kegley, 1991). Typically severe trace mineral deficiencies (Graham, 1991) or toxicities (NASEM, 2016) cause impaired maintenance, growth, and production in beef cattle, but dietary trace minerals provided to cows in the current study and in previous studies discussed did not reach those extremes.

Initial cow plasma glucose, serum urea N, and serum NEFA were not different ($P \geq 0.11$) among treatments (Table 2.5). Gestational treatment did not affect ($P \geq 0.35$) plasma glucose, serum urea N, or serum NEFA during late gestation or at 1 h post-

calving. This indicates that treatment did not affect cow metabolic status, which further supports the lack of differences in gestational BW and BCS. There was, however, a main effect ($P \leq 0.02$) of day for all gestational metabolites (data not shown). This could be explained by differences in the basal diet, as quality varied among the 3 hays fed throughout the treatment period (Table 2.1). This could also be due to physiological changes in metabolism as gestation progresses and exponential fetal growth occurs (NASEM, 2016) resulting in effects such as increased hepatic gluconeogenesis and adipose tissue mobilization (Bell, 1995).

Gestational treatment did not affect ($P \geq 0.13$) cow BW or BCS during lactation or at weaning (Table 2.4). This was expected as late gestational cow performance was not affected during the treatment period and cows were on a similar plane of nutrition from treatment termination to weaning.

Mineral Status

Initial cow liver Cu, Zn, and Mn concentrations were not different ($P \geq 0.41$) among treatments (Table 2.6). Late gestational Cu, Zn, and Mn source and inclusion affected ($P = 0.03$) post-calving cow liver Cu concentrations, where cows fed CTM had greater ($P \leq 0.01$) liver Cu than cows fed CON and RR, and tended ($P = 0.07$) to have greater liver Cu than ITM fed cows. Treatment did not affect ($P \geq 0.28$) cow liver Zn or Mn concentrations post-calving.

In a similar study, mature beef cows provided ad libitum access to inorganic or organic (proteinates) Cu, Zn, and Mn during late gestation and lactation had greater liver Cu, Zn, and Mn at d 110 of lactation than cows that did not receive supplement (Ahola et

al., 2004). Also in that study, cows fed the organic source of Cu, Zn, and Mn had greater liver Cu than cows fed the inorganic source, indicating the organic source had greater bioavailability as average daily mineral disappearance was similar among treatments (Ahola et al., 2004). Marques and others (2016) reported that beef cows supplemented inorganic or organic sources (amino acid complexes) of Cu, Zn, and Mn and Co glucoheptonate during late gestation had greater pre-calving liver Co, Cu, and Zn in supplemented cows compared with control cows. Dairy cows provided daily boluses of organic sources of Cu, Zn, Mn (amino acid-complexed trace minerals) and Co (Co glucoheptonate) during late gestation had greater liver Cu and Mn at d 10 post-calving compared with cows provided inorganic sources, but there was no difference between liver Zn concentrations (Osorio et al., 2016). Although duration of supplementation, source of organic trace minerals, breed type, and sampling times differed among these studies, these data demonstrate that liver Cu, Zn, and Mn can be affected by dietary trace minerals.

Liver is the main storage organ for copper (McDowell, 1992), making liver Cu concentration the best indicator of Cu status (Herdt and Hoff, 2011). Therefore, our data suggest that MMHAC Cu was more bioavailable to the cow than Cu sulfate. Initial CON, ITM, and CTM treatment means for liver Cu concentrations indicate adequate Cu status (125 to 600 mg/kg [DM]), whereas the treatment mean for RR treatment indicates marginally deficient Cu status (33 to 125 mg/kg [DM]; Kincaid, 2000). For postpartum liver Cu treatment means, ITM and CTM indicate adequate Cu status and CON and RR indicate marginal Cu deficiency. These data support that liver Cu is representative of Cu status, as cows fed CON had adequate liver Cu on average at study initiation but were

marginally deficient after consuming a Cu-deficient diet throughout late gestation. This also suggests that late gestational dietary Cu recommendations may not be accurate because RR-fed cows were marginally Cu-deficient at study initiation and remained in this state after consuming a Cu-adequate diet.

Both initial and post-calving liver Zn treatment means indicated adequate Zn status, and both initial and post-calving liver Mn treatment means indicated marginally deficient Mn status (Kincaid, 2000). The basal diet provided adequate Mn concentrations for the majority of the treatment period, so similar liver Mn concentrations were anticipated. However, the discrepancy between dietary Mn concentrations and liver Mn status could indicate that either hepatic Mn reference ranges or dietary Mn recommendations are not accurate for gestating beef cows. Liver Zn and Mn concentrations are less responsive to changes in dietary trace minerals than liver Cu (Kincaid, 2000; Herdt and Hoff, 2011) which may explain the lack of Zn status differences in the current study. Two-year-old beef cows provided supranutritional Cu, Zn, Mn, and Co from calving until breeding had similar liver Zn and Mn concentrations (Olson et al., 1994). In studies previously discussed, liver Zn was not affected by trace mineral treatment in the last 30 d of gestation in dairy cows (Osorio et al., 2016), but was affected when the treatment was the last ≥ 80 d of gestation in beef cows (Ahola et al., 2004; Marques et al., 2016). These data could indicate length of treatment period or breed type could be a factor in liver Zn result differences among these studies and the current study.

Antioxidant Status

Cow serum TBARS, GPX, GSH, GSSG, and GSH/GSSG ratio were not different ($P \geq 0.11$) among treatments at study initiation (Table 2.7). Gestational trace mineral treatment tended ($P = 0.10$) to affect gestational serum TBARS, where CTM-fed cows had less ($P = 0.01$) circulating TBARS than CON-fed cows. Late gestational Cu, Zn, and Mn supplementation did not affect ($P \geq 0.10$) cow serum GPX, GSH, GSSG, or GSH/GSSG ratio throughout the treatment period. During lactation, serum TBARS tended to be affected ($P = 0.09$) by the interaction of treatment x day, where cows fed RR during late gestation had greater ($P = 0.04$) serum TBARS at d 35 of lactation than CON cows (Table 2.7). At d 60 of lactation, cows fed CTM during late gestation had greater ($P \leq 0.05$) serum TBARS than CON and ITM fed cows and tended ($P = 0.09$) to have greater serum TBARS than RR fed cows. Despite this, cow serum GPX, GSH, GSSG, and GSH/GSSG ratio during lactation were not affected by gestational treatment ($P \geq 0.20$).

Thiobarbituric acid reactive substances are products of membrane phospholipid peroxidation by free radicals and therefore a marker of oxidative stress (Al-Qudah, 2011). Superoxide anion radical is one of the most common types of free radicals (Phaniendra et al., 2015) and is converted to hydrogen peroxide by superoxide dismutase, an enzyme containing Cu, Zn, or Mn depending on the cellular compartment (Fukai and Ushio-Fukai, 2011). Glutathione peroxidase, a selenoenzyme (Flohe et al., 1973), is responsible for converting hydrogen peroxide into water while converting GSH to GSSG (Weydert and Cullen, 2009). Thus, a reduction in GSH/GSSG ratio indicates greater oxidative stress. The discrepancies between TBARS and GSH/GSSG ratio results could be due to the presence of free radicals other than superoxide anion causing membrane damage.

These results suggest that trace mineral supplementation may reduce oxidative stress in gestational beef cows. However, lactational results suggest that feeding beef cows a chelated source of Cu, Zn, and Mn during gestation increased oxidative stress during lactation. In dairy cows provided daily boluses that contained inorganic or organic Cu, Zn, Mn, and Co from 30 d prepartum to 30 d postpartum, blood reactive oxygen metabolites and total antioxidant capacity were not affected by trace mineral supplementation (Osorio et al., 2016). Yasui and others (2014) reported transition dairy cows fed inorganic Cu, Zn, and Mn had less total antioxidant capacity and greater TBARS compared with cows fed a more bioavailable source, hydroxy Cu, Zn, and Mn. These previous studies have more consistent oxidative stress data than the current study which could be due to total antioxidant capacity encompassing more antioxidant enzymes than only GPX (Ghiselli et al., 2000). Differences among studies could also be due to breed type differences because diet and general management differ, and metabolic demands are greater in dairy cows than beef cows. In the current study, greater circulating TBARS in cows fed CTM during lactation was unexpected because CTM cows had improved Cu status compared with all other treatments post-calving. However, lactational data were collected after treatment termination when cows had ad libitum access to an inorganic trace mineral supplement. That could lead to variable intakes of trace minerals and could also explain the inconsistencies in these results. These data may also indicate that antioxidant capacity was not altered but free radical concentrations were inconsistent which could lead to variable serum TBARS results.

Colostrum Yield and Quality

Late gestational inclusion and source of Cu, Zn, and Mn tended to affect ($P \leq 0.10$) pre-suckling, single rear-quarter colostrum volume and weight. Cows fed CTM had greater ($P \leq 0.05$) volume than ITM and RR cows but were not different ($P = 0.18$) than CON cows (Table 2.8). Similarly, weight of colostrum from CTM cow was greater ($P = 0.02$) than CON cows and tended ($P = 0.06$) to be greater than ITM cows. These results were unforeseen because the cows that were fed a marginally Cu and Zn deficient diet throughout late gestation did not have less colostrum yield. Also, typical factors that affect colostrum yield, age, BCS, and intakes of energy and protein, were similar among treatments (Table 2.4).

These results were not supported by previous data from late gestational trace mineral supplementation studies in ruminants. Karkoodi and others (2012) also observed that source (different proportions of inorganic and organic to meet recommendations) of Cu, Zn, Mn and Se supplementation in the last 3 wk of pregnancy did not alter pre-suckling colostrum yield in dairy cows. Colostrogenesis begins several weeks prior to calving (Barrington et al., 2001), therefore treatments in the previous study may not have been implemented early enough in gestation to detect colostrum yield differences. The colostrum yield tended to be different among treatments in the current study, which indicates these differences could also be due to inability to allocate cows to treatments by colostrum yield potential, as it was unknown.

Although treatment did not affect ($P \geq 0.17$) colostrum protein or urea N concentration or content, lactose concentration and content were affected ($P = 0.04$) by treatment (Table 2.8). Colostrum lactose concentration was greater ($P = 0.04$) in CTM cows than ITM and RR but was not different ($P = 0.28$) than CON cows. Control cows

tended ($P = 0.06$) to have greater colostrum lactose concentrations than RR but were not different ($P = 0.25$) than ITM cows. Similarly, total colostrum lactose was greater ($P \leq 0.03$) in CTM cows than ITM and RR but was not different ($P = 0.13$) than CON. Source and inclusion of Cu, Zn, and Mn in late gestation also tended ($P = 0.09$) to affect colostrum triglyceride concentration where RR cows had greater ($P \leq 0.04$) triglycerides concentration compared with CON and CTM cows but were not different ($P = 0.36$) than ITM cows. Gestational trace mineral treatment did not affect ($P = 0.50$) colostrum triglycerides content.

Differences in macronutrients were not expected as gestational metabolic status was not affected by late gestational trace mineral treatment. However, colostrum lactose differences may explain differences in colostrum yield because lactose accounts for half of the osmotic pressure of milk. As a result, greater lactose concentration causes a greater influx of water into mammary secretions which increases volume (McGrath et al., 2016). Because total colostrum triglycerides were not different among treatments, the colostrum triglycerides concentration results could be driven by RR cows yielding less colostrum than CTM cows, making the triglycerides more concentrated. However, that does not explain colostrum triglycerides concentration differences between RR and CON cows because colostrum yields were not different between those 2 treatments and this did not occur for any other colostrum macronutrient.

Varying proportions of inorganic and organic trace mineral supplement sources fed to meet recommendations during late gestation did not affect pre-suckling colostrum macronutrient composition in either dairy (Karkoodi et al., 2012) or beef cows (Price et al., 2017). However, Kinal and others (2007) reported greater colostrum lactose

concentrations in dairy cows fed inorganic trace mineral (Cu, Zn, and Mn) compared with cows fed a diet including just organic (chelates) or both organic and inorganic Cu, Zn, and Mn. They also reported greater colostrum crude fat concentration in cows supplemented both inorganic and organic or just organic trace mineral compared with inorganic. These results differ from the current study where cows provided both inorganic and organic Cu, Zn, and Mn (RR) had greater colostrum triglyceride concentration than cows provided organic trace minerals (CTM) but were not different than cows provided ITM. Trace mineral supplementation's role in colostrum lactose and triglycerides synthesis is unclear and further investigation is needed.

Inclusion and source of Cu, Zn, and Mn in the diet did not affect ($P \geq 0.25$) colostrum Cu, Zn, and Mn concentration or content (Table 2.8). Trace mineral sequestration by the mammary gland and secretion into the alveoli lumen is highly regulated; therefore major dietary changes are required to alter Cu, Zn, and Mn concentrations in milk (Lönnerdal et al., 1981; Kelleher and Lönnerdal, 2005). This regulation likely exists in colostrogenesis too.

Dairy cows fed all inorganic or 20% or 30% organic (bound to amino acids or peptides) Cu, Zn, and Mn to meet recommendations during the last 6 wk of gestation had greater colostrum Zn concentrations as inclusion of organic trace minerals in the diet increased (Kinal et al., 2007). In the previous study, inclusion of organic trace minerals in the diet resulted in greater colostrum Cu concentrations. Conversely, dairy cows supplemented organic (Cu, Zn, and Mn amino acid complexes and Co glucoheptonate) trace minerals for the last 21 d of gestation had less colostral Zn concentrations compared with cows supplemented inorganic trace minerals (Kincaid and Socha, 2004). These

results could differ from the current study because dairy cows have greater colostrum yields which makes their nutritional demand greater than beef cows, therefore colostrum trace mineral may be more sensitive to changes in the diet.

In beef cows, neither inorganic nor organic (metal amino acid complex) Cu supplementation to a marginally Cu deficient diet during the last 45 d of gestation improved pre-suckling colostrum Cu concentrations (Muehlenbein et al., 2001). Price and others (2017) reported beef cows supplemented organic trace minerals (Se-yeast and Cu, Zn, Mn, and Co proteinates) in late gestation had greater colostrum Zn concentrations compared with cows supplemented inorganic trace minerals but colostrum Cu was not detectable (<1 mg/kg) in all samples. Differences in colostrum trace mineral results between the previous studies and the current study could be due to different mineral statuses of cows at study initiation. That would dictate the size of endogenous trace mineral stores cows have to mobilize in order to deliver similar amounts of trace minerals to the mammary gland.

Colostral IgG, IgA, and IgM concentration and content were not affected ($P \geq 0.21$) by gestational treatment (Table 2.8). In a study previously discussed, late gestational Cu supplementation did not affect colostrum IgG (the main Ig isoform) concentration in beef cows (Muehlenbein et al., 2001). Beef cows provided organic Cu, Zn, Mn, and Co supplement during late gestation had greater colostrum IgM concentrations compared with cows provided inorganic trace mineral supplement (Price et al., 2017). Kincaid and Socha (2004) reported supplementing dairy cows with organic Cu, Zn, Mn and Co the last 21 d of gestation resulted in greater colostrum IgG concentration compared with cows supplemented inorganic trace minerals. However,

both Jacometo et al. (2015) and Karkoodi et al. (2012) observed that source of Cu, Zn, Mn, and Co, or Cu, Zn, Mn, and Se, respectively, provided during the late gestation did not alter colostrum IgG concentrations. This variation in results indicates that the relationship between gestational trace mineral supply and colostrum Ig concentration is complex and further investigation is needed.

Milk Yield and Quality

Milk yields (mass and volume) at d 35 and 60 of lactation were not affected ($P \geq 0.62$) by gestational treatment (Table 2.9). Trace mineral supplementation of dairy cows starting in late gestation and continuing into lactation resulted in variable milk yield treatment differences during the treatment period (Yasui et al., 2014; Osorio et al., 2016) in previous studies. However, trace mineral treatments were terminated 11 d post-calving in the current study; therefore, similar milk yields at d 35 and 60 of lactation were expected among treatments.

Milk lactose, triglycerides, protein, and urea N concentration and content (Table 2.9) at d 35 and 60 of lactation were not affected ($P \geq 0.15$) by treatment. Milk lactose data support the lack of milk yield differences among treatments, as lactose drives yield (McGrath et al., 2016). Supplementing dairy cows different sources of trace minerals during late gestation and lactation resulted in variable milk macronutrient yield differences in the first 30 d of lactation (Osorio et al., 2016) and no milk macronutrient difference at d 84 of lactation (Yasui et al., 2014). However, trace mineral treatments were terminated approximately 20 d prior to the first milk sampling in the current study. Therefore similar macronutrient concentrations and content were expected because

gestational metabolic status was not affected by treatment, and cows were managed similarly.

Source and inclusion of Cu, Zn, and Mn in late gestation tended to affect (P = 0.07) milk Zn content at d 60 of lactation, where cows fed CTM had greater ($P \leq 0.04$) total milk Zn than ITM and RR cows but were not different ($P = 0.34$) from cows fed CON (Table 2.9). Milk Zn concentrations at d 60, however, were not affected ($P = 0.18$) by late gestational treatment. Gestational trace mineral treatment did not affect ($P \geq 0.18$) milk concentration or content of Cu, Zn, or Mn at d 35 of lactation or milk Cu and Mn at d 60 of lactation.

Milk Zn treatment differences at d 60 but not d 35 of lactation were not expected and are not supported by previous data. Dairy cows provided a greater proportion of organic vs. inorganic trace mineral supplement during late gestation and lactation had greater milk Zn concentrations in the first month but not in the second and third month of lactation (Kinal et al., 2007). Price and others (2017) reported beef cows supplemented inorganic or organic sources of trace mineral during late gestation and lactation had similar milk trace mineral concentrations at d 30 of lactation. Similarly, supplementing dairy cows an inorganic or organic sources of Cu, Zn, Mn, and Se from late gestation to 1 wk post-calving resulted in similar milk trace mineral concentrations at d 20 of lactation (Krys et al., 2009). There are limited data available reporting milk micronutrients long after trace mineral treatment termination, therefore it is difficult to determine if the total 60 d milk Zn data in the current study were an anomaly or biological change. In the current study, 60 d milk yield and Zn concentrations were not different. Hence, it may be assumed that numerical differences in milk yield and Zn concentration led to the

differences in 60 d milk Zn content, which highlights the importance of collecting milk yield data.

Previous data discussed supports the lack of Cu and Mn milk differences in the current study. This is likely due to milk samplings occurring long after treatment termination and high regulation of trace mineral homeostasis in the mammary gland (Lönnerdal et al., 1981; Kelleher and Lönnerdal, 2005).

CONCLUSION

In summary, inclusion of chelated Cu, Zn, and Mn to supply 133% of recommendations in the diet of late gestation beef cows resulted in improved Cu status and greater colostrum yield and lactose. Marginal Cu and Zn deficiency during late gestation resulted in greater circulating oxidative stress markers during gestation but did not during lactation. Mineral data differences did not consistently follow amount or bioavailability of Cu, Zn, and Mn in the diet, which suggests that trace mineral recommendations for late gestational beef cows are inaccurate. Further investigation into how gestation and lactation disrupt dam trace mineral homeostasis is needed to better understand recommendations and benefits of improved trace mineral status. Because the fetal and neonatal calf completely rely on the dam for supply of trace minerals, they could potentially benefit from improved dam trace mineral status.

Table 2.1. Nutrient composition and feeding duration of hays fed during treatment period

Variable	Hay 1	Hay 2	Hay 3[1]
Days fed to AI breeding group[2]	37	32	18 to 53
Days fed to NS breeding group	16	32	42 to 51
DM, %	87.0	82.1	86.9
		---DM basis---	
CP, %	11.9	14.6	10.6
NDF, %	67.2	57.1	62.4
ADF, %	43.7	43.9	40.9
NE_m, Mcal/kg[3]	0.95	0.94	1.04
Cu, mg/kg	3.59	7.43	6.63
Zn, mg/kg	6.05	14.57	7.90
Mn, mg/kg	46.0	32.7	73.4

[1]Hay 3 feeding duration was dependent on when cows calved, therefore a range is presented.
[2]Breeding groups: AI bred cows, NS = cows bred by natural service.
[3]Calculated value: 2.392 – 0.033*(% ADF)

Table 2.2. Dry matter intakes and dietary trace minerals provided during the treatment period

	Treatment[1]			
Variable	CON	ITM	CTM	RR
DMI, kg				
d 0 to 28 of study	10.7	11.4	10.9	10.9
d 29 to 56 of study	12.2	12.7	12.2	12.2
d 57 to 74 of study	12.4	13.0	12.2	12.5
Calving pens[2]	12.9	13.5	12.9	13.1
Dietary Cu, mg/kg DM				
Target[3]	—	13.0	13.0	10.0
d 0 to 28 of study	4.5	12.0	15.2	9.7
d 29 to 56 of study	6.7	13.8	15.9	11.1
d 57 to 74 of study	7.0	14.7	16.7	12.4
Calving pens	8.8	17.7	19.6	15.5
Dietary Zn, mg/kg DM				
Target	—	40.0	40.0	30.0
d 0 to 28 of study	9.8	30.5	32.1	23.0
d 29 to 56 of study	15.2	37.0	39.4	29.7
d 57 to 74 of study	16.9	40.6	41.7	33.7
Calving pens	13.8	36.8	39.7	31.7
Dietary Mn, mg/kg DM				
Target	—	53.3	53.3	40.0
d 0 to 28 of study	43.2	60.3	64.7	51.3
d 29 to 56 of study	36.2	55.5	61.7	45.0
d 57 to 74 of study	47.4	61.1	65.6	56.8
Calving pens	65.9	65.5	64.1	64.8

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]Cows were housed in dry lot calving pens by treatment starting d 75 of study (average) until 11.0 ± 3.2 d post-calving, offered ad libitum hay, and pen-fed supplement. Intakes were calculated using estimated hay DMI (1.2% BW NDF intake) and pen-fed supplement DMI.

[3]Target concentrations of diets based on NASEM (2016) gestating cow Cu, Zn, and Mn recommendations and treatment goals.

Table 2.3. Final animal and sample numbers included in analysis

	Treatment[1]			
Variable	CON	ITM	CTM	RR
Cow-calf pairs	11	11	10	12
Blood samples				
Gestational	11	11	10	12
1 h post-calving	11	9	7	10
Lactational	11	11	9	12
Liver minerals				
Pre-study	11	11	10	12
Post-calving	11	11	9	12
Colostrum	10	9	7	9
35 d milk	11	11	9	11
60 d milk	11	10	9	10

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements.

Table 2.4. Effect of Cu, Zn, and Mn source and inclusion during late gestation on cow BW, BCS, and age at calving

	Treatment[1]					
Variable	CON	ITM	CTM	RR	SEM	*P*-value
BW, kg						
Gestational						
Initial[2]	643	653	643	653	27	0.98
d 28 of study	659	670	655	665	28	0.98
d 56 of study	680	700	678	691	29	0.93
Pre-calving[3]	692	722	693	709	30	0.85
Lactational						
d 35 post-calving	618	659	635	637	30	0.74
d 60 post-calving	629	673	635	650	29	0.62
Weaning[4]	573	594	590	582	26	0.92
BCS[5]						
Gestational						
Initial	5.2	5.4	5.2	5.3	0.2	0.87
d 28 of study	5.2	5.4	5.2	5.4	0.1	0.47
d 56 of study	5.4	5.6	5.3	5.5	0.2	0.53
Lactational						
d 35 post-calving	5.1	5.7	5.2	5.4	0.2	0.13
d 60 post-calving	5.1	5.5	5.1	5.4	0.2	0.41
Weaning	4.7	5.0	4.7	4.8	0.2	0.60
Age at calving, yr	4.0	4.4	4.2	4.3	0.4	0.88

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[2]Study initiation = 91.2 ± 6.2 d pre-calving.
[3]Pre-calving = 17.5 ± 8.0 d pre-calving, d 75 of study (average).
[4]Weaning = 195.3 ± 8.3 d post-calving.
[5]BCS assigned based on a 9 point scale (1 = emaciated; 9 = obese).

Table 2.5. Effects of Cu, Zn, and Mn source and inclusion during late gestation on cow plasma glucose, serum urea N, and serum NEFA during gestation and 1 h post-calving

	Treatment[1]					*P*-value		
Variable	CON	ITM	CTM	RR	SEM	Trt	Day	Trt x Day
Gestational								
Glucose, mg/dL						0.61	0.02	0.82
Initial[2]	70.9	69.7	73.3	69.3	1.8			
d 28 of study	65.8	66.6	68.2	67.4	1.8			
d 56 of study	70.3	67.4	67.2	68.6	1.9			
Pre-calving[3]	70.1	68.2	68.7	67.9	1.9			
Urea N, mg/dL						0.97	0.02	0.95
Initial	5.72	5.49	5.42	5.82	0.56			
d 28 of study	6.29	6.10	6.25	6.20	0.30			
d 56 of study	6.88	6.80	7.27	6.48	0.43			
Pre-calving	6.52	6.59	6.43	6.57	0.24			
NEFA, mEq/L						0.54	<0.001	0.88
Initial	570	475	548	601	88			
d 28 of study	392	380	338	334	50			
d 56 of study	448	346	361	383	45			
Pre-calving	446	357	445	417	67			
1 h post-calving								
Glucose, mg/dL	101.8	95.9	91.7	95.9	9.5	0.85		
Urea N, mg/dL	6.03	6.44	5.68	6.27	0.35	0.35		
NEFA, mEq/L	775	679	742	853	91	0.40		

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[2]Initiation = 91.2 ± 6.2 d pre-calving.
[3]Pre-calving = 17.5 ± 8.0 d pre-calving, d 75 of study (average).

Table 2.6. Effects of Cu, Zn, and Mn source and inclusion during late gestation on cow liver Cu, Zn, and Mn concentration

	Treatment[1]					
Variable	CON	ITM	CTM	RR	SEM	*P*-value
Cu, mg/kg DM						
Initial[2]	143	144	147	101	25	0.44
Post-calving[3]	100[b]	131[b]	214[a]	88[b]	33	0.03
Zn, mg/kg DM						
Initial	141	146	123	115	16	0.41
Post-calving	151	112	149	136	20	0.43
Mn, mg/kg DM						
Initial	8.93	8.73	8.94	7.53	0.78	0.45
Post-calving	9.70	7.94	9.83	8.45	0.90	0.28

[a,b]Indicates treatment means differ ($P < 0.10$)

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]Initial = 94.2 ± 5.5 d pre-calving; prior to gestational treatment initiation.

[3]Post-calving = 11.0 ± 3.2 d post-calving.

Table 2.7. Effects of Cu, Zn, and Mn source and inclusion during late gestation on cow serum thiobarbituric acid reactive substances (TBARS), glutathione peroxidase (GPX), reduced (GSH) and oxidized glutathione (GSSG), and GSH/GSSG ratio

	Treatment[1]					*P*-value		
Variable	CON	ITM	CTM	RR	SEM	Trt	Day	Trt x Day
Gestational								
TBARS[2], μM	7.20[a]	6.87[ab]	6.48[b]	6.82[ab]	0.20	0.10	<0.001	0.93
Initial[3]	9.21	8.97	8.48	8.74	0.52			
d 28 of study	6.74	6.34	6.27	7.03	0.39			
d 56 of study	5.92	5.40	5.04	5.64	0.24			
Pre-calving[4]	6.90	6.76	6.12	5.88	0.58			
GPX, mg/g protein						0.64	0.04	0.13
Initial	2.74	2.51	2.64	2.78	0.16			
d 28 of study	2.93	2.62	2.84	2.82	0.14			
d 56 of study	2.68	2.54	2.76	2.54	0.16			
Pre-calving	2.69	2.71	2.91	2.37	0.20			
GSH, μg/g protein						0.76	0.01	0.48
Initial	94.4	124.9	66.6	68.4	27.6			
d 28 of study	62.4	75.7	51.4	60.5	13.9			
d 56 of study	68.0	75.2	55.7	62.6	17.6			
Pre-calving	67.6	62.1	77.5	75.9	17.4			
GSSG, ng/g protein						0.29	<0.001	0.87
Initial	181	149	175	160	17			
d 28 of study	205	183	199	179	15			
d 56 of study	140	112	153	108	20			
Pre-calving	128	104	141	90	21			
GSH/GSSG ratio[5]						0.77	<0.001	0.48
Initial	606	888	466	530	190			
d 28 of study	366	433	308	451	109			
d 56 of study	644	854	496	857	231			
Pre-calving	869	817	809	1,123	339			
1 h post-calving								
TBARS, μM	8.09	6.87	7.45	7.26	0.83	0.62		
GPX, mg/g protein	2.32	2.23	2.56	2.24	0.28	0.78		
GSH, μg/g protein	38.1	22.1	26.5	32.7	12.3	0.69		
GSSG, ng/g protein	162	120	184	143	25	0.24		
GSH/GSSG ratio	300	218	186	239	115	0.85		
Lactational								
TBARS, μM						0.26	0.45	0.09
d 35 post-calving	5.62[b]	6.05[ab]	5.89[ab]	6.68[a]	0.41			
d 60 post-calving	5.53[b]	5.56[b]	6.69[a]	5.74[b]	0.42			
GPX, mg/g protein						0.40	<0.001	0.34
d 35 post-calving	2.51	1.95	2.38	2.19	0.25			
d 60 post-calving	1.44	1.19	1.57	1.30	0.21			

GSH, µg/g protein						0.20	0.13	0.65
d 35 post-calving	42.4	24.9	23.1	18.1	9.4			
d 60 post-calving	40.0	34.1	33.8	25.1	7.8			
GSSG, ng/g protein						0.28	<0.001	0.91
d 35 post-calving	194	156	199	165	22			
d 60 post-calving	155	119	150	116	22			
GSH/GSSG ratio						0.73	<0.001	0.64
d 35 post-calving	264	166	186	124	63			
d 60 post-calving	338	368	328	280	99			

[a,b]Indicates treatment means differ ($P < 0.10$)

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]Treatment means listed above interactive means.

[3]Initiation = 91.2 ± 6.2 d pre-calving.

[4]Pre-calving = 17.5 ± 8.0 d pre-calving, d 75 of study (average).

[5]Ratio of ng GSH to ng GSSG.

Table 2.8. Effects of Cu, Zn, and Mn source and inclusion during late gestation on colostrum yield and composition

	Treatment[1]					
Variable	CON	ITM	CTM	RR	SEM	*P*-value
Colostrum weight[2], g	1,135[ab]	910[b]	1,552[a]	757[b]	259	0.10
Colostrum volume, mL	1,065[ab]	834[b]	1,481[a]	707[b]	247	0.09
Macronutrients						
Protein, %	12.2	14.3	10.9	13.9	1.3	0.17
Urea N, mg/dL	4.43	4.58	4.16	4.41	0.33	0.79
Lactose, %	3.27[ab]	2.83[b]	3.71[a]	2.54[b]	0.33	0.04
Triglycerides, g/dL	4.21[b]	5.51[ab]	4.24[b]	6.41[a]	0.83	0.09
Protein total, g	127	120	128	93	20	0.42
Urea N total, mg	50.2	40.2	66.4	32.9	12.4	0.18
Lactose total, g	37.4[ab]	27.5[b]	55.8[a]	21.0[b]	9.6	0.04
Triglycerides total, g	42.3	39.3	55.7	36.7	10.3	0.50
Micronutrients						
Cu, µg/kg	168	212	190	169	27	0.46
Zn, mg/kg	18.0	19.0	17.4	19.0	2.7	0.95
Mn, µg/kg	41.5	69.7	62.8	48.8	16.8	0.47
Cu total, µg	204	177	320	146	75	0.31
Zn total, mg	16.7	15.1	18.2	12.6	3.5	0.61
Mn total, µg	58.6	51.1	112.7	44.0	28.7	0.25
Immunoglobulins (Ig)						
IgG, g/L	173	193	124	190	23	0.21
IgA, g/L	8.41	11.72	7.51	10.81	2.74	0.56
IgM, g/L	7.52	10.07	8.25	9.84	1.87	0.59
IgG total, g	153	157	146	122	21	0.63
IgA total, g	7.06	8.82	6.48	6.89	1.99	0.79
IgM total, g	7.23	8.09	7.42	5.80	1.51	0.63

[a,b]Indicates treatment means differ ($P \leq 0.10$)

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]A single, rear-quarter of colostrum was completely milked pre-suckling (57.5 ± 17.5 min post-calving).

Table 2.9. Effects of Cu, Zn, and Mn source and inclusion during late gestation on 4-h milk yield[1] and nutrient composition

	Treatment[2]					
Variable	CON	ITM	CTM	RR	SEM	*P*-value
d 35 of lactation						
Milk weight, g	1,648	1,603	1,745	1,702	129	0.84
Milk volume, mL	1,710	1,609	1,748	1,706	137	0.87
Macronutrients						
Protein, %	2.27	2.37	2.25	2.31	0.08	0.68
Urea N, mg/dL	17.4	17.0	15.1	16.2	1.1	0.36
Lactose, %	6.39	6.61	6.40	6.71	0.24	0.66
Triglycerides, g/dL	4.38	4.63	4.43	4.63	0.37	0.92
Protein total, g	37.5	37.5	39.3	39.3	3.1	0.94
Urea N total, mg	302	276	267	279	32	0.85
Lactose total, g	104	105	111	114	8	0.74
Triglycerides total, g	76.6	75.7	77.9	76.2	10.7	0.99
Micronutrients						
Cu, µg/kg	77.3	76.0	70.7	141.5	32.5	0.26
Zn, mg/kg	2.91	2.77	2.88	3.07	0.25	0.81
Mn, µg/kg	49.5	38.7	39.6	87.8	22.3	0.26
Cu total, µg	128	122	130	252	63	0.29
Zn total, mg	4.73	4.47	4.97	5.40	0.66	0.70
Mn total, µg	85.6	66.8	75.2	157.3	43.3	0.32
d 60 of lactation						
Milk weight, g	1,656	1,553	1,635	1,527	109	0.62
Milk volume, mL	1,668	1,558	1,635	1,534	112	0.62
Macronutrients						
Protein, %	2.17	2.25	2.15	2.24	0.09	0.76
Urea N, mg/dL	15.2	12.8	14.6	14.2	0.9	0.23
Lactose, %	6.13	6.27	6.26	6.11	0.23	0.93
Triglycerides, g/dL	3.68	4.00	3.59	3.90	0.30	0.69
Protein total, g	36.3	35.1	35.9	34.3	2.9	0.92
Urea N total, mg	249	194	220	212	24	0.15
Lactose total, g	101.2	95.8	101.8	93.4	7.8	0.70
Triglycerides total, g	62.0	62.5	60.6	60.8	7.5	0.99
Micronutrients						
Cu, µg/kg	84.2	75.2	89.1	68.4	12.4	0.59
Zn, mg/kg	2.70	2.35	2.91	2.47	0.21	0.18
Mn, µg/kg	42.4	47.2	47.8	37.8	6.9	0.65
Cu total, µg	144	115	172	108	27	0.18
Zn total, mg	4.46[ab]	3.63[b]	4.97[a]	3.78[b]	0.47	0.07
Mn total, µg	71.3	74.5	84.6	59.8	15.2	0.55

[a,b]Indicates treatment means differ ($P \leq 0.10$).

[1]All quarters were milked completely, cow was separated from calves, and yield was determined by milking 4 h later.

[2]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

CHAPTER 3

EFFECTS OF COPPER, ZINC, AND MANGANESE SOURCE AND INCLUSION DURING LATE GESTATION ON BEEF CALF FETAL GROWTH, NEONATAL MINERAL AND METABOLIC STATUS, AND PRE-WEANING PERFORMANCE

ABSTRACT

To determine the effects of source and inclusion of Cu, Zn, and Mn supplementation during late gestation on neonatal calf size and vigor, mineral and metabolic status, and calf pre-weaning performance, multiparous, fall-calving, Sim-Angus cows (n = 48; 649 ± 80 kg BW; 5.3 ± 0.5 BCS; 4.3 ± 1.2 yr) were individually-fed tall fescue-based hay (12.2% CP, 61.5% NDF) and supplemented to meet or exceed all nutrient recommendations except Cu, Zn, and Mn. From 91.2 ± 6.2 d pre-calving to 11.0 ± 3.2 d post-calving, cows received: no additional Cu, Zn, or Mn (CON); Cu, Zn, and Mn sulfates supplying 133% NASEM recommendations (ITM); Cu, Zn, and Mn metal methionine hydroxy analogue chelates (MMHAC, MINTREX, Novus International) supplying 133% recommendations (CTM); or Cu, Zn, and Mn sulfates and MMHAC supplying 100% recommendations (reduce and replace, RR). Data were analyzed with treatment and breeding group as fixed effects, animal as experimental unit, and sampling time as a repeated effect for plasma mineral and circulating metabolites. Gestational trace mineral treatment did not affect ($P \geq 0.13$) calf birth BW, size, vigor, or placental size measures. Calves born to cows fed CTM had greater ($P \leq 0.06$) liver Cu concentration at d 11 of age compared with all other treatments. Calves born to cows fed RR had less ($P \leq$

0.08) liver Mn than all other treatments. Liver Zn and relative metallothionein mRNA expression were not affected ($P = 0.58$) by treatment. Calf plasma Zn was maintained ($P \geq 0.15$) from 0 to 48 h of age in ITM and CTM calves but decreased ($P \leq 0.03$) during that period in CON and RR. Neonatal calf plasma Cu and cotyledonary Cu, Zn, and Mn were not affected ($P \geq 0.17$) by treatment. Treatment affected ($P = 0.05$) neonatal calf serum Ca where CTM calves had greater ($P \leq 0.05$) serum Ca than all other treatments. Other calf serum chemistry parameters, and plasma cortisol and triglycerides at 0 and 48 h of age were not affected ($P \geq 0.17$) by treatment. Treatment did not affect ($P \geq 0.46$) pre-weaning calf BW, ADG, and circulating glucose, NEFA, and urea N. Inclusion of chelated Cu, Zn, and Mn to 133% of recommendations in late gestational beef cow diets improved neonatal calf liver Cu. Supplying late gestational cows 133% of Cu, Zn, and Mn recommendations resulted in improved neonatal calf Zn metabolism without altering fetal growth, pre-weaning growth, or metabolic status of the calf.

INTRODUCTION

The majority of pre-weaning beef calf death loss occurs in the first 3 wk of life (APHIS, 2010), demonstrating how challenging the neonatal period is for calves. They need to stand, find the udder, and suckle shortly after birth in order to receive adequate nutrients and immunoglobulins (**Ig**) from colostrum. This is also a major time of transition in which the calf has to mature in order to breath, thermoregulate, and digest nutrients on its own as well as handle a rapid increase in pathogen load. Therefore, proper fetal growth and development is important not only to ensure appropriate structure and vigor to stand and suckle, but also to ensure organ systems are prepared for this

transition.

Essential trace minerals Cu, Zn, and Mn are components of and cofactors in many different enzyme systems that span a wide range of biological mechanisms (McDowell, 1992). These minerals are important for bone formation, lipid metabolism, glucose utilization, DNA synthesis and transport, Fe transport, and antioxidant function which are required proper fetal development (Hostetler et al., 2003).

Dam nutrition greatly impacts the maternal environment which can have long-term effects on the offspring by altering fetal development (Fowden et al., 2006; Wu et al., 2006). Chelated trace minerals are hypothesized to be more bioavailable than inorganic forms of trace minerals (Nuzback et al., 2019). Because fetal and neonatal ruminants are completely dependent on the dam for trace mineral supply (Hidiroglou and Knipfel, 1981), we hypothesized that providing chelated Cu, Zn, and Mn to late gestational beef cows would improve cow mineral status, which would improve calf mineral status and fetal development. Our specific objective was to determine the effects of concentration and source of Cu, Zn, and Mn in late gestation on fetal growth, calf vigor at birth, neonatal mineral and metabolic status, and calf pre-weaning performance.

MATERIALS AND METHODS

All animal procedures were approved by the University of Missouri Animal Care and Use Committee (Protocol #9045) and took place at University of Missouri Beef Teaching and Research Farm (Columbia, MO).

Animal Management and Diets

Forty-eight crossbred beef cows (Sim-Angus, average initial BW = 649.3 ± 79.6 [SD throughout] kg, average initial BCS = 5.30 ± 0.46, age at calving range: 3 to 7 yr [average = 4.25 ± 1.20 yr]) bred by AI (n = 36, due date: September 17, 2017) or natural service (n = 12) were fed tall fescue-based hay and a pelleted supplement to meet or exceed NE_m, CP, Ca, Na, vitamin A, vitamin D, vitamin E, I, Se, and Co recommendations (NASEM, 2016). Cows were allocated by BW, BCS, age, and breeding group (AI or natural service) to 1 of 4 gestational trace mineral treatments from 91.2 ± 6.2 d pre-calving to 11.0 ± 3.2 d post-calving: 1) basal diet with no additional Cu, Zn, or Mn (control; **CON**); 2) basal diet with Cu, Zn, and Mn sulfates to supply 133% NASEM (2016) recommendations (inorganic trace minerals; **ITM**); 3) basal diet with Cu, Zn, and Mn metal methionine hydroxy analogue chelates (**MMHAC**; MINTREX, Novus International, St. Charles, MO) to supply 133% NASEM recommendations (chelated trace minerals, **CTM**); and 4) basal diet with Cu, Zn, and Mn sulfates and MMHAC to supply 100% NASEM recommendations (reduce and replace strategy; **RR**). Hays and corresponding supplements formulated to deliver trace mineral treatments are described in Chapter 2.

Cows were housed in partially-covered 3.7 x 15.8 m pens (n = 4/pen) with electronic feeding gates (American Calan, Northwood, NH) for individual feeding of diets.

Dry matter intakes and dietary Cu, Zn, and Mn provided to each treatment are presented in Table 3.1 along with target dietary trace minerals for each treatment. Feed

sampling, nutrient analysis, and intake calculations are described in Chapter 2. Actual Cu, Zn, and Mn provided in the diet varied from target concentrations for ITM-, CTM-, and RR-fed cows because hay offered differed from the initial core samples used to formulate the supplements. This was expected because trace mineral content of forages is highly variable (McDowell, 1992). Cows fed the CON diet received average dietary Cu and Zn below NASEM recommendations (2016) throughout the treatment period and less average dietary Mn from d 29 to 56 of study.

After treatment termination (11.0 ± 3.2 d post-calving), all cow-calf pairs were housed in an additional calving pen for ≥1 wk for monitoring, provided ad libitum hay, and allowed access to a pressed, molasses-based supplement that provided vitamins and inorganic minerals (MLS #12 Minera-lix, Midcontinent Livestock Supplements, Moberly, MO). Pairs were then moved to and rotated through 3 tall fescue-based pastures, provided ad libitum access to water and the same vitamin and mineral source, and monitored until weaning at 195.3 ± 8.3 d post-calving.

Neonatal Data Collection and Sampling

Calving monitoring and data collection. Prior to calving (17.3 ± 7.0 d pre-calving) cows were moved to 18 x 61 m dry lot calving pens (Duncan and Meyer, 2018) by treatment. In the calving pens, ad libitum hay was provided, treatment supplements were pen-fed at 1800, and cows had free access to water. A shed was shared between 2 adjacent pens as a creep shade area for calves as well as a stadium light to allow for continuous monitoring of cows and calves throughout the night. Cows were closely monitored while in the calving pens by trained personnel to detect when cows were in

stage II of parturition by walking through pens at least once every hour except 0200 to 0400 during heavy calving. Once stage II was detected, the cow was continuously monitored to record time of birth. If >1 h had passed since fetal membranes were observed or calf was presenting abnormally, the cow was moved to the chute and assistance was provided (n = 2, 2, and 1 for CON, CTM, and RR, respectively). At 10 min of age, the calf was assigned a vigor score: 1 = very weak to 5 = extremely vigorous (Duncan, 2018). The calf was then monitored to record time of standing (defined as calf being up on all 4 feet for 5 consecutive seconds). Calf birth BW was measured using a hanging scale by placing the sling under the abdomen of the calf and lifting until all 4 feet were not touching the ground. Body size measurements were measured using a flexible measuring tape. Shoulder to tailhead length was measured from the front of the shoulder blades to the end of the tailhead along the spine. Heart girth was measured as the body circumference immediately posterior to the shoulder blades and front legs and perpendicular to the spine. Abdominal girth was measured as the body circumference perpendicular to the spine and over the umbilicus. Girth at the flank was measured as the body circumference immediately anterior to the hooks and perpendicular to the spine. Cannon circumference was measured at the smallest circumference of a rear cannon bone. Next, the calf was identified with an ear tag and the navel was sprayed with 1:40 chlorhexidine solution until saturated.

Neonatal blood collection. Calves were restrained and jugular blood samples were collected at 0 h of age, which was post-standing but pre-suckling (34.3 ± 20.2 min of age). At 48 h of age (48.2 ± 0.5 h of age), calves were restrained again for jugular blood sample collection. Blood was collected into 4 tubes at each time point (2

Vacutainer serum collection tubes containing no additives [10 mL draw; Becton Dickinson, Franklin Lakes, NJ], 1 Monoject plasma collection tube containing 0.10 mL of 15% K_3EDTA [10 mL draw; Covidien, Mansfield, MA], and 1 Vacutainer plasma collection containing 10.8 mg of K_2EDTA [6 mL draw; Becton Dickinson, Franklin Lakes, NJ] for mineral determination).

All blood tubes were inverted, placed on ice (serum tubes were allowed to clot before being placed on ice), and centrifuged for 30 min at 1,500 x *g* at 4°C within 8 h of sampling. Plasma and serum were aliquoted into plastic 2 ml microcentrifuge tubes and stored at -20°C until analysis.

Placenta collection and processing. Placentas were collected upon expulsion and immediately stored at 4°C. Placentas were rinsed (13.8 ± 11.3 h post collection) by being agitated gently in water to loosen organic matter contamination (mud, manure, limestone, hay, etc.) 2 to 3 separate times. After rinsing, placentas were stored at 4°C until dissection.

Prior to dissection (23.2 ± 11.2 h after rinsing), placentas were examined to determine if the placenta was complete (had both horns and all 4 umbilical vessels). Umbilical vessels were cut distal to the first visible branching from the initial site of rupture at parturition, and cross-sectional diameter of cut vessels were measured using calipers. Cotyledons were dissected from intercotyledonary tissue using scissors if cotyledons were >20 mm in diameter. Number of cotyledons was recorded, and 3 large cotyledons were frozen for mineral analysis. The wet weights of the mineral subsample and remaining cotyledonary tissue were recorded, then the subsample and remaining cotyledonary and intercotyledonary tissues were frozen separately at -20°C until further

analysis. Cut vessels were excluded from intercotyledonary tissue.

After all placentas were collected and processed, cotyledonary and intercotyledonary tissues were completely thawed and dried at 100°C for ≥48 h. After drying, tissue weights were recorded. The mineral analysis subsample was included in cotyledonary tissue dry weight by adding wet weight of the subsample multiplied by the overall DM concentration of cotyledonary tissue. Average cotyledon weight was calculated by dividing total cotyledonary dry weight by number of cotyledons.

Liver biopsies. Liver biopsies were performed on calves at 11.0 ± 3.2 d of age to prevent hyperthermia caused by the general anesthetic Xylazine (EMEA, 1999). Biopsies occurred in in September and October, when temperature humidity index was high, so target biopsy age range was 7 to 14 d of age as to not further stress neonatal calves while their ability to thermoregulate is still developing.

The biopsy protocol for calves was based on Swanson and others (2000). Calves received 29.9 mg/kg oxytetracycline and 2.0 mg/kg flunixin meglumine prior to biopsy (Hexasol, Norbrook Inc., Overland Park, KS) subcutaneously as prophylaxis.

Calves were sedated with 0.10 to 0.50 mg/kg BW Xylazine HCl (depending on calf size and vigor; AnaSed, Akorn Animal Health, Lake Forest, IL) intravenously. After the calf was successfully sedated, organic matter was removed from the surgical area, the hair was clipped, and the area was scrubbed in concentric, non-overlapping circles with iodopovidine followed by 70% alcohol. The scrubbing cycle was repeated ≥3 times until the surgical area was clean of dirt and hair. One milliliter lidocaine (2% lidocaine HCl, MWI Veterinary Supply, Boise, ID) was injected subcutaneously at the surgical site and an approximately 1 cm incision was made in the intercostal space between the 11th and

12th ribs on their right side approximately 15 cm from dorsal midline. A small trocar and cannula (bone marrow biopsy/aspiration needle, Jamshidi 8-gauge, 10 cm tapered distal tip, Becton Dickinson, New Franklin, NJ) was inserted through the peritoneal tissue then the trocar was removed, and the cannula was advanced into the liver with a twisting motion while angling the cannula towards the left elbow joint. The cannula was retracted, and the liver sample was placed in a plastic 2 mL microcentrifuge tube, then was flash frozen on dry ice and stored at -80°C for mineral analysis at a later date.

After biopsy, a single chromic suture was used to close the hide at the incision site, then the incision and surrounding skin were sprayed with 5% permethrin (Prozap Screw Worm Aerosol, Neogen Coorporation, Pleasantville, IA). Anesthesia was reversed using 2 mg/kg Tolazoline (Akorn Animal Health) administered intravenously. Cow-calf pairs were monitored in a shaded area until the calf was fully recovered from anesthesia, then they were moved to an extra calving pen and treatment was terminated.

Pre-weaning Data Collection and Sampling

Calf 1-d BW were recorded at d 35, 60, and 125 of age (34.6 ± 1.8 d, 60.5 ± 4.3 d, and 125.9 ± 3.6 of age, respectively) and 2-d BW were recorded 2 separate days immediately prior to weaning (195.3 ± 8.3 d of age). Jugular blood samples were also collected during these sampling times into 4 tubes (2 Vacutainer serum collection tubes containing no additive, 1 Monoject plasma collection tube containing 0.10 mL of 15% K_3EDTA, and 1 Vacutainer plasma collection tube containing 15 mg of sodium fluoride and 12 mg of potassium oxalate [6 mL draw; Becton Dickinson] for glucose determination). Blood samples were handled and processed as previously described.

Mineral Analysis

All mineral analyses were performed at Novus International Inc. Neonatal calf plasma and cotyledon samples were analyzed for Cu, Zn, and Mn concentration by inductively coupled plasma (**ICP**) optical emission spectrometry with a cyclonic spray chamber and high solids gemcone nebulizer (PerkinElmer, Waltham, MA). Liver samples were analyzed for Cu, Zn, and Mn concentration by ICP mass spectrometry (Agilent 7500, Agilent Technologies, Santa Clara, CA). Samples were digested using HNO_3 acid and heat (260°C). After acid digestion, an internal standard (0.2 ml of 250 ppm yttrium solution) was added, all samples were brought to a common volume using deionized water and mixed thoroughly. Once cooled to room temperature, samples were centrifuged at 3,650 rpm for 15 min and filtered through a 0.2 μm nylon filter. A calibration curve was generated for each mineral. Samples were injected, normalized to the internal standard, and analyzed in triplicate. Analysis was acceptable if the tested value and theoretical value of the internal standard had a relative percent difference < 10%. Neonatal calf plasma Mn concentrations were not detectable (0.05 ppm detection limit) in any sample.

Serum Chemistry Analysis

One aliquot of 0 and 48 h calf serum (1 mL) was stored at 4°C and transported to the University of Missouri Veterinary Medical Diagnostic Laboratory (**VMDL**) on the day of collection, unless the sample was collected on an evening or weekend. Samples were then refrigerated for ≤48 h and transported to VMDL for analysis as soon as

possible. Serum glucose, urea N, creatinine, total protein, globulin, albumin, sodium, calcium, chloride, phosphorus, potassium, magnesium, anion gap, bicarbonate, direct and total bilirubin, and activities of liver enzymes, aspartate transaminase (**AST**), gamma-glutamyl transferase (**GGT**), and creatine kinase (**CK**) were analyzed using a Beckman Coulter AU 400e Chemistry Systems (Beckman Coulter Inc., Brea, CA).

Circulating Metabolite and Hormone Analysis

Neonatal calf plasma was thawed at 4°C, then analyzed for triglycerides using a commercially available Infinity Triglycerides kit (Thermo Scientific) based on the glycerol-3-phosphate oxidase method and modifications from Fossati and Prencipe (1982) and McGowan and others (1983). Neonatal and pre-weaning calf serum was thawed at 4°C, then analyzed for NEFA using a modified procedure of the NEFA C kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) based on the acyl-CoA synthetase-acyl-CoA oxidase method. Pre-weaning calf serum was thawed at 4°C, then analyzed for urea N using a commercially available urea N kit (Urea Nitrogen Procedure Number 0580; Stanbio Laboratory, Boerne, TX) based on the diacetylmonoxime method. Lastly, pre-weaning calf plasma was thawed at 4°C, then analyzed for glucose concentration using the Infinity glucose hexokinase commercially available kit (Fisher Diagnostics, Middletown, VA) based on the glucose-6-phosphate dehydrogenase method.

For each assay, samples were analyzed in duplicate in 96-well polystyrene plates (Corning Inc., Corning, NY) on a microplate reader (Biotek Synergy HT, Biotek Instruments Inc., Winooski, VT). For plasma triglycerides, plates were read at 500 and 660 nm and final results were calculated by subtracting the 660 nm reading from the 500

nm reading. Plates were read at 550, 520, and 340 nm for NEFA, urea N, and glucose assays, respectively.

Pooled neonatal or pre-weaning calf plasma or serum was used as an internal control depending on type of sample being analyzed in each assay. The intraassay and interassay CV for serum triglycerides were 2.48% and 2.83%, respectively. The intraassay and interassay CV for neonatal calf serum NEFA were 3.79% and 4.64%, respectively, and for pre-weaning calf serum NEFA were 2.78% and 5.38%, respectively. The intraassay and interassay CV for serum urea N were 3.26% and 3.77%, respectively. The intraassay and interassay CV for plasma glucose were 3.07% and 3.90%, respectively.

Neonatal calf plasma cortisol was analyzed using a commercial coated-tube RIA kit (MP Biomedicals, Irvine, CA) in duplicate as described previously (Foote et al., 2016). The intraassay CV was 1.67%.

Liver Metallothionein mRNA

Subsamples (5 to 10 mg) of liver subsamples were added to 600 μL buffer provided in an RNAeasy Mini kit (Qiagen, Hilden, Germany) with β-mercaptoethanol. After sample was added to the buffer, it was homogenized, and total mRNA was extracted following manufacturer instructions. The final elution volume was 50 μL. Extracted RNA was kept at -80°C until cDNA synthesis. Quantity of each RNA sample was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Quality of each RNA sample was assessed using gel electrophoresis. Samples of RNA were run through a 1% agarose gel in Tris/borate/EDTA buffer with

ethidium bromide. The gel ran for 30 min at 140 V, then an image of the gel was captured with ChemiDoc Touch Imaging System (Bio-Rad Laboratories Inc., Hercules, CA). Bands for 18S and 28S rRNA were present for each RNA sample.

Extracted RNA samples were thawed at 4°C prior to cDNA synthesis with QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). Approximately 100 ng of RNA were put into each 14 µL genomic DNA elimination reaction. Reactions were prepared on ice in 0.2 mL DNase/RNase-free tubes and performed on a thermal cycler (T100 Thermal Cycler, Bio-Rad Laboratories Inc., Hercules, CA) at 42°C for 2 min. After the genomic DNA elimination reaction, samples were immediately placed on ice then 6 µL of a mastermix containing reverse transcriptases (Ominiscript and Sensiscript, both expressed in *E. coli*), buffer, deoxynucleotide triphosphates, and both oligo-dt and random hexamer primers was added to each reaction resulting in a reaction volume of 20 µL. Reverse transcription reactions were performed on the same thermal cycler as the genomic DNA elimination reaction using the following protocol: 42°C for 30 min, 95°C for 3 min, then cooled to 4°C. Eighty microliters of DNase/RNase-free water was added to each reaction to make a 1:5 dilution of cDNA, then a subsample of cDNA was taken from a subset of samples to make a pool of cDNA to use as an internal control. Samples and control were stored at -20°C until mRNA expression analysis using real-time PCR.

Samples of cDNA were thawed at 4°C and analyzed for mRNA expression of metallothionein 1A and ribosomal protein subunit 9 (**S9**) using real-time PCR. Primer sequences (listed 5' to 3') were: bovine metallothionein-1A forward, CTGCTCCTGCCCCAC (56.2°C melting temperature [$\mathbf{T_m}$]), reverse, CAGCCCTGGGCACAC (56.9°C T_m); S9 forward, GAAGCTGATCGGCGAGTATG

(55.6°C T_m), reverse, CGCAACAGGGCATTACCTTC (56.6°C T_m; Richard, 2008). Efficiencies for metallothionein-1A and S9 primers were 99.1% and 102.1%, respectively. Amplifications were performed in duplicate on 384-well PCR microplates (Corning Inc., Union City, CA) and contained 4 µL of cDNA (1:5 dilution with DNase/RNase-free water) and 11 µL mastermix which contained 1 µL forward primer (7.5 µM), 1 µL reverse primer (7.5 µM), 7.5 µL iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA), and 1.5 µL DNase/RNase-free water with a final reaction volume of 15 µL. DNase/RNase-free water was used as a no template control (NTC) for each gene to ensure there was no contamination. Pooled cDNA sample was used to make a 4-point dilution curve (1:1 to 1:1000 dilutions) for each gene to calculate primer efficiency. After samples and mastermix were pipetted on the plate, the plate was sealed, spun briefly using an MPS 1000 Mini PCR Plate Spinner (Labnet International, Edison, NJ), and reactions were performed using a C1000 Touch thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA). Conditions for the PCR reactions were 95°C for 30 s, 40 cycles of 95° for 5 s followed by 56°C for 30 s, then a melt curve analysis was performed by increasing the temperature from 65 to 95°C in 0.5°C increments for 5 s each. Data were recorded and analyzed with CFX Manager Software 3.1 (Bio-Rad Laboratories Inc., Hercules, CA).

Primer sets on each plate were considered acceptable if there was no NTC amplification, or if NTC amplified but the threshold cycle was >30 and melt curve was not similar to samples. Sample duplicates were considered acceptable if CV was <5%. Derivative melt curves for each reaction were checked for indicators of contamination or poor cDNA quality (i.e. peak at different temperature, >1 peak, small peak, etc.) and

samples with compromised derivative melt curves were repeated. If the standard curve for each gene had $R^2 > 0.900$ and calculated primer efficiencies between 90 and 110%, it was considered acceptable. Intraassay CV for metallothionein 1A and S9 were 0.82 and 1.04%, respectively. All samples for each gene fit on one plate and there were no repeats, therefore there were no interassay CV to report. Expression of metallothionein 1A was calculated relative to the reference gene and the pooled sample control using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Three cows were removed from the study due to late gestational abortion, and 1 cow was removed from the study because she was later bred than all other natural service cows resulting in 44 cows that calved. One cow had twins (1 stillborn and 1 live); therefore neonatal calf data were excluded from analysis. However, that cow-calf pair was kept with the herd and pre-weaning calf data were included in analysis. After post-calving liver biopsies, 1 cow-calf pair was removed due to cow temperament resulting in 43 cow-calf pairs that were followed through weaning. Final sample numbers are provided in Table 3.2. Measures with fewer sample numbers are due to cows calving overnight which prevented personnel from collecting data and samples or cow temperament which prevented personnel from safely collecting data and samples. Incomplete placentas were not included in placental size analysis but were subsampled for mineral analysis.

For mRNA expression data, if a $2^{-\Delta\Delta Ct}$ was > 3 standard deviations away from the mean, it was considered an outlier and removed from the data set. One outlier was

removed from the RR treatment.

Data were analyzed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) with treatment and breeding group as fixed effects and cow as experimental unit. For all measures, calf sex was kept in the model if $P < 0.25$. Calf birth BW and age at biopsy were used as a covariates for calf liver mineral concentrations and relative metallothionein 1A mRNA expression. For serum chemistry and circulating metabolites and hormones over time, treatment, sampling time, and their interaction were considered fixed effects. Sampling time was considered a repeated effect for these measures, using the best-fit covariate structure (chosen from compound symmetry, heterogeneous compound symmetry, autoregressive, and heterogenous autoregressive). Means were separated if the treatment or treatment x day $P \leq 0.10$. Means were considered different if $P \leq 0.05$ and were considered tendencies if $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Neonatal Calf Size, Vigor, and Placental Size

Source and inclusion of Cu, Zn, and Mn in the maternal diet during late gestation did not affect ($P \geq 0.28$) calf birth BW and size measurements (Table 3.3). Typically severe gestational trace mineral deficiencies result in impaired growth, skeletal malformations, and neurological abnormalities if the deficiencies do not result in loss of pregnancy (Hidiroglou, 1980; Hurley, 1981; Hostetler et al., 2003), and may lead to impaired fetal growth and vigor of the offspring. However, CON diets were not severely deficient in Cu and Zn; therefore, impaired fetal growth and vigor at birth were not expected (Graham, 1991).

Supranutritional trace mineral supplementation of gestational cows has not been reported to increase fetal growth (Stanton et al., 2000; Jacometo et al., 2015; Marques et al., 2016; Price et al., 2017). Conversely, calves born to beef heifers receiving subcutaneous organic trace mineral injections throughout gestation had lower birth BW compared with calves born to heifers receiving saline injections (Stokes et al., 2018). Dairy calves born to cows given daily boluses of organic trace minerals the last 30 d of gestation had greater wither height in the first 7 wk of life compared with calves born to cows that received inorganic trace mineral boluses (Jacometo et al., 2015). The previous studies and current study results likely differ due to route of administration of trace minerals, dam parity, breed type, and length of supplementation differences.

Gestational trace mineral treatment did not affect ($P \geq 0.13$) calf vigor measures (Table 3.3). There are limited data available on how gestational supranutritional trace mineral supplementation affects neonatal ruminant vigor; however, late gestational ewe Fe, Cu, and Co supplementation did not affect lamb vigor at birth in one study (Norouzian et al., 2014). Hansen and others (2006) reported calves born to beef heifers fed a Mn-deficient diet throughout gestation had greater incidence of unsteadiness or weakness at birth which likely impaired calf vigor.

Placental size parameters were not affected ($P \geq 0.55$) by gestational treatment (Table 3.3). These data support the lack of treatment differences in calf birth BW and size. There are few data available on the effects of gestational trace mineral supplementation on ruminant placental size. In mice, pups born to dams fed a marginally Zn-deficient diet during gestation had lower placental weights compared with mice fed a Zn adequate diet during gestation (Wilson et al., 2017). However, there are many

differences between species that could explain inconsistencies in results between the previous study and the current study like different placental types, gestation length, and trace mineral requirements which makes it difficult to compare between species.

Neonatal Calf Mineral Status

Liver mineral. Late gestational treatment affected ($P = 0.05$) calf liver Cu, where calves born to cows fed RR had greater ($P \leq 0.05$) liver Cu concentrations compared with ITM and CON calves and CTM calves tended ($P = 0.06$) to have greater liver Cu concentrations compared with CON calves (Table 3.4). All treatment means were within the liver Cu reference range for neonates (125 to 600 mg/kg DM; Herdt and Hoff, 2011) which indicated calf Cu status was adequate on average. Liver is the main Cu storage organ and is responsive to dietary changes, thus it represents Cu status of the animal (Herdt and Hoff, 2011); therefore results indicate calves born to cows fed RR and CTM had improved Cu status compared with those fed a marginally Cu and Zn deficient diet during gestation. Cows fed RR were supplemented Cu to meet NASEM recommendations (2016), whereas cows fed ITM and CTM were supplemented to exceed Cu recommendations by 33%. Thus, greater liver Cu in CTM calves than CON calves was expected but greater liver Cu in RR calves than ITM calves was unexpected based on mineral inclusion in the maternal diet alone. Also, CTM cows had greater liver Cu concentrations than all other treatments at the same sampling time (data presented in Chapter 2). This could indicate gestational supplementation of both inorganic and chelated Cu, Zn, and Mn together (RR) altered perinatal Cu metabolism, benefiting the neonate but not benefiting the dam.

Fetuses of beef heifers supplemented to meet Cu recommendations during the last 2/3 of gestation had greater liver Cu than fetuses of beef heifers deficient in dietary Cu (Fry et al., 2013). Calf liver Cu at birth was also greater in calves born to beef heifers provided adequate dietary trace minerals and subcutaneous trace mineral injections throughout gestation compared with calves born to heifers that received saline injections (Stokes et al., 2018). Supranutritional supplementation of organic forms of Cu, Zn, Mn, and Co to beef cows in late gestation resulted in greater calf liver Cu at birth than calves born to cows with no trace mineral supplementation (Marques et al., 2016). Similarly, in year 2 of a 2-yr study, calves born to cows supplemented an organic form of Cu in late gestation had greater liver Cu at 10 d of age than calves born to marginally Cu-deficient dams; however, there were no calf liver Cu differences in year 1 (Muehlenbein et al., 2001). Both Marques et al. (2016) and Muehlenbein et al. (2001) reported calves born to cows that were not supplemented and those born to cows supplemented with inorganic Cu had similar liver Cu concentrations. Although these data support the calf liver Cu differences between CTM and CON calves and lack of differences between ITM and CON calves in the current study, there are limited data available to support or refute the hypothesis of altered perinatal Cu transfer when dams were provided both inorganic and chelated Cu together in late gestation.

Source and inclusion of Cu, Zn, and Mn in the diet during late gestation did not affect ($P = 0.58$) calf liver Zn concentrations (Table 3.4). The liver Zn reference range for neonates is 120 to 400 mg/kg DM (Herdt and Hoff, 2011). This indicated calf Zn status was adequate on average because all treatment means were within the reference range. Post-calving cow liver Zn was not affected by treatment (data presented in Chapter 2),

therefore similar calf liver Zn concentrations among treatments were anticipated. Calf liver Zn was not altered at 10 d of age by supranutritional Zn supplementation compared with no supplementation in late gestational beef cows (Muehlenbein et al., 2001) or at birth by trace mineral or saline injections to beef heifers throughout gestation (Stokes et al., 2018). However, Holstein calves supplemented various sources of Zn for approximately 100 d postnatally had greater liver Zn than nonsupplemented calves (Wright and Spears, 2004) which indicates liver Zn can be altered. Marques and others (2016) reported greater liver Zn concentrations at birth in calves born to cows supplemented supranutritional organic Zn during late gestation compared with calves born to nonsupplemented cows. Liver Zn concentrations were also greater in fetuses collected from beef heifers fed adequate Cu compared with a Cu-deficient diet throughout mid- and late gestation (Fry et al., 2013). Data from the previous studies likely differ from the current study because timing of trace mineral treatments and liver sampling differ. Although the liver stores Zn, circulating Zn may be a better representation of trace mineral status because it is more responsive to dietary changes and Zn is widely distributed throughout the body (Herdt and Hoff, 2011). Therefore, neonatal plasma Zn may be more representative of Zn status in the current study.

Calf liver Mn concentrations tended ($P = 0.09$) to be affected by gestational treatment, where calves born to cows fed RR had less ($P \leq 0.04$) liver Mn compared with ITM and CON calves, and tended ($P = 0.08$) to have less liver Mn than CTM calves (Table 3.4). All treatment means were within the liver Mn reference range for neonates (3.5 to 15 mg/kg DM; Herdt and Hoff, 2011) which indicated calf Mn status was adequate on average. These results were unexpected as cow liver Mn was not affected by

gestational treatment (data presented in Chapter 2) and the basal diet provided adequate Mn for the majority of the treatment period. Beef heifers fed a Mn-deficient diet throughout gestation gave birth to neonatal calves with less liver Mn than heifers fed a Mn-adequate diet (Howes and Dyer, 1971). Despite this, liver Mn is poorly responsive to dietary changes; therefore, neonatal calf liver Mn is rarely altered by gestational trace mineral treatment (Stanton et al., 2000; Marques et al., 2016; Stokes et al., 2018). The data in the current study could also support the hypothesis that providing both inorganic and chelated Cu, Zn, and Mn to beef cows in late gestation alters perinatal trace mineral transfer, because it takes large differences in Mn supply to negatively impact liver Mn (Herdt and Hoff, 2011). However, it is unclear why this disturbance in perinatal transfer would be advantageous to calf liver Cu and disadvantageous to calf liver Mn.

The age of calves at time of sampling should be taken into consideration because neonates mobilize Cu, Zn, and Mn stores in early life to manage the challenges that come with this stressful, transitionary time (Aggett, 1998). For example, calf liver Cu and Zn concentrations were greater at 10 d of age compared with 30 d of age regardless of gestational trace mineral treatment (Muehlenbein et al., 2001). This could explain inconsistencies of fetal and neonatal calf liver mineral results among different studies. Neonates also consume dietary trace minerals through colostrum and milk during this time, which affects the amount of trace mineral stores that need to be mobilized. In the current study, colostrum Cu, Zn, and Mn concentration and content were similar among treatments (data presented in Chapter 2); therefore calves were likely consuming similar amounts of these trace minerals. Therefore, it may be hypothesized that calf liver mineral differences were mainly due to differences in mineral accretion in late gestation because

calves in the current study were born into similar conditions and likely consumed similar dietary Cu, Zn, and Mn.

Liver metallothionein mRNA expression. Gestational trace mineral treatment did not affect ($P = 0.58$) relative mRNA expression of metallothionein-1A in calf liver. Metallothionein is a protein that can both store and donate Cu and Zn ions, making it essential in Cu and Zn metabolism (Cousins, 1985). Synthesis of metallothionein is induced by presence of Cu, Zn, or Cd in the cell (Dunn et al., 1987). It has been hypothesized that calf hepatic metallothionein is more responsive to Zn status than Cu status (López-Alonso et al., 2005), however, there are few data available on the effects of gestational trace mineral supply on liver metallothionein in offspring, especially in livestock species. Fetuses collected in late gestation from beef cows fed a Cu-deficient diet the last two thirds of pregnancy had similar metallothionein 1A expression compared with fetuses from cows fed a Cu-adequate diet (Fry et al., 2013). When rhesus monkey dams were fed a Zn-deficient diet during gestation and the 1st month of lactation, neonatal liver metallothionein content at 30 d of age was similar to infants born to dams fed a Zn-adequate diet (Keen et al., 1989). However, in rat pups born to Zn-deficient dams liver metallothionein content was lower than liver metallothionein in pups born to Zn-adequate dams (Gallant and Cherian, 1986). Differences in offspring hepatic metallothionein data could be due to differences in trace mineral deficiencies, species, or sampling time between these 3 previous studies.

Hepatic metallothionein is responsive to dietary changes in Cu and Zn (Cousins, 1985), therefore these data could also indicate that dietary Cu and Zn were similar among calves. Colostrum mineral data support this hypothesis, as pre-suckling colostrum Cu and

Zn were similar among treatments (data presented in Chapter 2). Rat pups hepatically injected with Zn at 1 d of age had greater liver metallothionein content at d 2 and 7 of age which suggests that neonatal liver metallothionein is responsive to greater Zn delivery (Chan and Cherian, 1993). These data may also indicate that Cu and Zn absorption was similar among treatments because after absorption in the small intestine, Cu and Zn are transported to the portal vein (Goff, 2018). Thus, the lack of differences among treatments of neonatal hepatic metallothionein transcription in the current study could be the result of similar amounts of Cu and Zn being delivered to the liver after intestinal absorption.

Placenta mineral. Cotyledonary tissue Cu, Zn, and Mn concentration and content were not affected ($P \leq 0.17$) by gestational treatment (Table 3.4). These data indicate that placental accretion of these minerals in late gestation were similar among treatments which could suggest that placental transfer of Cu, Zn, and Mn prior to expulsion may be similar among treatments. Marques and others (2016) observed supplementing beef cows supranutritional organic trace minerals during the last third of gestation resulted in greater Cu concentrations in cotyledons from expelled placentas compared with no trace mineral supplementation, but cotyledonary Zn and Mn were not affected. Cotyledonary Cu and Mn results corresponded to neonatal calf liver Cu and Mn results; however, cotyledonary Zn and calf liver Zn were not analogous (Marques et al., 2016). Placentomes collected from beef heifers fed a Cu-deficient diet during the last two thirds of gestation had less Cu than placentomes collected from heifers fed a Cu-adequate diet, but placentome Zn was not affected (Fry et al., 2013). Placentome Cu results were analogous to treatment difference in fetal liver Cu collected from these heifers; however, Zn concentration

results did not correspond (Fry et al., 2013). The placentome includes the caruncle and cotyledon, thus the current study represents how much Cu, Zn, or Mn was transferred to the fetal tissue and the previous study represents trace minerals in both maternal and fetal tissue, which could explain differences in data. Inconsistencies between cotyledonary and neonatal calf liver trace mineral concentrations in the current study and in the previous study could indicate that regulation of placental and fetal accretion of trace minerals are different.

Plasma mineral. Neonatal plasma Cu was not affected ($P \geq 0.60$) by gestational treatment (Table 3.5). Sampling hour affected ($P < 0.001$) plasma Cu concentrations where calf plasma Cu increased ($P < 0.001$) from 0 to 48 h of age. This was likely due to an increase in ceruloplasmin, the main Cu transporter in the blood, production that occurs in the first weeks of life (Underwood and Suttle, 1999).

Plasma Cu is indicative of the Cu transport pool (Herdt and Hoff, 2011) which was not affected by gestational trace mineral treatment. Similar results of calf plasma or serum Cu at birth have been reported in calves born to cows fed Cu-deficient compared with Cu-adequate diet in late gestation (Muehlenbein et al., 2001) or dams provided supranutritional trace minerals compared with the basal diet adequate for trace minerals (Jacometo et al., 2015; Stokes et al., 2018). Similarly, in the study published by Jacometo and others (2015), calf plasma Cu was also not affected by gestational trace mineral treatment at 1 d of age. The other studies discussed did not collect blood samples at a later age.

There tended ($P = 0.10$) to be a treatment by sampling hour interaction for plasma Zn concentrations (Table 3.5). Gestational trace mineral treatment did not affect ($P \geq$

0.13) calf plasma Zn within sampling hour, however from 0 to 48 h of age plasma Zn concentrations of ITM and CTM calves were maintained ($P \geq 0.15$), whereas CON and RR plasma Zn concentrations decreased ($P \leq 0.03$). Circulating Zn is responsive to dietary changes which makes it a good indicator of Zn status (Herdt and Hoff, 2011). In this study, neither colostral Zn (data presented in Chapter 2) nor calf liver Zn were affected by treatment which suggests this maintenance of plasma Zn in calves born to dams fed Cu, Zn, and Mn concentrations greater than recommendations was not a function of calves mobilizing more Zn from endogenous stores or consuming more Zn. Liver mineral concentrations at birth and plasma Zn concentrations at time of biopsy would give better insight into this hypothesis. These data also suggest that gestational Zn recommendations are underestimated as there were similar plasma Zn results in calves born to dams that were fed a marginally Cu and Zn deficient diet and those born to dams fed to meet Cu, Zn, and Mn recommendations.

Supranutritional Zn provided to cows (Muehlenbein et al., 2001) or heifers (Stokes et al., 2018) did not alter calf plasma or serum Zn at birth in previous studies. However, dairy calves whose dams received daily organic trace mineral boluses the last 30 d of gestation had greater plasma Zn at birth compared with 1 d of age, and calves born to cows that received inorganic trace mineral boluses had less plasma Zn at birth compared with 1 d of age (Jacometo et al., 2015). As the calves had similar colostrum mineral intakes, Jacometo and others (2015) hypothesized that maternal diet altered Zn metabolism. However, the basal diet for the dairy cows in the previous study discussed was adequate for trace minerals, therefore the boluses were supranutritional and beyond the dietary mineral concentrations provided to ITM and CTM cows in the current study,

which may explain why calf results differ from the current study.

Manganese concentrations were not detectable in any neonatal calf plasma samples because the detection limit was 0.05 ppm and the reference range for neonatal calf serum Mn is 0.001 to 0.004 ppm (Herdt and Hoff, 2011).

Neonatal Calf Metabolic Status

Source and inclusion of Cu, Zn, and Mn in late gestational beef cow diets did not affect ($P \geq 0.17$) circulating energy metabolites (glucose, NEFA, or triglycerides) or protein metabolites (urea N, creatinine, total protein, globulin, or albumin) in the neonatal calf (Table 3.6). Serum albumin is the primary mode of Zn transportation in the blood (Giroux et al., 1976) which indicates albumin differences are not responsible for differences in neonatal calf plasma Zn. Neonatal calf circulating liver enzymes (AST, CK, or GGT), anion gap, bicarbonate, or direct or total bilirubin were not affected ($P \geq 0.19$) by treatment. Although trace minerals are important for metabolic functions (Spears, 1999), there are few data available regarding the effects of Cu, Zn, and Mn supply during gestation on neonatal ruminant offspring metabolic status. Dairy calves born to cows that received organic trace mineral boluses the last 30 d of gestation had less circulating glucose prior to suckling compared with calves born to cows that received inorganic trace mineral, but there were no differences 24 h post-colostrum consumption (Jacometo et al., 2015). Calf circulating NEFA, creatinine, urea N, bilirubin, GGT, and albumin were not affected by gestational trace mineral supplementation in the previous study (Jacometo et al., 2015); however, it was unclear if the metabolic status of the cow was affected by trace mineral supplementation which could explain the differences in calf

circulating glucose. In the current study, cow metabolic status was not affected during gestation (data presented in Chapter 2); therefore, it was expected that neonatal calf metabolic status was minimally affected by gestational treatment.

Treatment did not affect ($P \geq 0.12$) circulating macrominerals Na, Cl, P, K, or Mg; however, treatment affected ($P = 0.05$) circulating Ca. Calves born to cows fed CTM had greater ($P \leq 0.05$) serum Ca than all other treatments. These differences in neonatal serum Ca could be explained by CTM calves receiving colostrum that had greater lactose compared with ITM and RR calves (data presented in Chapter 2). In adult humans, the inclusion of lactose in the diet resulted in delayed but prolonged dietary Ca absorption compared with those fed a lactose-deficient diet (Cochet et al., 1983). They hypothesized this was due to delayed gastric emptying. However, sampling time did not affect ($P \geq 0.14$) serum Ca of these calves, which suggests differences in lactose consumption driven by colostrum composition differences would not be a factor in these results. These data also indicate that greater pre-suckling colostrum lactose or triglyceride concentration (data presented in Chapter 2) did not alter energy metabolites in calves at 48 h of age.

Gestational trace mineral treatment did not affect ($P \geq 0.33$) neonatal plasma cortisol (Table 3.6). These data indicate differences observed in neonatal calves were not likely due to a difference in stress response to parturition and early life. Late gestational trace mineral supplementation resulted in greater calf circulating cortisol 3 d post-weaning compared with calves born to cows that received no trace mineral supplement (Marques et al., 2016). However, there are limited data available on gestational Cu, Zn, and Mn supply and neonatal circulating cortisol.

Pre-Weaning Calf Growth and Metabolic Status

Source and inclusion of Cu, Zn, and Mn in late gestation did not affect ($P \geq 0.54$) pre-weaning BW or growth (Table 3.7). In previous studies, calf weaning BW or pre-weaning ADG were not affected by gestational (Stokes et al., 2018), lactational (Olson et al., 1999), or gestational and lactational trace mineral supply (Muehlenbein et al., 2001; Sprinkle et al., 2006). However, supranutritional supplementation of organic trace minerals during late gestation (Marques et al., 2016) or during gestation and lactation (Stanton et al., 2000; Price et al., 2017) resulted in greater calf weaning BW compared with calves born to dams that received no trace mineral supplement or inorganic trace mineral supplement, respectively. Conversely, calves born to cows that received supranutritional trace mineral supplement during late gestation and lactation had lower weaning BW than calves born to cows that received no trace mineral supplement in both years of a 2-yr study (Ahola et al., 2004). There are many differences among these studies such as, length and source of supplementation, basal diet trace mineral content, and calf age at weaning, which could be the cause of variability in these results. However, in the current study, pre-weaning calf growth was expected to be similar among treatments because there were no differences in calf birth BW and pairs were managed similarly and intensely after treatment termination at 11 d post-calving.

Pre-weaning calf circulating glucose, serum urea N, and NEFA were not affected ($P \geq 0.46$) by treatment (Table 3.7). These data support the lack of pre-weaning calf growth treatment differences. Trace minerals are involved in many metabolic pathways (Spears, 1999) but maternal trace mineral supplementation did not affect neonatal

metabolic status and treatments ceased when calves were 11 d of age therefore it is unlikely that metabolic status would be affected later in life.

CONCLUSION

In summary, inclusion of chelated Cu, Zn, and Mn in late gestational beef cow diets improved neonatal calf Cu status. Neonatal calf Zn metabolism was improved when Cu, Zn, and Mn were supplied to 133% of recommendations during late gestation. Fetal growth, calf vigor, and pre-weaning growth were not affected by gestational trace mineral treatment. There were multiple discrepancies among cow and calf mineral status results which suggest perinatal trace mineral transfer is complex and unclear. As calf growth and performance were not altered, further investigation to determine the production response to improved neonatal Cu status and Zn metabolism is needed.

Table 3.1. Final animal and sample numbers included in analysis

	Treatment[1]			
Variable	CON	ITM	CTM	RR
Cow-calf pairs at calving	11	11	10	12
Cow-calf pairs at weaning	11	11	9	12
Blood samples				
0 h	11	10	10	10
48 h	11	8	9	11
Pre-weaning	11	11	9	12
Placentas				
Placental minerals	6	6	7	11
Placental size	5	5	7	8
Liver samples	11	10	10	11

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements.

Table 3.2. Cow dry matter intakes and dietary trace minerals provided during the treatment period

	Treatment[1]			
Variable	CON	ITM	CTM	RR
DMI, kg				
d 0 to 28 of study	10.7	11.4	10.9	10.9
d 29 to 56 of study	12.2	12.7	12.2	12.2
d 57 to 74 of study	12.4	13.0	12.2	12.5
Calving pens[2]	12.9	13.5	12.9	13.1
Dietary Cu, mg/kg DM				
Target[3]	—	13.0	13.0	10.0
d 0 to 28 of study	4.5	12.0	15.2	9.7
d 29 to 56 of study	6.7	13.8	15.9	11.1
d 57 to 74 of study	7.0	14.7	16.7	12.4
Calving pens	8.8	17.7	19.6	15.5
Dietary Zn, mg/kg DM				
Target	—	40.0	40.0	30.0
d 0 to 28 of study	9.8	30.5	32.1	23.0
d 29 to 56 of study	15.2	37.0	39.4	29.7
d 57 to 74 of study	16.9	40.6	41.7	33.7
Calving pens	13.8	36.8	39.7	31.7
Dietary Mn, mg/kg DM				
Target	—	53.3	53.3	40.0
d 0 to 28 of study	43.2	60.3	64.7	51.3
d 29 to 56 of study	36.2	55.5	61.7	45.0
d 57 to 74 of study	47.4	61.1	65.6	56.8
Calving pens	65.9	65.5	64.1	64.8

[1]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]Cows were housed in dry lot calving pens by treatment starting d 75 of study (average) until 11.0 ± 3.2 d post-calving, offered ad libitum hay, and pen-fed supplement. Intakes were calculated using estimated hay DMI (1.2% BW NDF intake) and pen-fed supplement DMI.

[3]Target concentrations of diets based on NASEM (2016) gestating cow Cu, Zn, and Mn recommendations and treatment goals.

Table 3.3. Effects of Cu, Zn, and Mn source and inclusion during late gestation on neonatal calf size, vigor, and placental size

	Treatment[1]					
Variable	CON	ITM	CTM	RR	SEM	*P*-value
Size at birth						
Birth BW, kg	37.9	37.5	35.8	38.1	1.3	0.57
Shoulder to tailhead length, cm	57.7	58.3	57.8	57.8	1.0	0.97
Heart girth, cm	75.9	75.5	73.3	75.7	1.1	0.28
Abdominal girth, cm	72.4	70.4	70.1	71.4	1.4	0.59
Girth at flank, cm	67.4	65.4	63.9	65.4	1.7	0.50
Cannon circumference, cm	12.1	12.2	12.2	12.2	0.2	0.97
Vigor at birth						
Time to stand[2], min	19.1	25.1	18.6	29.4	4.3	0.13
Vigor score[3]	3.07	3.11	3.09	3.36	0.17	0.54
Placental size						
Dry cotyledonary weight, g	155	149	176	152	21	0.66
Dry intercotyledonary weight, g	162	173	179	193	19	0.55
Dry total placental weight, g	318	319	354	343	34	0.73
Average dry cotyledonary weight[4], g	2.08	2.17	2.35	2.29	0.30	0.86
Number of cotyledons	76.9	71.7	77.3	71.7	10.5	0.94
Average umbilical vessel diameter[5], mm	9.43	9.35	9.20	9.07	0.65	0.96

[1]Calves born to cows individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[2]Calf standing on all 4 feet for 5 consecutive seconds.
[3]Assigned at 10 min of age; scale ranging from 1 (very weak) to 5 (extremely vigorous).
[4]Cotyledon weight divided by cotyledon number.
[5]Vessels measured just distal to first vascular branch.

Table 3.4. Effects of Cu, Zn, and Mn source and inclusion during late gestation on neonatal calf liver and cotyledon mineral concentrations

	Treatment[1]					
Variable	CON	ITM	CTM	RR	SEM	*P*-value
Liver[2] mineral						
Cu, mg/kg DM	174[c]	182[bc]	221[ab]	228[a]	18	0.05
Zn, mg/kg DM	191	166	148	179	24	0.58
Mn, mg/kg DM	8.55[a]	8.53[a]	8.36[a]	7.24[b]	0.47	0.09
Metallothionein 1A relative mRNA expression, $2^{-\Delta\Delta Ct}$	1.85	1.40	0.82	1.95	0.66	0.58
Cotyledonary[3] mineral						
Cu, mg/kg	5.99	5.46	6.74	7.59	1.14	0.35
Zn, mg/kg	64.0	43.9	57.4	49.4	7.7	0.17
Mn, mg/kg	2.39	7.46	7.47	16.41	8.50	0.46
Cu total[4], mg	0.96	0.86	1.23	1.23	0.26	0.64
Zn total, mg	9.16	6.91	10.29	8.30	1.60	0.36
Mn total, mg	0.82	1.26	1.49	1.31	0.42	0.58

[a,b]Indicates treatment means differ ($P < 0.10$)

[1]Calves born to cows individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]Liver biopsies collect at 11.0 ± 3.2 d of age.

[3]Large cotyledons (n = 3) subsampled.

[4]Cotyledonary mineral concentration multiplied by cotyledonary dry weight.

Table 3.5. Effects of Cu, Zn, and Mn source and inclusion during late gestation on neonatal plasma mineral concentrations[1]

	Treatment[2]					*P*-value		
Variable	CON	ITM	CTM	RR	SEM	Trt	Hour	Trt x Hour
Plasma Cu, ppm						0.93	<0.001	0.60
0 h[3]	0.246	0.239	0.240	0.224	0.027			
48 h	0.346	0.360	0.391	0.379	0.032			
Plasma Zn, ppm						0.87	0.008	0.10
0 h	1.14[a]	1.02[ab]	0.97[ab]	1.14[a]	0.10			
48 h	0.842[b]	0.836[b]	1.055[ab]	0.888[b]	0.114			

[a-c]Indicates treatment means differ ($P \leq 0.10$).

[1]Mn concentrations were below detection limit (0.05 ppm) in all samples.

[2]Calves born to cows individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[3]Samples collected post-standing but pre-suckling (34.3 ± 20.2 min of age).

Table 3.6. Effects of Cu, Zn, and Mn source and inclusion during late gestation on neonatal calf serum chemistry profile and plasma triglycerides and cortisol concentrations at 0 and 48 h of age

	Treatment[1]					*P*-value		
Variable	CON	ITM	CTM	RR	SEM	Trt	Hour	Trt x Hour
Glucose, mg/dL						0.52	<0.001	0.63
0 h	41.8	38.0	44.8	48.2	6.2			
48 h	119	122	130	119	7			
NEFA, mEq/L						0.26	0.02	0.17
0 h	469	453	214	480	104			
48 h	235	258	283	332	34			
Triglycerides, mg/dL						0.75	<0.001	0.86
0 h	9.15	9.18	5.77	10.20	1.75			
48 h	65.6	73.4	69.5	77.2	10.5			
Urea N, mg/dL						0.51	0.002	0.26
0 h	6.18	6.37	6.57	6.03	0.39			
48 h	10.27	7.32	7.57	8.46	1.40			
Creatinine, mg/dL						0.46	<0.001	0.53
0 h	3.91	4.29	4.91	4.22	0.47			
48 h	1.15	1.11	1.18	1.07	0.06			
Total protein, g/dL						0.93	<0.001	0.65
0 h	4.29	4.23	4.40	4.37	0.08			
48 h	6.94	7.28	7.02	6.92	0.32			
Globulin, g/dL						0.79	<0.001	0.50
0 h	1.43	1.39	1.54	1.54	0.05			
48 h	4.59	5.09	4.69	4.61	0.34			
Albumin, g/dL						0.53	<0.001	0.71
0 h	2.89	2.86	2.89	2.87	0.05			
48 h	2.38	2.27	2.39	2.37	0.06			
Aspartate transaminase, U/L						0.74	<0.001	0.82
0 h	16.7	15.1	14.1	14.9	1.1			
48 h	47.4	44.5	46.0	47.4	2.9			
Creatine kinase, U/L						0.23	0.66	0.50
0 h	149.8	81.6	79.9	108.0	31.7			
48 h	120	82	107	138	21			
Gamma-glutamyl transferase, U/L						0.19	<0.001	0.19
0 h	12.8	9.3	11.2	11.2	1.5			
48 h	717	829	1219	653	213			
Anion gap, mEq/L						0.63	<0.001	0.47
0 h	21.6	21.4	23.2	22.5	1.2			
48 h	19.4	19.2	19.4	17.8	0.7			
Bicarbonate,						0.68	0.06	0.31

mEq/L								
0 h	28.6	28.6	26.6	27.2	0.8			
48 h	26.0	26.9	26.9	26.7	0.9			
Direct bilirubin, mg/dL						0.51	0.04	0.47
0 h	0.112	0.122	0.102	0.133	0.012			
48 h	0.121	0.138	0.168	0.157	0.028			
Total bilirubin, mg/dL						0.43	0.04	0.32
0 h	0.31	0.35	0.27	0.35	0.03			
48 h	0.32	0.37	0.43	0.44	0.07			
Sodium, mEq/L						0.57	<0.001	0.29
0 h	146	146	144	145	1			
48 h	140	140	140	139	1			
Calcium[2], mg/dL	11.4[b]	11.3[b]	11.8[a]	11.4[b]	0.2	0.05	0.14	0.68
0 h	11.2	11.2	11.7	11.4	0.2			
48 h	11.5	11.3	11.9	11.4	0.2			
Chloride, mEq/L						0.45	0.01	0.51
0 h	102	102	100	101	1			
48 h	100	99	99	100	1			
Phosphorus, mg/dL						0.60	0.66	0.44
0 h	7.48	7.38	7.73	7.68	0.35			
48 h	8.10	7.42	7.85	7.29	0.38			
Potassium, mEq/L						0.12	0.97	0.63
0 h	5.37	5.31	5.32	5.09	0.14			
48 h	5.54	5.22	5.19	5.12	0.15			
Magnesium, mg/dL						0.23	0.03	0.20
0 h	2.25	2.37	2.48	2.38	0.09			
48 h	2.29	2.13	2.41	2.22	0.09			
Cortisol, nmol/L						0.33	<0.001	0.42
0 h	349	330	387	333	23			
48 h	59.8	59.8	59.6	54.6	11.3			

[a,b]Indicates treatment means differ ($P \leq 0.10$)

[1]Calves born to cows individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]Treatment means listed above interactive means.

Table 3.7. Effects of Cu, Zn, and Mn source and inclusion during late gestation on pre-weaning calf growth and circulating metabolites

	Treatment[1]					*P*-value		
Variable	CON	ITM	CTM	RR	SEM	Trt	Day	Trt x Day
BW[2], kg								
d 35	80.6	78.9	79.0	78.4	2.7	0.89		
d 60	108	110	107	104	3	0.54		
d 125	186	181	185	179	5	0.63		
Weaning[3]	235	235	241	229	7	0.68		
Pre-weaning ADG, kg/d	1.02	1.02	1.07	1.00	0.03	0.54		
Plasma glucose, mg/dL						0.70	<0.001	0.74
d 35	112	116	117	113	3			
d 60	105	105	104	102	3			
d 125	86.4	88.5	83.1	86.1	2.8			
Weaning	82.1	83.2	85.0	82.6	2.8			
Serum urea N, mg/dL						0.74	<0.001	0.59
d 35	8.15	9.06	7.75	8.44	0.58			
d 60	9.22	9.41	8.91	9.23	0.51			
d 125	11.3	11.5	11.4	12.1	0.6			
Weaning	10.9	10.1	10.2	10.4	0.6			
Serum NEFA, mEq/L						0.46	<0.001	0.93
d 35	325	362	355	319	39			
d 60	329	316	360	295	37			
d 125	227	230	225	220	20			
Weaning	446	387	420	371	43			

[1]Calves born to cows individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[2]At d 35, 60, and 125 of age 1-d BW was collected; 2-d BW was collected at weaning.
[3]Calves were 195 ± 8 d of age at weaning.

CHAPTER 4

EFFECTS OF COPPER, ZINC, AND MANGANESE SOURCE AND INCLUSION DURING LATE GESTATION ON NEONATAL BEEF CALF PASSIVE TRANSFER AND IMMUNE RESPONSIVENESS

ABSTRACT

To determine the effects of source and inclusion of Cu, Zn, and Mn during late gestation on neonatal calf passive transfer and immune responsiveness, multiparous, fall-calving, Sim-Angus cows (n = 48) were individually fed tall fescue-based hay and supplemented to meet or exceed nutrient recommendations except Cu, Zn, and Mn. From 91.2 ± 6.2 d pre-calving to 11.0 ± 3.2 d post-calving, cows received: no additional Cu, Zn, or Mn (CON); Cu, Zn, and Mn sulfates supplying 133% NASEM recommendations (ITM); Cu, Zn, and Mn metal methionine hydroxy analogue chelates (MMHAC, MINTREX, Novus International) supplying 133% recommendations (CTM); or Cu, Zn, and Mn sulfates and MMHAC supplying 100% recommendations (reduce and replace, RR). Calf serum at 48 h of age was used for immunoglobulin (Ig) G, A, and M analysis using ELISA. Whole blood samples from 6 calves per treatment were used for ex vivo stimulation with toll-like receptor agonists lipopolysaccharide (LPS), lipoteichoic acid (LTA), and peptidoglycan (PGN) at low and high concentrations for 4 h at 37°C. Relative expression of inflammation-related genes was determined using real-time PCR (reference gene: S9) and $2^{-\Delta\Delta Ct}$ were calculated. Data were analyzed with treatment and breeding group as fixed effects. When treatment $P < 0.15$, LS means were separated. Treatment did not affect ($P \geq 0.57$) calf serum Ig at 48 h. Expression of pro-inflammatory cytokines

interleukin (IL) -1β and IL-8 was greater ($P \leq 0.10$) in calves born to RR cows than all other treatments when exposed to PGN-low. Expression of IL-1β was greater ($P \leq 0.09$) in CTM and CON than ITM when exposed to LPS-low and in RR than ITM when exposed to LTA-low. Expression of inducible nitric oxide synthase (iNOS) in CTM calves was greater ($P \leq 0.09$) than ITM and CON when exposed to LTA-low and PGN-high and all other treatments when exposed to LPS-low. When exposed to PGN-low, iNOS was greater ($P \leq 0.06$) in CTM and RR calves than CON. Treatment did not affect ($P \geq 0.22$) IL-6 and tumor necrosis factor α expression. Data demonstrate that late gestational chelated trace mineral supplementation did not alter passive transfer of Ig but may alter neonatal calf whole blood TLR stimulation response. These changes may increase innate immune responsiveness to bacterial pathogens during the challenging neonatal period, potentially increasing survival.

INTRODUCTION

In beef calves, pre-weaning death loss is estimated at 5.5% (APHIS, 2017). About 2/3 of this loss occurs in the first 3 weeks of life (APHIS, 2010). Of the known reasons, digestive (scours, bloat, enterotoxemia, etc.) and respiratory problems (pneumonia or other respiratory infections) are in the top 4 causes of early calf death (APHIS, 2007). This is a challenging time for neonatal calves because they have to handle a rapid increase in pathogen load (Barrington and Parish, 2001) while their immune system is still developing (Chase et al., 2008). During the neonatal period, the calf immune system is immature due to circulating hormones during late gestation, other immunosuppressors in the colostrum, no placental transfer of immunoglobulins (**Ig**), and lack of or decreased

functionality of immune cells (Chase et al., 2008). Therefore, the calf relies primarily on passive immunity from the dam via colostrum which not only provides Ig, but also provides maternal immune cells and cytokines that are active for 1 to 3 wk (Chase et al., 2008; Cortese, 2009).

Deficiencies in essential trace minerals Cu, Zn, and Mn can result in decreased presence of immune cells, antibody production, and cytokine response (Keen et al., 2004; Cunningham-Rundles et al., 2009). However, supplementation of more bioavailable sources of Cu, Zn, and Mn resulted in improved immune response in dairy cows (Osorio et al., 2016) and steer calves (Chirase and Greene, 2001). Therefore, we hypothesized supplementing beef cows a chelated, or more bioavailable, source of Cu, Zn, and Mn in late gestation would improve mineral status of both cows and calves and subsequently, calf immune function. Our objective was to determine the effects of inorganic and chelated trace mineral inclusion in late gestational beef cow diets on neonatal calf passive immune transfer and immune responsiveness.

MATERIALS AND METHODS

All animal procedures were approved by the University of Missouri Animal Care and Use Committee (Protocol #9045) and took place at University of Missouri Beef Teaching and Research Farm (Columbia, MO).

Animal Management and Diets

Forty-eight crossbred beef cows (Sim-Angus, average initial BW = 649.3 ± 79.6 [SD throughout] kg, average initial BCS = 5.30 ± 0.46, age at calving range: 3 to 7 yr

[average = 4.25 ± 1.20 yr]) bred by AI (n = 36, due date: September 17, 2017) or natural service (n = 12) were fed tall fescue-based hay and a pelleted supplement to meet or exceed all nutrient recommendations (NASEM, 2016) except Cu, Zn, and Mn. Cows were allocated by BW, BCS, age, and breeding group (AI or natural service) to 1 of 4 gestational trace mineral treatments from 91.2 ± 6.2 d pre-calving to 11.0 ± 3.2 d post-calving: 1) basal diet with no additional Cu, Zn, or Mn (control; **CON**); 2) basal diet with Cu, Zn, and Mn sulfates to supply 133% NASEM (2016) recommendations (inorganic trace minerals; **ITM**); 3) basal diet with Cu, Zn, and Mn metal methionine hydroxy analogue chelates (**MMHAC**; MINTREX, Novus International, St. Charles, MO) to supply 133% NASEM recommendations (chelated trace minerals, **CTM**); and 4) basal diet with Cu, Zn, and Mn sulfates and MMHAC to supply 100% NASEM recommendations (reduce and replace strategy; **RR**). A description of cow management and composition and formulation of treatment supplements is provided in Chapter 2.

Cows were individually fed hay and respective treatment supplements using electronic feeders (American Calan, Northwood, NH). On d 72 and 79 of study for AI and natural service, respectively (average: 17.3 ± 7.0 d pre-calving), cows were moved to 18 x 61 m dry lot calving pens (Duncan and Meyer, 2018) by treatment in order to collect neonatal calf data. In the calving pens, ad libitum hay was provided, treatment supplements were pen-fed, and cows had free access to water. Three stadium lights allowed for continuous monitoring of cows throughout the night and a shed was shared between 2 adjacent pens as a creep shade area for calves.

Dry matter intakes, dietary trace mineral provided, and target dietary trace mineral concentrations for each treatment are presented in Table 4.1. Actual Cu, Zn, and

Mn provided in the diet varied from target concentrations for ITM, CTM, and RR because hay fed differed from the initial hay core samples used to formulate treatment supplements. This was likely due to high variability of trace minerals in forages (McDowell, 1992). Cows fed CON had dietary Cu and Zn concentrations below the NASEM (2016) recommendations throughout the treatment period and had dietary Mn concentrations below recommendations from d 29 to 56 of study.

Calf Serum Collection and Immunoglobulin Analysis

Calves were restrained and jugular blood samples (n = 39) were collected at 48 h (48.2 ± 0.5 h) of age into 1 Vacutainer serum collection tube containing no additive (10 mL draw; Becton Dickinson, Franklin Lakes, NJ). Tubes were inverted, allowed to clot, placed on ice, and centrifuged for 30 min at 1,500 x *g* at 4°C within 8 h of sampling. Serum was aliquoted into 2 mL plastic microcentrifuge tubes, then stored at -20°C until analysis.

Serum samples were thawed at 4°C then analyzed for IgG, IgA, and IgM using an enzyme-linked immunosorbent assay (Bovine IgG ELISA Quantitation Set, Bovine IgA ELISA Quantitation Set, and Bovine IgM ELISA Quantitation Set, respectively, Bethyl Laboratories, Inc., Montgomery, TX). Bovine reference serum (24 mg/mL IgG, 0.11 mg/mL IgA, and 1.8 mg/mL IgM) provided in the kit was used to make a 7-point standard curve ranging from 7.8 to 500 ng/mL for IgG and 15.675 to 1000 ng/mL for IgA and IgM assays to generate a 4-parameter curve fit. Pooled 48-h calf serum was used as an internal control and placed into wells on the top half and bottom half of the plate to control for plate location differences and time relative to plating samples. Samples and

internal controls were diluted with a dilution factor of 1:250,000 for IgG, 1:7,500 for IgA, and 1:20,000 for IgM. Standards were analyzed in triplicate, and samples and internal control were analyzed in duplicate in 96-well polystyrene plates (Corning Inc., Corning, NY) and read on a microplate reader (Biotek Synergy HT, Biotek Instruments Inc., Winnoski, VT) at 450 nm. Standards, samples, and internal controls CV < 15% were considered acceptable. If a standard had a CV > 15% due to 1 value in the triplicate being an outlier, then that value was masked. Standard curves were considered acceptable if R^2 > 0.99. The intraassay and interassay CV were 4.13% and 7.73% for IgG, respectively, were 4.94% and 5.67% for IgA, respectively, and were 4.48% and 8.20% for IgM, respectively.

Neonatal Calf Whole Blood Stimulations

Jugular whole blood samples were collected from a subset of calves (n = 24, 6 per treatment) at 48.2 ± 0.5 h of age into 1 Monoject plasma collection tube containing 0.10 mL of 15% K_3EDTA (10 mL draw; Covidien, Mansfield, MA). The tube was inverted after collection and kept at room temperature in a lateral position for 120 ± 62 min post-collection (range: 35 to 272 min post-collection) until ex vivo stimulation. Subsamples (500 µL) of whole blood from each calf were incubated in 2-mL plastic microcentrifuge tubes after addition of Toll-like receptor (**TLR**) agonists liposaccharide (**LPS**; from *Escherichia coli* O111:B4, Sigma-Aldrich, Co., St. Louis, MO), lipoteichoic acid (**LTA**; from *Staphylococcus aureus*, Sigma-Aldrich, Co., St. Louis, MO), or peptidoglycan (**PGN**; from *Staphylococcus aureus*, Sigma-Aldrich Co., St. Louis, MO) at low concentrations (5 ng/mL, 10 µg/mL, and 1 µg/mL, for LPS, LTA, and PGN,

respectively) or high concentrations (5, 100, and 10 μg/mL for LPS, LTA, and PGN, respectively) for 4 h at 37°C. One whole blood subsample was incubated without a TLR agonist and was used as the control condition. The TLR agonists used were suspended in PBS and different volumes of agonist were needed to deliver the appropriate amount to each condition. In the LTA-high condition, 50 μL of agonist was added. To make all other conditions, including control, isovolumetric, sterile PBS was added to result in a final volume of 550 μL. After incubation, samples were returned to room temperature, 500 μL DNA/RNA Shield (Zymo Research, Irvine, CA) was added to each tube, and tubes were vortexed. Samples were then stored at -80°C until RNA extraction.

RNA Extraction and cDNA Synthesis

Stimulated whole blood samples were thawed at room temperature, and total RNA was extracted from each subsample using Quick-RNA Whole Blood kit (Zymo Research, Irvine, CA). The protocol was adjusted for 500 μL of blood instead of 200 μL. Proteinase K (20 μL) was added to each sample, samples were vortexed, and incubated at room temperature for 30 min. After incubation, 1,020 μL isopropanol (equal volume to the current volume of the sample) were added to each sample and vortexed. Samples were then run through a series of columns, and various buffers provided in the kit were added to recover, prepare, and wash the RNA before elution in 15 μL DNase/RNase-free water. Extracted total RNA was kept at -80°C. This protocol was first completed on 4 additional stimulated whole blood samples which were then used to assess quality of extracted RNA using this protocol. First, concentration was assessed using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the 260/280 ratio

was used to determine RNA quality. Total RNA concentrations of the additional stimulated whole blood samples ranged from 137 to 178 ng/μL and the 260/280 ratio ranged from 2.00 to 2.02. Then, quality of RNA from each additional sample was assessed after electrophoresis through a 1% agarose gel in Tris/borate/EDTA buffer with ethidium bromide. The gel ran for 30 min at 140V, then an image of the gel was captured with ChemiDoc Touch Imaging System (Bio-Rad Laboratories Inc., Hercules, CA). Bands for 18S and 28S rRNA were present for each additional RNA sample. Due to a limited amount of RNA from each sample to be used in actual analysis, it was assumed RNA concentration and quality for the rest of the RNA extracted from stimulated whole blood samples using the same protocol was adequate based on these data.

Extracted RNA samples were thawed at 4°C prior to cDNA synthesis with QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). The same volume (8 μL) of RNA sample was added to each 14 μL genomic DNA elimination reaction. Reactions were prepared on ice in 0.2 mL DNase/RNase-free tubes and performed on a thermal cycler (T100 Thermal Cycler, Bio-Rad Laboratories Inc., Hercules, CA) at 42°C for 2 min. After the genomic DNA elimination reaction, samples were immediately placed on ice, then 6 μL of a mastermix containing reverse transcriptases (Ominiscript and Sensiscript, both expressed in *E. coli*), buffer, deoxynucleotide triphosphates, and both oligo-dt and random hexamer primers was added to each reaction bringing the reaction volume to 20 μL. Reverse transcription reactions were performed on the same thermal cycler as the gDNA elimination reaction using the following protocol: 42°C for 30 min, 95°C for 3 min, and then cooled to 4°C. Eighty microliters of DNase/RNase-free water were added to each reaction to make a 1:5 dilution of cDNA, then a subsample of

cDNA was taken from a subset of samples to make a pool of cDNA to use as a control. Samples and control were stored at -20°C until gene expression analysis using real-time PCR.

Real-time PCR

Samples of cDNA were thawed at 4°C and analyzed for mRNA expression of interleukin (**IL**) -1β, -6, -8, tumor necrosis factor α (**TNFα**), and inducible nitric oxide synthase (**iNOS**) using real-time PCR. Ribosomal protein subunit 9 (**S9**) was used as the reference gene. Forward and reverse primer sequences, melting temperatures, and primer efficiencies for each gene are listed in Table 4.2. Primer sequences for IL-1β, IL-6, IL-8, TNFα, iNOS, and S9 were previously published by Bruno and others (2010). Amplifications were performed in duplicate on 384-well PCR microplates (Corning Inc., Union City, CA), and each well contained 4 μL of cDNA (1:5 dilution with DNase/RNase-free water) and 11 μL mastermix containing 1 μL forward primer (7.5 μM), 1 μL reverse primer (7.5 μM), 7.5 μL iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA), and 1.5 μL DNase/RNase-free water with a final reaction volume of 15 μL. Multiple genes were analyzed on each plate to minimize the number of freeze/thaw cycles for cDNA samples. DNase/RNase-free water was used as a no template control (**NTC**) for each gene on each plate to ensure there was no contamination. The pooled cDNA sample was used to make a 4-point dilution curve (1:1 to 1:1000 dilutions) for each gene on each plate to calculate primer efficiency, and the 1:1 dilution on the curve was used as the sample control to calculate interassay CV. After samples and mastermix were pipetted, the plate was sealed, spun using MPS 1000 Mini

PCR Plate Spinner (Labnet International, Edison, NJ), and reactions were performed using C1000 Touch thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA). Conditions for the PCR reactions were 95°C for 30 s, 40 cycles of 95° for 5 s followed by 56°C for 30 s, and then a melt curve analysis was performed by increasing the temperature from 65 to 95°C in 0.5°C increments for 5 s each. Data were recorded and analyzed with CFX Manager Software 3.1 (Bio-Rad Laboratories Inc., Hercules, CA).

Primer sets on each plate were considered acceptable if there was no NTC amplification, or if NTC amplified but the threshold cycle was >30 and melt curve was not similar to samples. Sample duplicates were considered acceptable if CV was <5%. Derivative melt curves for each reaction were checked for indicators of contamination or poor cDNA quality (i.e. peak at different temperature, >1 peak, small peak, etc.). Samples with compromised derivative melt curves were repeated. If 1 well had a small extra peak at a lower temperature than the main peak and the other well had a derivative melt curve that looked normal and the sample CV was <5%, then the sample was considered acceptable. If the gene standard curve on a plate had $R^2 > 0.900$ and calculated primer efficiencies between 90 and 110%, it was considered acceptable. Interleukin 6 and TNFα were not as greatly expressed as other genes, consequently on 3 plates analyzing IL-6 and 1 plate analyzing TNFα, the 1:1000 dilution on the curve had to be masked in order to accurately represent the efficiency and R^2 of the curve. Interassay CV for IL-1β, IL-6, IL-8, TNFα, iNOS, and S9 were 1.55, 0.80, 2.17, 0.65, 1.33, and 2.74%, respectively. Intraassay CV for IL-1β, IL-6, IL-8, TNFα, iNOS, and S9 were 0.901, 0.824, 0.986, 0.778, 0.884, and 1.057 %, respectively. Expression of IL-1β, IL-6, IL-8, TNFα, and iNOS for each sample in each condition was calculated relative to the reference gene and

the control condition using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

For mRNA expression data, if a $2^{-\Delta\Delta Ct}$ was > 3 standard deviations away from the mean within a condition, it was considered an outlier and removed from the dataset. In the LPS-low condition, there was 1 iNOS and 1 IL-6 outlier removed from RR and CTM, respectively. In LPS-high condition, there was 1 outlier for all genes removed from CON. In LTA-low condition, there was 1 iNOS outlier removed from RR treatment. In LTA-high condition, there was 1 iNOS outlier removed from RR and 1 outlier for all genes removed from ITM. In the PGN-low condition, there was 1 outlier for all genes removed from RR, 1 IL-6 outlier removed from ITM, and 1 TNFα outlier removed from RR. Finally, in the PGN-H condition, there was 1 IL-1β, 1 iNOS, and 2 IL-6 outliers removed from RR.

Data were analyzed using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) with treatment and breeding group as fixed effects. For calf serum Ig, calf sex was included in the model if $P < 0.25$. Means were separated if treatment $P \leq 0.15$ and means were considered different if $P \leq 0.10$.

RESULTS

Serum Immunoglobulins

Inclusion and source of Cu, Zn, and Mn in late gestation did not affect ($P \geq 0.57$) calf serum IgG, IgA, or IgM concentrations at 48 h of age (Table 4.3).

Neonatal Calf Whole Blood Stimulation mRNA Expression

Fetal growth and mineral status of calf subset. Mineral status of the subset of calves sampled for ex vivo whole blood stimulation differed from the data presented in Chapter 3 that included all calves in the data set. Calf birth BW and liver Zn concentrations at 11 d of age were not affected ($P \geq 0.66$) by gestational trace mineral treatments (Table 4.4). In this subset, treatment did not affect ($P \geq 0.18$) calf liver Cu or Zn. However, calves born to cows fed CTM and RR had numerically greater Cu concentrations than CON and ITM calves, which is analogous to liver Cu data when all calves are included in the data set (presented in Chapter 3). Liver Mn was affected ($P = 0.04$) by gestational trace mineral supplementation where CTM calves had greater ($P \leq 0.07$) Mn concentrations than all other treatments. Similar to results from the whole data set, plasma Cu was not affected ($P \geq 0.31$) by treatment. Treatment did not affect ($P \geq 0.38$) calf plasma Zn, but if the F test is unprotected, calves born to cows fed CON and ITM had decreased ($P \leq 0.07$) plasma Zn from 0 to 48 h of age whereas CTM and RR calves maintained ($P \geq 0.45$) plasma Zn.

Interleukin 1β. Source and inclusion of Cu, Zn, and Mn in late gestation affected ($P \leq 0.10$) relative expression of IL-1β mRNA when 48-h calf whole blood was exposed to low concentrations of LTA and PGN (Table 4.5). Calves born to cows fed RR had greater ($P = 0.01$) IL-1β expression than ITM calves when exposed to LTA-low and greater ($P \leq 0.10$) IL-1β expression than all other treatments when exposed to PGN-low. In the LPS-low condition, IL-1β expression tended ($P = 0.14$) to be affected by treatment, where calves born to CON- and CTM-fed cows had greater ($P \leq 0.09$) expression than ITM calves. Expression of IL-1β was not affected ($P \geq 0.30$) by gestational trace mineral

treatment when 48-h calf whole blood was exposed to high concentrations of LPS, LTA, or PGN.

Interleukin 6. Relative mRNA expression of IL-6 was not affected ($P \geq 0.51$) by source and inclusion of Cu, Zn, and Mn in late gestation when 48-h calf whole blood was exposed to low or high concentrations of LPS, LTA, or PGN (Table 4.5).

Interleukin 8. Gestational trace mineral treatment affected ($P = 0.04$) relative mRNA expression of IL-8 in the PGN-low condition, where calves born to cows fed RR had greater ($P \leq 0.04$) IL-8 expression compared with all other treatments (Table 4.5). When 48-h calf whole blood was exposed to high concentrations of LPS, LTA, or PGN, or low concentrations of LPS or LTA, treatment did not affect ($P \geq 0.22$) IL-8 expression.

Tumor necrosis factor α. When 48-h calf whole blood was exposed to low or high concentrations of LPS, LTA, or PGN, relative mRNA expression of TNFα was not affected ($P \geq 0.28$) by gestational trace mineral treatment (Table 4.5).

Inducible nitric oxide synthase. Source and inclusion of Cu, Zn, and Mn in late gestation affected ($P = 0.09$) relative iNOS mRNA expression when 48-h calf whole blood was exposed to LTA-low condition (Table 4.5). In this condition, calves born to cows fed CTM had greater ($P \leq 0.04$) iNOS expression than CON and ITM calves. Gestational trace mineral treatment tended ($P \leq 0.14$) to affect iNOS expression in LPS-low, PGN-low, and PGN-high conditions. Relative expression of iNOS was greater ($P \leq 0.09$) in calves born to CTM-fed cows than all other treatments in LPS-low condition and greater ($P \leq 0.07$) than CON and ITM calves in PGN-high condition. In the PGN-low condition, CTM and RR calves had greater ($P \leq 0.06$) iNOS expression than CON calves. When calf whole blood was exposed to high concentrations of LPS or LTA, treatment did

not affect ($P \geq 0.26$) relative iNOS mRNA expression.

DISCUSSION

There are few data available on the role of trace minerals in developmental programming in beef calves. The studies regarding gestational and sometimes lactational trace mineral supply that report improved calf growth and performance hypothesize that improvements are due to improved immune system function (Gengelbach et al., 1994; Ahola et al.; Marques et al., 2016; Stokes et al., 2018). However, this hypothesis is typically paired with passive transfer data or morbidity and mortality data, not data that indicates actual immune responsiveness. To the author's knowledge, this study is the first example of how gestational trace mineral supply may affect innate immune response of the neonatal beef calf.

Passive Immune Transfer

Calf serum Ig concentrations were not affected by gestational trace mineral treatment, which indicates passive transfer was not altered. All treatment means were considered to be adequate (> 24 g/L; Waldner and Rosengren, 2009). Boland and others (2005) reported lambs born to ewes consuming high concentrations of trace minerals had less IgG absorption compared with lambs born to control ewes, even if those lambs received colostrum from control dams. This indicates trace minerals supplied to the fetus can affect Ig absorption capacity. Neonatal beef calves born to cows fed a Cu-deficient diet had less serum Ig compared with calves born to cows fed organic Cu supplement in yr 1, however in yr 2 calves born to Cu-deficient dams had greater serum Ig than calves

born to cows fed organic Cu (Muehlenbein et al., 2001). In yr 1 of that study, cows supplemented inorganic Cu had greater colostrum Ig concentrations compared with Cu-deficient cows, and in yr 2 gestational Cu supply did not affect colostrum Ig. Neonatal calves born to beef cows supplemented organic trace minerals (Co, Cu, Zn, and Mn) during the last 3rd of pregnancy had greater serum IgA than calves born to cows that received inorganic trace mineral (Price et al., 2017). In that study, colostrum IgM was greater in cows fed organic trace mineral than cows fed inorganic trace mineral. These data may indicate that neonatal calf serum Ig and colostrum Ig are not parallel when dam are fed adequate or deficient gestational trace mineral diets.

In the current study, colostrum Ig concentration and content (data presented in Chapter 2) and calf vigor (data presented in Chapter 3) were not affected by gestational treatment. This suggests that calf colostrum consumption was likely similar among treatments. However, previous studies suggest colostrum Ig are not always analogous with calf serum Ig; therefore, there may be other factors contributing to these results such as differences in Ig absorption capacity.

Inflammation-related mRNA Expression in Stimulated Whole Blood

Toll-like receptors are evolutionarily conserved transmembrane molecules that are present in macrophages, dendritic cells, and granulocytes (Ozato et al., 2002). They are part of the first line of defense against invasive microbial pathogens and do not require the animal's immune system to be previously exposed because the receptors bind to highly conserved components of these pathogens; thus, TLR are considered part of the innate immune response (Ozato et al., 2002; Takeda and Akira, 2005). The additives used

to stimulate calf whole blood in this method not only bind to TLR but also are representative of pathogens that neonatal beef calves encounter in early life. Lipopolysaccharide is a component in the cell wall of Gram-negative bacteria and binds to TLR4 (Takeuchi et al., 1999). Peptidoglycan and LTA are cell wall components of Gram-positive bacteria and both bind to TLR2 (Takeuchi et al., 1999). Activation of these TLR stimulate transcription of inflammatory cytokines (e.g. IL-1β, IL-6, IL-8, and TNFα) and iNOS through a similar signaling pathway in order to produce an inflammation response (Takeda and Akira, 2005). The inflammation response is reflective of innate immunity; therefore, greater expression of inflammation-related mRNA indicates greater innate immune response.

Trace mineral supply. In the current study, treatment differences in mineral status of the subset of calves sampled for whole blood stimulations were similar to treatment differences in inflammation-related genes. Mineral results suggest calves born to cows fed CTM or RR had improved Cu status and Zn metabolism, and CTM calves had improved Mn status. Likewise, if treatment affected gene expression, CTM and RR calves typically had greater IL-1β, IL-8, and iNOS expression, except for CON calf expression of IL-1β in LPS-low condition. These data suggest that providing a more bioavailable source of Cu, Zn, and Mn in late gestation affects TLR response, or innate immunity, of the neonatal calf.

Trace minerals Cu, Zn, and Mn have different roles in the innate immune response. Zinc is important for the TLR pathway because the pathway relies on rapid Zn signals and Zn finger proteins which are crucial in inflammatory cytokine production regulation (O'Reilly and Moynagh, 2003; Maares and Haase, 2016). Both low and high

concentrations of intracellular Zn have been shown to both reduce and increase the inflammation response of stimulated immune cells; therefore Zn homeostasis has been hypothesized to be crucial for appropriate immune responsiveness (Maares and Haase, 2016). Although the role of Cu is minimal for TLR signaling, Cu status of the animal greatly affects innate immunity through immune cell production and maturation as Cu deficiency can decrease circulating leukocytes, primarily neutrophils, and impair leukocyte maturation and function (Percival, 1998; Stafford et al., 2013). The involvement of Mn in TLR pathways is unclear. High concentrations of Mn that mimicked Mn toxicity resulted in greater iNOS expression in cells from chicken testes (Du et al., 2015) and greater IL-6, TNF, and iNOS production in LPS-stimulated microglial cells (macrophages found in the central nervous system) compared with adequate Mn exposures (Chang et al., 2006; Crittenden and Filipov, 2008). However, there are limited data available on the effects of Mn deficiency on TLR pathways or circulating immune cells.

Trace minerals supplied to immune cells affect TLR pathways differently depending on physiological state of the animal, cell stimulation, and immune cell tissue. Most of the data available on the effects of trace minerals on TLR-activated cytokine expression or production revolve around Zn supplementation or deficiency because the role of Zn in TLR pathways or general immune function is more established than the role of Cu or Mn (Haase and Rink, 2009; Sloup et al., 2017). Feeding weaned pigs a chelated source of Zn compared with Zn sulfate resulted in less expression of TLR4, mediators in the TLR pathway, and inflammatory cytokine production in the jejunum (Hu et al., 2018). In humans, Zn supplementation resulted in less plasma IL-6 (Kahmann et al.,

2008; Bao et al., 2010), greater plasma IL-6 (Mariani et al., 2006; Mocchegiani et al., 2008), and no difference in plasma TNFα (Mocchegiani et al., 2008) in healthy, elderly adults compared with control. Blood immune cells collected from Zn-supplemented humans and stimulated with a TLR agonist resulted in greater inflammatory cytokine production in healthy adult males (Aydemir et al., 2006), elderly adults (Kahmann et al., 2008), and children (Sandstead et al., 2008) than control cells. However, stimulated peripheral blood mononuclear cells (**PMBC**) collected from Zn-supplemented adults had less TNFα and IL-1β expression (Prasad et al., 2004), and from Zn-supplemented healthy, elderly adults had less TNFα production (Prasad et al., 2007) than control PMBC. These studies differed in length and amount of Zn supplementation, whether or not diet of the subject was controlled before trial, and which immune cell cytokine production was measured, which may explain differences in results. In previous data discussed, length of stimulation and cell type differed which could also explain some of the variability. However, it is clear in these studies that Zn greatly affects TLR response, which suggests that altered Zn homeostasis of calves in the current study could have mediated altered calf TLR responsiveness.

Murine macrophages pre-treated with bioactive Cu- or Zn-containing glass had less TNFα production compared with cells that were not exposed to Cu- or Zn-containing glass when stimulated with LPS (Varmette et al., 2009). This study suggests that Cu can also affect TLR response, therefore differences in calf Cu status in the current study could also alter TLR responsiveness.

There are few data available on gestational trace mineral supply and innate immunity of the calf to support data in the current study. Jacometo and others (2015)

reported greater TLR2 expression, but decreased TLR pathway mediators and IL-1β expression in isolated neutrophils collected from neonatal dairy calves whose dams received organic Cu, Zn, Mn, and Co the last 30 d of gestation compared with calves born to cows that received inorganic trace mineral. They hypothesized organic trace mineral calves had a more efficient innate immune system as upregulation of TLR2 was not followed by upregulation of the pro-inflammatory pathway. However, they did not report any liver mineral concentrations, and plasma Cu, Zn, and Mn were not affected by source of trace minerals supplied to the cow. Interpretation of these data differ from interpretation of data in the current study because these cells were not stimulated in an ex vivo setting. Therefore, it could be hypothesized that greater expression of TLR2 in the previous study could lead to greater inflammation-related gene expression if cells were subjected to a TLR2 agonist, which would support some of the data in the current study.

Conditions. Comparing stimulations with low and high concentration of TLR agonists is difficult when species, agonists, and immune cell types differ among studies discussed. However, it is worth addressing that in the current study, high concentrations of TLR agonists used in stimulations only resulted in one treatment effect for iNOS expression in the PGN-high condition. This could indicate that a large pathogen load elicits similar innate immunity responses regardless of gestational trace mineral supply, and a smaller pathogen load is where innate immune responses would differ among treatments.

Neonatal response. Both innate and adaptive immunity are immature in neonates, which is especially true in ruminants (Chase et al., 2008). Therefore, in the current study, interpretation of greater pro-inflammatory cytokines and iNOS must be considered in

terms of an immunologically immature animal.

Neonatal and adult immune cell TLR stimulation and cytokine expression or production have been compared in humans and rodents. When exposed to LPS, whole blood from human adults had greater IL-1β and TNFα production compared with neonatal whole blood (Li et al., 2015), adult whole blood had greater TNFα and IL-12 expression than umbilical cord blood and whole blood from neonates at 1 mo of age (Belderbos et al., 2009), and adult mice macrophages had greater IL-1, IL-12, and TNFα production and expression than neonatal pup macrophages (Chelvarajan et al., 2004). Although data from these studies differ in terms of which cytokines were upregulated or produced in greater quantity, they reached a similar conclusion that less inflammatory cytokine production or expression in stimulated cells indicates impaired TLR response in neonates. Conversely, LPS stimulation of neonatal immune cells for ≥ 20 h has also resulted in greater cytokine production or expression in humans (Levy et al., 2009; Caron et al., 2010), mice (Zhao et al., 2008), and goats (Tourais-Esteves et al., 2008). These studies concluded greater inflammatory cytokines indicated impaired TLR response of neonates due to poor regulation of inflammation cytokine transcription. However, in these studies the LPS stimulation was longer than the previous studies listed; therefore, that is likely an effect of late-phase NF-κB, a mediator in TLR pathways, stimulation instead of early-phase NF-κB stimulation (Kawai and Akira, 2007). In the current study, whole blood was stimulated for 4 h, thus the more accurate interpretation would be greater expression of inflammation-related genes indicates improved TLR response.

In the studies previously discussed, it was hypothesized that impaired TLR response could be a partly responsible for greater neonatal susceptibility to pathogens but

there were no data to support that claim. Wynn and others (2007) injected neonatal and young adult mice with a cecal slurry to mimic polymicrobial sepsis; they found greater susceptibility to peritoneal infection and less plasma IL-1β and TNFα 12 h post-injection in neonatal mice than adult mice. Injection of cecal slurry to these animals likely activated TLR pathways because the slurry contained bacterial pathogens. Therefore, these data indicate less inflammatory cytokine production after stimulation denotes impaired immune function of the neonates, as they were more susceptible to sepsis. Thus, these data provide a connection between impaired TLR response in neonates and greater pathogen susceptibility.

Whole blood TLR stimulation may be a better representation of immune function in neonates than isolated cells. Kollmann and others (2009) reported neonatal whole blood was less responsive to LPS stimulation than adult whole blood, but there were no cytokine production differences between neonatal and adult isolated immune cells (PMBC or conventional dendritic cells). This indicates that there are important regulatory factors present in whole blood that can affect the innate immune response. This is further supported by Levy and others (2004) where TNFα release was greater in neonatal hemocytes subjected to adult plasma than in adult hemocytes subjected to neonatal plasma. Therefore, measuring whole blood inflammatory cytokine and iNOS mRNA expression in the current study more accurately represents innate immune responsiveness in an ex vivo setting.

CONCLUSION

Overall, inclusion of chelated trace minerals in the maternal diet during late

gestation resulted in greater expression of pro-inflammatory cytokines IL-1β and IL-8, and iNOS when neonatal calf whole blood was exposed to low concentrations of TLR agonists. However, passive transfer was not affected by gestational trace mineral supply. Data also suggest inclusion of chelated trace mineral in the diet improved indicators of Cu status, Mn status, and Zn metabolism of calves. This could indicate improved innate immune responsiveness of calves born to cows supplemented chelated trace mineral is partly due to improved trace mineral status of the animal. This improvement in innate immune responsiveness to bacterial pathogens could increase calf survival by decreasing susceptibility to these pathogens typically found in the neonatal environment.

Table 4.1. Cow dry matter intakes and dietary trace mineral provided during treatment period

	Treatment[1]			
Variable	CON	ITM	CTM	RR
DMI, kg				
d 0 to 28 of study	10.7	11.4	10.9	10.9
d 29 to 56 of study	12.2	12.7	12.2	12.2
d 57 to 74 of study	12.4	13.0	12.2	12.5
Calving pens[2]	12.9	13.5	12.9	13.1
Cu intake, mg/kg DM				
Target[3]	—	13.0	13.0	10.0
d 0 to 28 of study	4.5	12.0	15.2	9.7
d 29 to 56 of study	6.7	13.8	15.9	11.1
d 57 to 74 of study	7.0	14.7	16.7	12.4
Calving pens	8.8	17.7	19.6	15.5
Zn intake, mg/kg DM				
Target	—	40.0	40.0	30.0
d 0 to 28 of study	9.8	30.5	32.1	23.0
d 29 to 56 of study	15.2	37.0	39.4	29.7
d 57 to 74 of study	16.9	40.6	41.7	33.7
Calving pens	13.8	36.8	39.7	31.7
Mn intake, mg/kg DM				
Target	—	53.3	53.3	40.0
d 0 to 28 of study	43.2	60.3	64.7	51.3
d 29 to 56 of study	36.2	55.5	61.7	45.0
d 57 to 74 of study	47.4	61.1	65.6	56.8
Calving pens	65.9	65.5	64.1	64.8

[1]Cows were individually-fed basal diet and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[2]Cows were housed in dry lot calving pens by treatment starting d 75 of study (average) until 11.0 ± 3.2 d post-calving, offered ad libitum hay, and pen-fed supplement. Intakes were calculated using estimated hay DMI (1.2% BW NDF intake) and pen-fed supplement DMI.
[3]Target concentrations of diets based on NASEM (2016) gestating cow Cu, Zn, and Mn recommendations and treatment goals.

Table 4.2. Real-time PCR primer sequences[1] for interleukin (IL) -1β, -6, and -8, tumor necrosis factor α (TNFα), inducible nitric oxide synthase (iNOS), and ribosomal protein subunit 9 (S9)

Gene	Sequence (5' to 3')	Melting temperature (°C)	Efficiency, %
IL-1β			98.4
Forward	GGCTTACTACAGTGACGAGAATGAG	56.7	
Reverse	AACCGAGGTCCAGGTGTTG	57.4	
IL-6			100.1
Forward	ATCAGAACACTGATCCAGATCC	54.0	
Reverse	CAAGGTTTCTCAGGATGAGG	52.7	
IL-8			97.4
Forward	GAAGAGAGCTGAGAAGCAAGATCC	57.1	
Reverse	ACCCACACAGAACATGAGGC	57.6	
TNFα			99.9
Forward	CTTCTGCCTGCTGCACTTCG	59.0	
Reverse	GAGTTGATGTCGGCTACAAC	53.4	
iNOS			105.5
Forward	GCAGCGGAGTGACTTTCCAA	58.0	
Reverse	GGATGCCAGGCAAGACTTG	56.5	
S9			101.4
Forward	GAAGCTGATCGGCGAGTATG	55.6	
Reverse	CGCAACAGGGCATTACCTTC	56.6	

[1]Primers previously published (Bruno et al., 2010)

Table 4.3. Effects of Cu, Zn, and Mn source and inclusion during late gestation on 48 h calf serum immunoglobulin (Ig) concentrations

	Treatment[1]					
Variable	CON	ITM	CTM	RR	SEM[2]	*P*-value
IgG, mg/mL	47.9	42.9	39.5	42.8	4.74	0.57
IgA, mg/mL	2.67	3.21	3.84	2.61	0.95	0.68
IgM, mg/mL	2.42	2.13	2.42	1.69	0.51	0.61

[1]Calves born to cows individually-fed basal diet and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[2]48-h calf serum sample n = 11, 8, 9, and 11 for CON, ITM, CTM, and RR, respectively.

Table 4.4. Effects of Cu, Zn, and Mn source and inclusion during late gestation on birth BW and neonatal liver and plasma mineral concentrations of calf subset sampled for ex vivo whole blood stimulation assay

	Treatment[1]					*P*-value		
Variable	CON	ITM	CTM	RR	SEM[2]	Trt	Hour	Trt x Hour
Calf birth BW, kg	37.1	36.5	36.4	38.4	1.9	0.78		
Liver[3] mineral								
Cu, mg/kg DM	196	191	253	251	29	0.18		
Zn, mg/kg DM	161	168	155	210	39	0.66		
Mn, mg/kg DM	8.05[b]	7.59[b]	9.19[a]	7.45[b]	0.48	0.04		
Plasma[4] Cu, ppm						0.92	<0.001	0.31
0 h	0.248	0.284	0.218	0.244	0.046			
48 h	0.342	0.376	0.400	0.394	0.047			
Plasma Zn, ppm						0.82	0.01	0.38
0 h	1.12	1.07	1.05	1.06	0.18			
48 h	0.84	0.69	1.02	0.95	0.15			

[a,b]Indicates treatment means differ ($P < 0.10$)

[1]Calves born to cows individually-fed basal diet supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.

[2]n = 6 calves per treatment.

[3]Liver biopsies collect at 11.0 ± 3.2 d of age.

[4]Mn concentrations were below the detection limit (0.05 ppm) in plasma samples.

Table 4.5. Effects of Cu, Zn, and Mn source and inclusion during late gestation on 48-h calf whole blood relative mRNA expression of interleukin (IL) 1β, 6, and 8, tumor necrosis factor α (TNFα), and inducible nitric oxide synthase (iNOS) when stimulated with Toll-like receptor agonists lipopolysaccharide (LPS), lipoteichoic acid (LTA), or peptidoglycan (PGN) at low and high concentrations

	Treatment[1]					
Variable	CON	ITM	CTM	RR	SEM[2]	*P*-value
IL-1β mRNA expression, $2^{-\Delta\Delta Ct}$						
LPS-low	18.4[a]	10.0[b]	20.8[a]	14.6[ab]	4.0	0.14
LPS-high	142	49	128	114	46	0.30
LTA-low	13.3[ab]	6.3[b]	14.0[ab]	21.1[a]	4.2	0.06
LTA-high	16.1	7.9	15.3	21.3	6.3	0.41
PGN-low	3.46[b]	2.91[b]	4.09[b]	6.47[a]	1.11	0.10
PGN-high	14.0	7.3	12.2	13.1	3.4	0.36
IL-6 mRNA expression, $2^{-\Delta\Delta Ct}$						
LPS-low	37.2	41.5	31.5	25.4	25.6	0.94
LPS-high	1,739	867	1,088	988	879	0.84
LTA-low	40.8	61.9	33.9	53.5	24.1	0.75
LTA-high	82.9	49.7	70.5	95.5	34.8	0.74
PGN-low	21.8	7.5	11.2	18.5	8.6	0.51
PGN-high	97.5	66.5	59.4	82.5	32.3	0.74
IL-8 mRNA expression, $2^{-\Delta\Delta Ct}$						
LPS-low	5.26	4.08	5.75	5.63	1.46	0.75
LPS-high	44.3	28.8	24.8	39.9	13.4	0.54
LTA-low	3.83	3.76	3.22	6.24	1.27	0.22
LTA-high	4.79	4.37	3.62	6.48	1.68	0.54
PGN-low	2.21[b]	1.85[b]	1.84[b]	3.91[a]	0.58	0.04
PGN-high	6.62	4.28	4.82	7.55	1.93	0.45
TNFα mRNA expression, $2^{-\Delta\Delta Ct}$						
LPS-low	15.0	10.5	16.0	9.4	3.5	0.33
LPS-high	19.2	14.1	17.1	16.1	6.3	0.91
LTA-low	10.5	5.6	10.9	11.7	2.9	0.28
LTA-high	8.67	8.19	10.47	10.65	3.66	0.92
PGN-low	2.63	3.33	3.10	4.02	1.09	0.79
PGN-high	7.90	6.36	8.45	8.88	3.12	0.91
iNOS mRNA expression, $2^{-\Delta\Delta Ct}$						
LPS-low	67[b]	137[b]	355[a]	107[b]	106	0.12
LPS-high	2,795	61	852	1,175	1,814	0.63
LTA-low	18.3[b]	52.3[b]	290.4[a]	169.4[ab]	94.8	0.09
LTA-high	25.9	60.6	358.3	180.6	150.1	0.26
PGN-low	-2.4[b]	29.6[ab]	51.8[a]	63.4[a]	22.7	0.14
PGN-high	19.4[b]	85.3[b]	267.9[a]	159.4[ab]	84.1	0.11

[a,b]$P < 0.10$

[1]Calves born to cows individually-fed basal diet and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both

inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[2]48-h whole blood sample n = 6 per treatment.

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Appendix Table 1. Effects of Cu, Zn, and Mn source and inclusion during late gestation on cow plasma mineral concentrations[1]

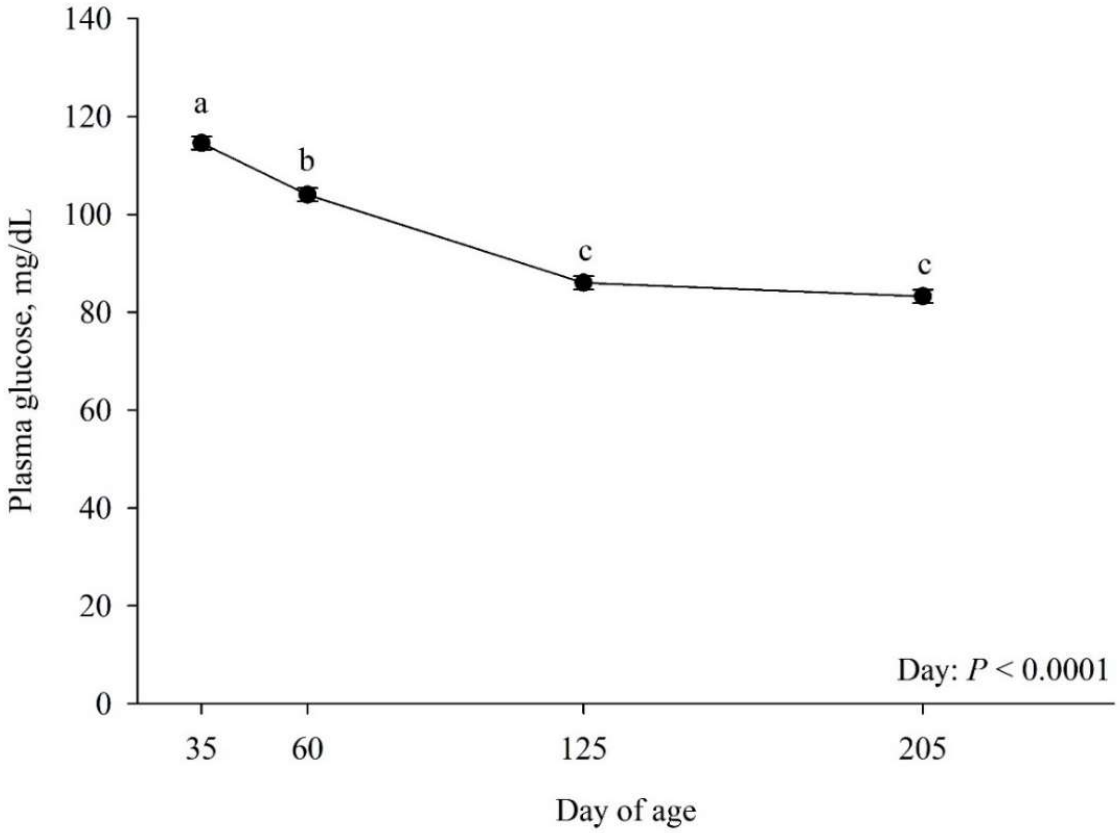
	Treatment[2]					*P*-value		
Variable	CON	ITM	CTM	RR	SEM	Trt	Day	Trt x Day
Gestational								
Cu, ppm						0.65	0.002	0.87
Initial[3]	0.683	0.727	0.663	0.651	0.045			
d 28 of study	0.806	0.742	0.697	0.859	0.104			
d 56 of study	0.765	0.767	0.736	0.776	0.040			
Pre-calving[4]	0.741	0.757	0.692	0.732	0.053			
Zn, ppm						0.22	<0.001	0.42
Initial	0.519	0.449	0.449	0.499	0.056			
d 28 of study	0.676	0.508	0.481	0.721	0.093			
d 56 of study	0.754	0.831	0.777	0.894	0.046			
Pre-calving	0.561	0.622	0.601	0.602	0.054			
1 h post-calving								
Cu, ppm	0.832	0.870	0.765	0.809	0.062	0.59	—	—
Zn, ppm	0.382	0.545	0.395	0.424	0.059	0.11	—	—
Lactational								
Cu, ppm						0.33	0.26	0.67
d 35 of lactation	0.876	0.969	0.881	0.898	0.056			
d 60 of lactation	0.914	0.991	0.979	0.883	0.056			
Zn, ppm						0.62	0.20	0.28
d 35 of lactation	0.599	0.720	0.600	0.681	0.069			
d 60 of lactation	0.623	0.614	0.572	0.550	0.069			

[1]Mn concentrations were below detection limit (0.05 ppm) in all samples.
[2]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.
[3]Study initiation = 91.2 ± 6.2 d pre-calving.
[4]Pre-calving = 17.5 ± 8.0 d pre-calving.

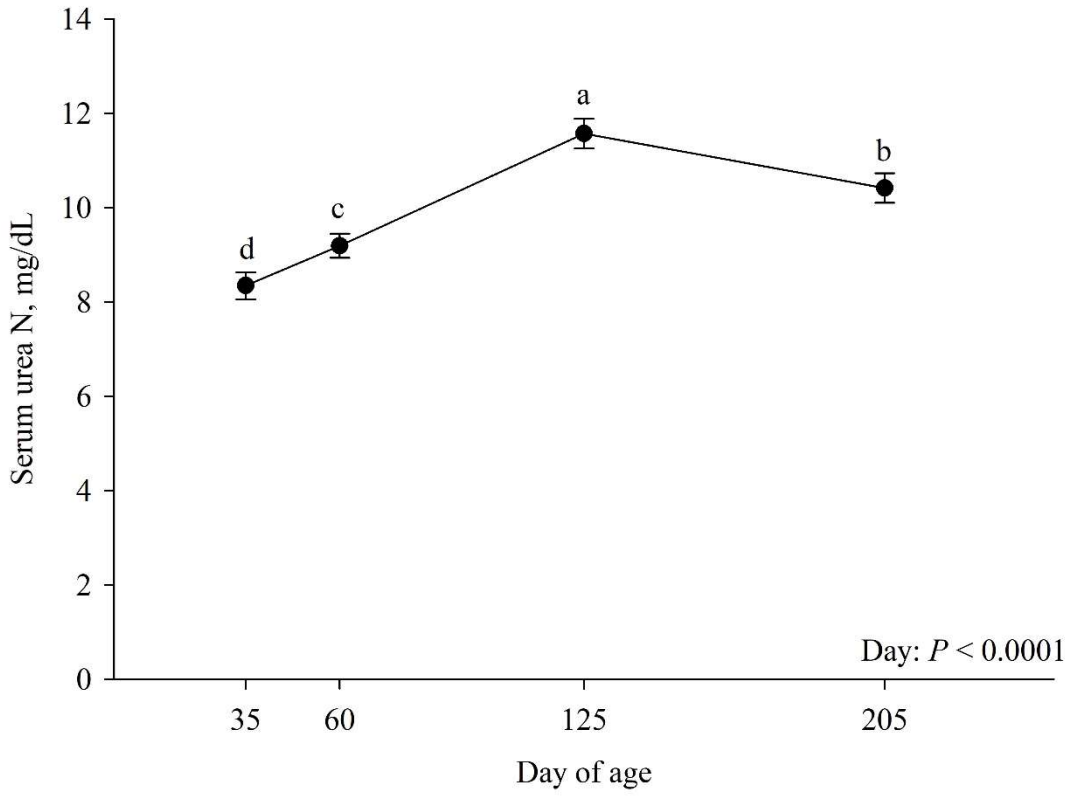
Appendix Table 2. Effect of Cu, Zn, and Mn source and inclusion during late gestation on pre-weaning calf plasma mineral concentrations[1]

	Treatment[2]					*P*-Value		
Variable	CON	ITM	CTM	RR	SEM	Trt	Day	Trt x Day
Cu, ppm						0.93	0.02	0.55
d 35 of age	0.698	0.705	0.679	0.624	0.081			
d 60 of age	0.516	0.580	0.593	0.606	0.049			
Zn, ppm						0.74	0.95	0.47
d 35 of age	0.531	0.498	0.613	0.504	0.088			
d 60 of age	0.547	0.480	0.521	0.586	0.045			

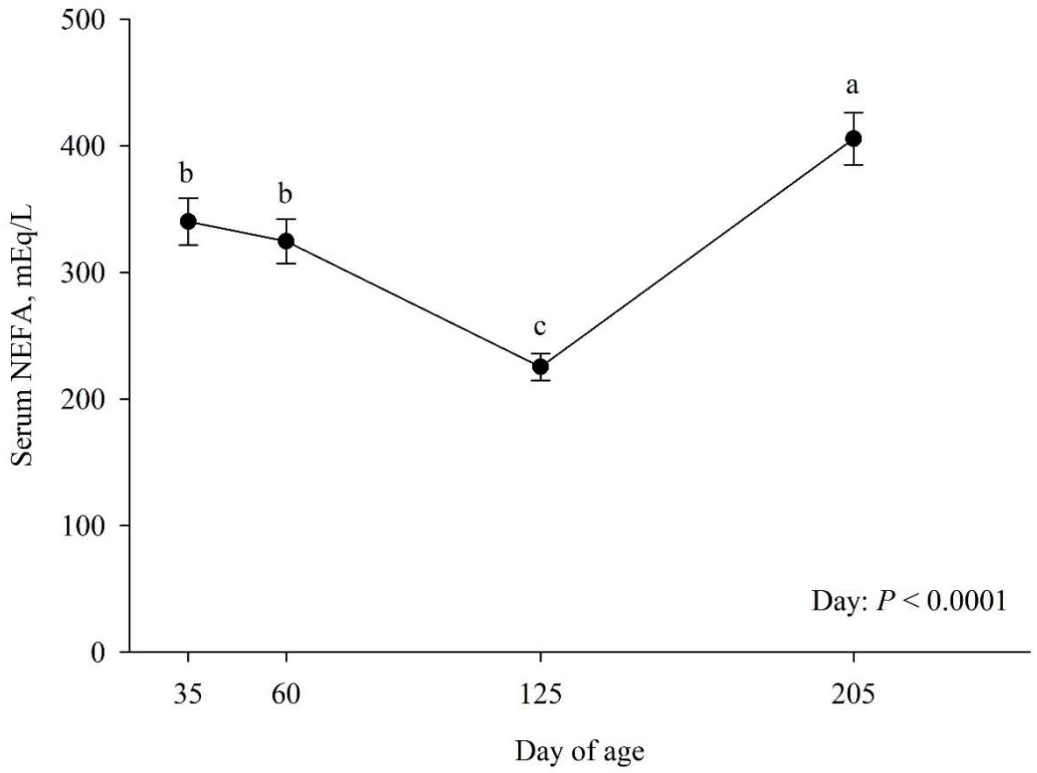
[1]Mn concentrations were below detection limit (0.05 ppm) in all samples.
[2]Cows were individually-fed hay and supplemented with: no additional Cu, Zn, or Mn (control, CON), sulfate-based Cu, Zn, and Mn (inorganic, ITM) or metal methionine hydroxy analogue chelate Cu, Zn, and Mn (chelated, CTM) to meet 133% of requirements, or both inorganic and chelated Cu, Zn, and Mn (reduce and replace, RR) to meet 100% of requirements from 91.2 ± 6.2 d pre-calving until 11.0 ± 3.2 d post-calving.



Appendix Figure 1. Effects of day of age on pre-weaning calf plasma glucose concentrations. Least square means ± SEM are presented (n = 43). [a-c]Indicates means differ ($P \leq 0.05$).



Appendix Figure 2. Effects of day of age on pre-weaning calf serum urea N concentrations. Least square means ± SEM are presented (n = 43). [a-d]Indicates means differ ($P \leq 0.05$).



Appendix Figure 3. Effects of day of age on pre-weaning calf serum NEFA concentrations. Least square means ± SEM are presented (n = 43). [a,b,c]Indicates means differ ($P \leq 0.05$).