Effects of mineral supplementation on growing cattle and *in vitro* fermentation by ruminal microbes

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Abstract

Three studies were conducted to assess effects of mineral supplementation on growing cattle performance, mineral status, and *in vitro* fermentation. Exp. 1 was a 3-part study that measured effects of Cu source and concentration on *in vitro* fermentation by mixed ruminal microbes. An initial *in vitro* experiment was performed to identify a Cu concentration (0, 100, 200, 300, 400, or 500 mg Cu/kg substrate DM) that would yield a 50% decrease in gas production. This concentration (100 mg Cu/kg substrate) was then used to evaluate varying Cu sources in the 3rd part of Exp.1. Titration of Cu (0, 10, 20, 30, 40, 50, 60, and 70 mg Cu/kg DM substrate) linearly decreased ($P < 0.01$) *in vitro* gas production, acetate, and propionate production. Inhibition of ruminal fermentation by Cu sources ($CuSO_4$, $CuCl_2$, $CuCO_3$, CuO, and tribasic copper chloride) also was evaluated using an *in vitro* fermentation system. Sources were incorporated into cultures at 100 mg Cu/kg substrate DM, a concentration great enough to elicit an inhibitory response. Copper sulfate and $CuCl_2$ were more inhibitory to *in vitro* fermentation, as indicated by decreases in gas production, VFA, and IVDMD, and increases in pH ($P < 0.01$). In Exp. 2, heifers were fed 3 different free-choice minerals: salt (S), a dry mineral basemix with salt (M), and a cooked molasses block (B); M and utilized the identical basemix. Mineral source had no effect on DMI, G:F, or concentrations of plasma P and Zn ($P > 0.10$). Average daily gain was greatest for M ($P = 0.03$), and not different between S and B ($P = 0.98$). Liver Cu concentrations were different among treatments ($P < 0.01$), with M having the greatest, B intermediate, and S having the least. Total dietary mineral intake also was different among treatments ($P < 0.01$), and was greatest for M, intermediate for B, and the least for S ($P < 0.01$). Experiment 3×4 factorial design and evaluated minerals added as different supplement types and trace mineral

concentrations (0, 1, 5, or 10×) in an *in vitro* batch culture fermentation. Cooked molasses mineral blocks were compared to a dry mineral premix, and a dry mineral premix + molasses block added separately. *In vitro* fermentation was not different between the two molasses block treatments ($P > 0.01$); however, addition of molasses blocks increased fermentation to a greater extent than dry mineral alone ($P < 0.01$). Increasing trace mineral concentration decreased fermentation linearly ($P < 0.01$). In conclusion, excesses of trace elements can adversely affect fermentation by ruminal microbes. Mineral status in growing cattle was reflective of mineral intake; however, block supplements may be a method to control mineral intake to minimize overconsumption.

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“I can do all things through Christ who strengthens me” Philippians 4:13

Chapter-1 Literature Review: Copper in Ruminant Nutrition

HISTORY OF COPPER

The discovery of Cu dates back to ancient times, and its use was more mechanical than medicinal in early years. Copper was first reported as being required in the diet for basic function by Hart et al. (1928). While seeking methods to alleviate anemia in rats, they discovered both dietary Cu and Fe were important for hemoglobin function and growth. Previously, knowledge of Cu in both plant and animal tissues existed, but no knowledge of its mode of action or function in either clinical medicine or nutrition existed. The first reported copper deficiency was in cattle grazing native rangeland in Florida, otherwise known as “Salt Sick” (Neal et al., 1931). Cattle were characterized by decreases in intake, growth, reproduction performance, and had high mortality rates unless treated with Cu supplementation. Shortly after, similar symptoms were observed in sheep in the Netherlands (lecksucht or “licking disease”; Sjollema, 1933) and Western Australia (enzootic neonatal ataxia; Bennetts and Chapman, 1937); where Cu therapy alleviated disease symptoms. Interactions between Cu and other elements exist, and Mo is the most widely known antagonist. Teart, a disease caused by high Mo intake, was treated with $CuSO_4$. Conversely, low Mo was reported to cause Cu toxicity (Suttle, 2010). Copper, Mo, and S also interact with one another, and relative concentrations of each must be considered when determining supplementation requirements and toxicity (McDowell, 2003). Copper has been reported to be required for enzyme function, Fe function, cardiovascular function, and other important metabolic activities. Because of this, Cu has been established as a required trace element, and should be supplemented in livestock.

PHYSIOLOGICAL ROLE OF COPPER

Enzyme Function

Copper is present in multiple cuproenzymes, although the function of Cu in some of these enzymes is elusive, and enzyme activity is not always a function of Cu status. Uricase is a Cu-containing enzyme that catalyzes oxidation of uric acid, but deficiency of Cu has no impact on concentration or function of uricase. Dopamine-B-hydroxylase is a cuproenzyme that catalyzes oxidation of dopamine to norepinephrine (Sourkes, 1972; Prasad, 1978) and its response to Cu deficiency is ambiguous. There no evidence suggesting Cu deficiency decreases dopamine concentrations (Prohaska et al., 1990); however, there is an overwhelming amount of literature providing evidence suggesting dopamine activity is compromised during a Cu deficient state (Freidman and Kaufman, 1965). Brain and heart norepinephrine are reduced during Cu deficiency (Prasad, 1978), and injection of dopamine in Cu deficient rats has resulted in decreased conversion of dopamine to norepinephrine in comparison to Cu adequate rats (Sourkes, 1972). Copper-containing cytochrome c oxidase is present in mitochondria and is necessary for cellular energy (O'Dell, 1976). Hepatocyte mitochondria are abnormally large and misshapen in moderately Cu deficient rats, and this response is intensified with severe, acute, and chronic Cu deficiency. In extreme cases, morphological changes in mitochondria can squeeze out the endoplasmic reticulum and other organelles (Gallagher et al., 1973). Superoxide dismutase, responsible for catalyzing free radical oxygen conversion into water and O_2, is Cu dependent as well. Although not linked to disorders in vertebrates, superoxide dismutase is present in the cytosol of strictly aerobic microorganisms. Its presence has been documented in the rumen in small amounts, although it is a predominantly anaerobic environment. *Streptococcus bovis*, a facultative ruminal

bacterium, contains superoxide dismutase (McCord et al., 1971). Superoxide dismutase activity has been detected in the cytosol of ruminal obligate anaerobes as well, but in low quantities (Fulghum and Worthington, 1984). This is especially important in maintaining anaerobiosis of the rumen by removal of toxic free radicals from oxygen reduction. Lastly, amine oxidases are a Cu-containing class of enzymes. These enzymes have been isolated from plasma, organs, and connective tissues. Amine oxidases are especially important in connective tissue metabolism, lysyl oxidase being the predominant one in cross-linking of collagen and for elastin integrity (Mertz, 1987; Prasad, 1978). Benzylamine oxidase, an enzyme active in various structural and visceral tissues of chicks, also has decreased activity during Cu deficiencies (O'Dell, 1976).

Cardiovascular System

Copper was first associated with cardiovascular disturbances in Western Australia with the onset of "Falling Disease". This fatal disease, characterized by lesions in cardiovascular tissue, was commonly observed in dairy cattle after strenuous exercise and resulted in sudden death in up to 40% of the cows in some herds (Bennetts and Hall, 1939). Further studies by Bennetts et al. (1948) linked Cu deficiency as the main culprit in Falling Disease. Similar findings have been observed among a wide range of species including swine, rats, mice, and chickens, with multiple forms of anatomical degeneration reported (Klevay, 2000). Chicks fed a Cu deficient diet presented with ruptured blood vessels in 78% of the treatment group, compared to no internal hemorrhaging observed in Cu sufficient chicks (O'Dell, 1961). Histological analyses of vasculature revealed disturbances in the cross-linking of elastin and collagen, with overall weakening of connective tissue (O'Dell, 1961). Starcher et al. (1964) observed that diets containing 25 mg/kg DM added Cu fed to Cu

deficient chicks increased elastin content of the aorta by 12%. Interestingly, Cu deficient chicks had three times the amount of aortic elastin lysine in comparison to control chicks. Lysyl oxidase removes lysine residues in amino acid chains of elastin (Harris, 1976), which explains the relationship between Cu and cardiac hemorrhaging. Superoxide dismutase concentrations are decreased in individuals with hypertension, angina pectoris, and tachycardia. Hypercholesterolemia has been observed in Cu deficient humans as well (Klevay, 2000; Mertz, 1987). It has been suggested that Cu deficiency, as well as energy and mitochondrial activity, may complement each other in the mode of action for Cu-induced cardiovascular disease.

Immune Function

The role of Cu in immune response has been established for quite some time, with early work using mice and rats as models to substantiate relationships between Cu and immunity. Copper deficiency decreases activity and populations of both innate and acquired immune cells, and is closely associated with reduced activity and production of metalloenzymes (McDowell, 2003). A review by Percival (1998) emphasized the need for Cu in multiple species due to manifestation of neutropenia in Cu deficient animals and people. Prohaska and Lukasewycz (1981) exposed Cu deficient mice to ovine erythrocytes to measure humoral immune response. A diminished number of antibody-producing cells in splenocytes as well as a positive correlation between ceruloplasmin and antibody-producing cells were reported in Cu deficient mice in comparison to control counterparts. This same group observed a decrease in liver and thymus weight, an increase in spleen weight, and decreases in both cytochrome oxidase and superoxide dismutase in mice with diet induced Cu deficiency versus Cu sufficient mice (Prohaska, et al., 1983). These data provide

morphological and biochemical bases for Cu-induced immunodeficiency. Copper/zinc superoxide dismutase is an essential metalloenzyme in immune function, as demonstrated by its release in the presence of LPS, as well as its role in TNF-α release, macrophage activation, and ultimately the inflammatory response (Marikovasky et al., 2003). Cell-mediated immune response also is impacted by Cu deficiency, exhibiting neutropenia and decreased lymphocyte activity. Interestingly, Cu deficient mice are characterized by increased B-cell numbers as well as impairment in lymphocyte activity (Lukasewycz et al., 1985). Authors attributed increased B-cell number to two possible mechanisms: 1) atrophy of the thymus impairs regulation of B-cell production or, 2) feedback signaling failure from IgM (Lukasewycz et al., 1985). *In vitro* studies yield similar reductions in neutrophilic and lymphocytic activity of rats (Hopkins and Failla, 1995). A study summarizing immune function in Cu-deficient mice reported the following immunological responses: 1) decreased antibody production, 2) decreased response to LPS and other mitogen assays, 3) increased mortality rates when exposed to tumor cells, 4) decreased IgG concentration, 5) increased IL-1, and 6) decreased IL-2 (Lukasewycz and Prohaska, 1990). Similar observations have been made in domestic animals; copper deficient Friesian steers had as much as a 25% reduction in the ability of neutrophils to kill phagocytized *Candida albicans* in comparison to Cu-sufficient steers (Boyne and Arthur, 1981).

Immune response to Cu in cattle feeding studies is contradictory, and even more so when analyzing source and type of immune challenge. Mohammadi and Sakhaee (2015) observed serum samples randomly obtained from dairy cows, and found that 94.8% of the bovine leptospirosis positive samples were from cows deficient in Cu. Effects of both concentration and source of Cu on immune function have also been widely researched.

Dorton et al. (2003) used phytohemagglutinin to measure cell-mediated immune response and both porcine red blood cell antigen and ovalbumin antigen to measure humoral immune response at 2 concentrations (10 and 20 mg/kg Cu) and 2 sources of Cu ($CuSO_4$ and a Cu-AA complex, Availa-Cu, Zinpro Cooperation, Eden Prairie, MN) in Angus steers. There were no differences in cell-mediated immune response in control steers (no supplemental Cu) compared to steers supplemented with Cu. However, greater cell-mediated immune response was observed in cattle supplemented 20 versus 10 mg Cu/kg diet DM. Humoral response in this study was dependent on time point, type of challenge, Cu source, and Cu concentration. Response to ovalbumin was greater in Cu supplemented cattle only at hour 7; however, at hours 7 and 21 immune response was greater with 20 compared to 10 mg/kg Cu. Total Ig response to porcine red blood cells differed with Cu source, but IgG and IgM concentrations were dependent on source, time, and concentration. It is important to note that plasma Cu concentration was sufficient for cattle in all treatments, indicating no hypocuprosis. In another study, growing cattle displayed no marked cell-mediated immune response; however, humoral response, measured as antibody titers, was greater with Cu supplementation (Ward and Spears, 1999). In contrast to previous research, cattle supplemented with Cu had greater immune response under stress, whereas unstressed cattle had a lower response when supplemented (Ward and Spears, 1999). As mentioned previously, Cu source also may impact immune response. Although no differences in source were observed with respect to therapeutic treatments, re-treatments, or morbidity, Salyer et al. (2004) noted the IgG titer differed in cattle challenged with ovalbumin based on Cu source. Cattle supplemented with $CuSO_4$ had greater IgG titers in comparison to cattle supplemented with a polysaccharide mineral complex. In contrast to many of the above studies, Ahola et al. (2005) observed no

effects of Cu source or Cu supplementation on cell-mediated or humoral immune response; however, when analyzing Cu status, it was apparent that the basal diet supplied adequate Cu, perhaps explaining lack of a supplementation effect.

There is a clear and definitive relationship between Cu and immune function, but mechanisms that explain the relationship remain elusive. Based on past and current literature, extreme Cu deficiency is the most conclusive method to study immune response, and may be necessary in further research with livestock. Cattle that are marginal or sufficient in Cu status but differ in overall bodily Cu concentration often are not immunosuppressed. Evaluating effects of Cu on immune function may not be detectable unless subjects are extremely Cu deficient.

Iron Metabolism

Copper is necessary for Fe absorption and transport, and is required for Fe to synthesize hemoglobin. Its role in hemoglobin synthesis and Fe absorption explain symptoms of anemia when Cu is deficient, and why Fe alone cannot alleviate these symptoms (Hart et al., 1928; Lee et al., 1968). Iron absorption is mediated by ceruloplasmin, also known as ferroxidase, a Cu-containing enzyme. There is evidence to suggest that ceruloplasmin is necessary for modification of Fe to the ferric form, facilitating transport by transferrin from the intestinal mucosa to blood (Prasad, 1978). Typically, administration of ceruloplasmin and/or high concentrations of Cu results in an almost instantaneous spike in blood Fe (Ragen et al., 1960; Lee et al., 1976).

Wilson's disease contradicts ceruloplasmin's sole role in anemia. Although characterized by low concentrations of Cu and ceruloplasmin, anemia and low plasma Fe are not associated with Wilson's disease (Lee et al., 1976). Lee et al. (1968) suggested another

mode of action might be associated with anemia in Cu deficiency. They observed accumulation of Fe in normoblasts during Cu deficiency, suggesting that Fe absorbed into blood was not incorporated into hemoglobin (Goodman and Dallman, 1969). Copper deficient rats (0.5 mg/kg dietary Cu) administered Fe intramuscularly had Fe present in storage vesicles of both erythroblast and reticulocytes; however, no Fe was detected in mitochondria. These results were consistent with work of Lee et al. (1968), who suggested two mechanisms: 1) the absence of ceruloplasmin, and 2) decreased cytochrome oxidase (Goodman and Dallman, 1969). Cytochrome oxidase is a catalyst for energy production necessary for Fe transport into mitochondria, the absence of which impairs heme production. A long-term study using Angus calves from birth to harvest reported that Cu deficiency induced lower Fe concentration in blood, higher tissue Fe, and lower ceruloplasmin. From a regulatory standpoint, hepatic hepcidin, a protein that regulates Fe metabolism, was decreased, and there was a tendency for decreased hepatic ferroportin with Cu deficiency (Hansen et al., 2010). Hepcidin is up regulated by Fe and binds to ferroportin, a critical Fe exporter necessary for intestinal Fe absorption, and decreases Fe metabolism (Gulec et al., 2014). Therefore, Cu deficiency not only impacts Fe metabolism by inhibiting Fe modification for absorption, but also has inhibitory effects on Fe transport mechanisms.

Hair and Wool

Copper is known to play a role in pigmentation, texture, and structure of hair and wool in rabbits, rats, cattle, sheep, dogs, and guinea pigs (Mcdowell, 2003; Mertz, 1987; Suttle, 2010). Melanin formation is initially catalyzed by tyrosinase, a Cu-containing enzyme (Hearing, 1991). Tyrosinase catalyzes oxidation of tyrosine to DOPA and from DOPA to dopaquinone. These are the initial reactions in pheomelanin and eumelanin production; the

melanins responsible for the reddish and black pigments, respectively (Seo, 2007). Hansen et al. (2009) observed that growing Angus calves both Cu deficient and Cu deficient with supplemental Mn (7 mg Cu/kg) had reductions in pigmentation of hair in comparison to control calves. Dutch rabbits also presented with achromotrichia when fed a Cu deficient diet consisting of milk and supplemental Fe and Mn; with no achromotrichia in rabbits supplemented with 0.4 mg Cu/day (Smith and Ellis, 1947).

The role Cu plays in the texture of hair and wool is through the process of keratinization. Copper deficiency decreases disulfide groups in wool, which are necessary for the conversion of prekeratin to keratin through keratin cross-linking. "Steely" wool is a condition where the wool is wiry and crimp of wool is diminished, symptomatically similar to Menkes' kinky-hair syndrome in people. Copper deficient sheep express markedly improved texture, density, and elasticity of wool when treated with Cu, and poultry that are given Cu supplements show improvements in feather quality (Marston, 1952).

ABSORPTION OF COPPER

Luminal Absorption

The absorption of Cu primarily occurs via luminal enterocytes in the small intestine, although absorption can also occur in the stomach and large intestine (Cousins, 1985). In ruminant animals, modification by microbes can have an impact on intestinal absorption of Cu, where a majority of Cu absorption occurs (McDowell, 2003). There are multiple protein transporters that facilitate movement of Cu the brush border membrane of enterocytes, the most important of which is Ctr1 (Cu transporter 1). In cell culture, Ctr1 plays a physiological role in Cu absorption, due to its high affinity for Cu. *In vivo*, Ctr1 knockout mice displayed a disruption in intestinal Cu absorption resulting in Cu deficiency, thus inferring the mediatory

role of Ctr1 in enterocyte Cu import (Nose et al., 2006). Copper transporter 1 regulation in the small intestine has been extensively studied, and Cu seems to play an integral role in its expression. Kuo et al. (2005) studied effects of Cu deficient and Cu adequate diets on presence of Ctr1 in multiple tissues of mice. Copper deficient mice displayed decreased Cu concentration in tissue and signs of anemia. Results of an immunohistochemical analysis revealed intestinal Ctr1 was markedly greater in Cu deficient mice compared to Cu adequate mice. More recently, work by Nose et al. (2010) reported that apical Ctr1 activity is increased under conditions of restricted Cu in mice in comparison to Cu adequate counterparts.

Other trans membrane proteins have been suggested (Prohaska, 2008; Van Den Berghe and Klomp, 2009) in Cu uptake, including Ctr2 (Cu transporter 2) and DMT1 (divalent metal transporter 1). Van Den Berghe et al. (2007) used lysosomes and endosomes in cell culture in an attempt to determine activity of Ctr2 with respect to Cu uptake. The authors observed that Ctr1 and Ctr2 reacted similarly to a reporter created to detect Cu sensitivity, and suggested Ctr2 may be a transporter important for intestinal absorption. Recently, Ctr2 has been linked to Cu uptake and regulation (Bertinato et al., 2008) as well as intracellular action (Van Den Berghe and Klomp, 2009). Divalent metal transporter 1, typically associated with Fe absorption and transport, also has been associated with Cu absorption along brush border membranes (Van Den Berghe and Klomp, 2009). In contrast, Knöpfel et al. (2005) suggested an ATP-associated transporter rather than DMT1 as an additional Cu transport mechanism. Copper transporter 2, DMT1, and ATP-driven transport are controversial, with research being variable, thus meriting further investigation. Factors affecting luminal Cu absorption include age, species, diet, amino acids, chemical form, and antagonistic substances such as ascorbic acid, phytates, S, Mo, Fe, Zn, and Ca. The Cu-

thiomolybdate complex, resulting from increased Mo, limits absorption of Cu the epithelium, resulting in increased fecal excretion of Cu in ponies (Cymbaluk et al., 1981). Sulfur has negative impacts on Cu bioavailability and absorption due to formation of copper sulfide (CuS), which is insoluble in the rumen and cannot be absorbed (Prasad, 1978; Suttle, 2010). Similar to Mo, high S results in increased Cu excreted as unabsorbed Cu. Antagonism of Fe is also of interest in Cu absorption due to high soil Fe concentration in some regions. Excess Fe supplementation decreases Cu absorption, increases Cu depletion (Humphries et al., 1983; Suttle and Peter, 1985) and decreases Cu status (Gould and Kendall, 2011). Mechanisms for Fe antagonism include insoluble Fe-Cu complexes (Suttle and Peter, 1985; Gould and Kendall, 2011) and negative impacts on DMT1 activity, possibly by competition between Cu and Fe for transport (Arredondo and Núñez, 2005). Similarly both ascorbic acid and phytates result in limited absorption due to their ability to complex with Cu (Prasad, 1978). Zinc and Ca both compete with Cu for binding sites along the brush border membrane of intestinal enterocytes (Cousins, 1985). Additionally, both protein and amino acids positively influence Cu absorption (Cousins, 1985).

Intracellular Regulation

Upon entry into intestinal enterocytes, intermediate modification, transport, as well as regulatory mechanisms occur. Copper ions are toxic, and therefore are bound to amino acids and peptides within the cell to alleviate toxic effects. There are two mechanisms for regulation of Cu export within cells: 1) metallothionein, and 2) ATP7A (copper-transporting ATPase 1). Metallothionein is present in both enterocytes and hepatocytes and is the oldest known mechanism for Cu and Zn regulation due to its nature as an inducible protein. Metallothionein is a 61 amino acid peptide mainly characterized by cysteine residues and is

found in both enterocytes and hepatocytes (Kägi and Kojima, 1987). Copper often binds to metallothionein, and when bound it cannot be exported from cells. Due to its inability to dissociate from metallothionein, Cu cannot bind to transporters. This regulatory mechanism prevents excessive Cu from plasma transport and storage in the liver (Cousins, 1985). Metallothionein bound Cu is excreted when enterocytes slough off into the intestine and cannot be reabsorbed (van den Berghe, 2009). The expression of metallothionein is increased by increasing Cu, and is down regulated when Cu concentrations are low (Andrews, 2000; Jacob et al., 1999). Similar activity is observed in hepatocytes. Sheep are more sensitive to Cu than other ruminants, and this is explained by differences in response to Cu in sheep metallothionein. Metallothionein in sheep is not produced in sufficient amounts to compensate for excess dietary Cu in the same way that occurs other ruminant animals. Saylor et al. (1980) observed an increase in sheep hepatic metallothionein as dietary Cu increased from deficient to normal concentrations; however, increasing Cu supplementation above normal concentrations resulted in no change in Cu associated with metallothionein. Intestinal metallothionein did not follow a similar trend. Very little Cu was associated with sheep intestinal metallothionein, further indicating a limited capacity for Cu tolerance in sheep.

Copper-transporting ATPase 1 is an intracellular transporter responsible for Cu transport to the trans-Golgi network, and relies on hydrolysis of ATP to be functional. Within the Golgi apparatus, Cu is used for biosynthesis of Cu-containing enzymes such as lysyl oxidase or cytochrome c oxidase (Prokaska, 2006). These processes are aided by a Cu chaperone ATOX1, and are necessary for Cu transport (Lutsenko et al., 2007). Copper-transporting ATPase 1 is also located on the basolateral membrane and its main function is Cu export.

Basal Membrane Export

As discussed above, ATP7A plays a critical role in export of Cu from enterocytes. Currently, it is the only known transporter responsible for Cu export, and is necessary to maintain adequate Cu status. Menkes' Disease is caused by a mutation in ATP7A expression, resulting in Cu deficiency in young children. Early work with Menkes' Disease revealed that patients lacked Mc1, a gene necessary for copper-transporting ATPase (Vulpe et al., 1993). The expression of Mc1 was abnormal in 70% of individuals diagnosed with Menkes' Disease (Kaler, 1998). There are no indications that Cu induces gene expression of ATP7A, but Cu does result in translocation of ATP7A from intracellular action to membrane-associated action. Petris et al. (1996) reported ATP7A (referred to as MNK in the literature) differed in its location within cells based on Cu concentration. Copper-transporting ATPase 1 was predominantly found near the Golgi apparatus, and was immediately re-located to the basolateral membrane under conditions of elevated Cu concentration. Once Cu concentration was restored to normal ranges, ATP7A was once again concentrated near the Golgi apparatus. Similarly, in an *in vivo- in vitro*-coupled study, ATP7A was concentrated within intracellular regions in close proximity to the Golgi apparatus under normal Cu conditions. However, an increase in Cu *in vivo* and *in vitro* resulted in redistribution of ATP7A from intracellular regions to basolateral membranes of enterocytes (Nyasae et al., 2007). Therefore, ATP7A is a dual function transporter that differs in region and function based upon Cu concentration within both the intestine and enterocyte, and has functions in intracellular regulation and basal membrane export of Cu.

COPPER METABOLISM

Transport

After Cu is transported across the basolateral membranes of enterocytes it is deposited in blood and transported to the liver. Plasma Cu is bound in the blood in three fractions: albumin-bound, amino acid-bound, or bound to ceruloplasmin (Cp). Early work with Cu transport by Gubler et al. (1953) discovered there was a loosely bound fraction of plasma Cu that reacted directly with sodium diethyldithiocarbamate, while ceruloplasmin did not react with this compound. When administered orally and intravenously, Cu increased this same fraction directly, whereas ceruloplasmin remained relatively constant. This fraction was later defined as albumin bound Cu; which is affected by dietary influx of Cu and transports excreted Cu from enterocytes directly to the liver. Cartwright and Wintrobe (1964) reported concentrations of 7 μg/100 mL of albumin-bound Cu in comparison to 33 μg/100 mL ceruloplasmin. These results are consistent with literature; ceruloplasmin accounts for a majority of plasma Cu, approximately 95%, in ruminants (Suttle, 2010). Neumann and Sass-Kortsak (1967) reported that Cu also binds to amino acids after being secreted by enterocytes. The addition of amino acids to albumin-free, centrifuged plasma resulted in an increase in bound Cu. This trend continued as amino acid concentrations increased. These results suggest a competition between albumin and amino acids to bind with Cu. In this same study, it was observed that histidine and glutamine bound with Cu to the greatest extent (Neumann and Sass-Kortsak, 1967) in comparison to other amino acids. Both of these minor fractions are responsible for transporting Cu to the liver to be stored in hepatocytes and bound, incorporated into ceruloplasmin, or used for synthesis of metalloenzymes.

Ceruloplasmin is a transporter protein responsible for tissue specific transport, and as discussed previously, comprises the majority of Cu found in blood (McDowell, 2003). The first reported existence of ceruloplasmin and its properties was by Holmberg and Laurell (1948) in Sweden. Their early work established ceruloplasmin as the major Cu-containing protein in plasma, which was further emphasized by Cousins (1985). In addition to Cu transport, ceruloplasmin oxidizes Fe (II) for iron transport, and oxidizes amines. Ceruloplasmin also functions as a free radical scavenger and plays a regulatory role in immune function through the inflammatory response (Cousins, 1985). Similar to intestinal enterocytes, Cu is stored in the liver by incorporation into metallalothionein or other Cu pools within hepatocytes. To export Cu from the hepatocytes, Cu is incorporated into ceruloplasmin. Terada et al. (1995) used liver samples from rats and determined which cellular compartment contained the highest concentrations of Cu and ceruloplasmin. Their results indicated that the Golgi apparatus is the site of Cu incorporation into ceruloplasmin. Copper initially is transported by the chaperone ATOX1, which then delivers Cu to ATP7B (Copper-transporting ATPase 2; Prohaska, 2008). Copper-transporting ATPase 2 transports Cu to the Golgi apparatus where it can be incorporated into ceruloplasmin and secreted from the hepatocyte. Translocation of ATP7B to the apical membrane occurs within the hepatocyte, analogous to ATP7A in enterocytes for Cu efflux (Fontaine et al., 2007). Mutation affecting the function of ATP7B is the primary factor causing Wilson's disease, which is characterized by toxic concentrations of Cu stored in the liver (Cox and Moore, 2002). The relationship between Cu status and ceruloplasmin concentrations has been investigated by Owen (1965) using laboratory rats. Upon intravenous exposure to Cu, tissue Cu concentrations were quickly allocated from blood to the liver. This rapid accumulation of

Cu in tissues corresponded to Cu incorporated into ceruloplasmin, in comparison to non-ceruloplasmin Cu. These observations suggest that ceruloplasmin is, in fact, responsible for tissue specific Cu transport. Further, serum Cu and ceruloplasmin were highly positively correlated in 790 healthy adult humans, which had a Cu/Cp ratio of 5.8. These same authors calculated that of the fractions of Cu in serum, less than 1.3 µmol/L is within the non-ceruloplasmin Cu fraction (Arredondo et al., 2008). Although ceruloplasmin appears to be the most common transporter of Cu, there are tissue specific preferences for the form in which Cu is presented. For example, in a review by Linder et al. (1998), many reproductive associated tissues (i.e. placenta, fetus, fetal liver, and uterus) prefer Cu in an alternate form. Serum Cu and Cp had correlation coefficients of 0.83 and 0.92, in both cattle and sheep, respectively. This correlation was observed at both low and high Cu concentrations. Bovine plasma Cu was also correlated to Cp, with a correlation coefficient of 0.60. However, liver Cu and Cp activity were not highly correlated, which was unexpected since the liver is the site of Cp production (Blakely and Hamilton, 1984)

Storage

The primary site of Cu storage is in the liver, whereas plasma Cu represents only a small fraction of total Cu within the body. Albumin- and amino acid-bound Cu is responsible for transport to the liver, and similar to enterocytes, transporter proteins regulate entry of Cu into hepatocytes. Copper is reduced and Ctr1 imports Cu into cells through apical membranes, whereas intracellular transporters such as COX17 and ATOX1 direct Cu to mitochondria or the Golgi apparatus, respectively (Roberts and Sarkar, 2008). Within hepatocytes Cu is stored in proteins, used to synthesize enzymes, or excreted via bile. Rats injected with Cu had rapid accumulation of liver Cu within metallothionein. Over time the

fraction of Cu associated with metallothionein was diminished as a result of incorporation into other enzymes of heavier molecular weight, implying a sequence of events in which metallothionein is the mediator of (Van den Hamar, 1975). Rapid accumulation of Cu into the liver also was observed by Owen (1965). Rats injected with radioactive Cu presented with a sharp increase in hepatic Cu within 30 min, with a maximum concentration observed from 2 to 4 h.

Copper accumulation in the liver makes it the optimal tissue for determining Cu status. Mulryan and Mason (1992) compared various plasma parameters to liver Cu to determine the reliability of other forms of Cu status detection. Using liver samples, 14 of the 98 cattle used in the study had > 20 mg/kg DM Cu, whereas of those same animals, only 5 could be detected as deficient with respect to plasma Cu (0.5 mg/L). Claypool et al. (1975) used the relationship between liver Cu and plasma Cu in an attempt to develop a best fit curve, and despite the fact that plasma Cu was unsatisfactory in predicting liver Cu, it can still be used to distinguish critically deficient animals. Although a difficult measurement to obtain in the field, liver Cu would be ideal for identifying deficient animals; however, plasma Cu frequently is used as a quick method to detect extreme Cu deficiencies in commercial settings.

Excretion

Copper is excreted predominantly through feces, and the source of fecal Cu is normally either unabsorbed Cu or absorbed Cu that has been excreted into bile (Mertz, 1987; McDowell, 2003). When normal human subjects were administered radioactive Cu via oral gavage, 72.4% of the Cu was found in the feces and only 0.1% was excreted in urine (Bush et al., 1955). In a study using human subjects, Cartwright and Wintrobe (1964) observed that

approximately 32% of Cu is absorbed, 26% is excreted in bile, 6% is unabsorbed and excreted directly into feces, and 1.2% of Cu is excreted in urine. Mahoney et al. (1955) used normal dogs, dogs with ligated and obstructed bile ducts, and dogs that had bile diverted into the urinary bladder to study route of Cu excretion. In this study, 7 to 10.8% of administered radioactive Cu was excreted in bile, 1.5% of fecal Cu was directly from Cu absorbed through the intestinal wall, and 0.6% was excreted in urine. In this same study, animals that had disruptions in the biliary system had increased excretion via the urinary tract (kidney) and through the intestinal wall. Similar results have been observed among multiple species, including pigs and poultry (Bowland et al., 1961; Beck, 1961). When cattle were fed a low Cu diet, they exhibited a decrease in rate of biliary Cu excretion in comparison to cattle fed a high Cu diet (0.13 mg $6h^{-1}$ and 0.19 mg $6h^{-1}$, respectively). In this same study, both Mo and S resulted in a greater biliary Cu excretion when fed in conjunction with a high Cu diet (Gooneratne et al., 1994). Similar results were observed in sheep that were supplemented with additional Mo and sulfate, resulting in increased fecal and urinary Cu loss (Smith et al., 1968). Increased Cu excretion may contribute to Cu deficiency symptoms seen in cattle that are fed high Mo or S diets. It is evident that Cu excretion is a function of dietary Cu concentration as well as concentrations of dietary antagonists, such as Mo, S, and Fe.

RUMINAL FERMENTATION

The roles that Cu plays in the rumen are numerous, and often are misunderstood and contradictory according to current literature. Not only do ruminants themselves require metals such as Cu, but Cu also is required by ruminal organisms. Copper can interact with Mo and S within the rumen, impact fermentation by influencing microbes, impact microbial enzyme formation, and can impact digestion. McNaught et al. (1950) observed decreased

protein synthesis from 12 to 0.9 mg N/100 g ruminal fluid when 10 mg/kg of Cu as $CuSO_4$ were added to *in vitro* cultures containing strained ruminal fluid. In the same *in vitro* study, 25 mg/kg Cu completely inhibited growth of ruminal microbes. To determine effects that Cu has on cellulose digestion in the rumen, Hubbert et al. (1958) conducted an *in vitro* fermentation study using ruminal microorganisms in a washed suspension that was collected from cannulated steers on a corncob ration. Hubbert et al. (1958) observed an added Cu concentration of 1.5 µg/mL resulted in a decrease in cellulose digestion, and 2.5 µg/mL added Cu inhibited cellulose digestion by 96.6%. Martinez and Church (1970) observed decreased cellulose digestion at 1 µg/mL, but observed no further effects when Cu concentration was increased to 30 µg/mL. Salsbury (1973) reported a reduction in methane production in a fermentation vessel inoculated with ruminal fluid at a concentration of 50 mg/kg Cu. Forsberg (1978) conducted a study to test complete fermentative activity (using gas production as the response variable) and microbial response from varying concentrations of added Cu as $CuCl_2$. Forsberg (1978) observed an LD50, based on fermentative activity, at 21 µg/mL. A concentration of 10 µg/mL inhibited *B. succinogens* and *B. amlophilus*, 20 µg/mL Cu inhibited *R. albus* and *E. ruminantium*, 30 µg/mL inhibited *B. fibrosolvens*, 100 µg/mL inhibited *S. ruminantium* and *M. elsdenii*, and *S. bovis* was the most resistant to Cu and wasn't inhibited until Cu concentration reach 250 µg/mL.

In an attempt to defaunante the rumen, Essig et al. (1972) fed $CuSO_4$ to cattle at 44 mg/kg BW (approximately 17.2 mg Cu/kg BW). Although defaunantion was not successful, those treated with $CuSO_4$ had reduced numbers of protozoa and decreased concentrations of acetate, propionate, butyrate, and total ruminal VFA. Solaiman et al. (2007) collected ruminal fluid using a stomach tube in goats on diets containing Cu added at 0, 100, or 200

mg/kg diet DM. In contrast to Essig et al. (1972), Solaiman et al. (2007) reported only a tendency for high concentrations of Cu to decrease ruminal protozoa numbers. They also reported no differences in total VFA or individual VFA proportions. The above studies represent non-physiological dietary concentrations of Cu in order to see a response in protozoa. More important are responses in fermentation under conditions of normal concentrations of supplemental Cu. Váradyová, et al. (2006) conducted a grazing study with sheep that were grazing pastures with soils contaminated by heavy metals, which were compared to sheep grazing uncontaminated pastures. Although the soil contained toxic concentrations of Cu, 232.9 mg Cu/kg soil DM, the forage contained 7.02 mg Cu/kg DM. Ruminal fluid was collected after 1 year of exposure during the slaughter of the sheep and incorporated into an *in vitro* fermentation model. In comparison to unexposed sheep, authors observed a decrease in gas production, methane production, IVDMD, total VFA, acetate, and butyrate in response to fermentation of ruminal fluid collected from sheep exposed to pastures contaminated with Cu. Ruminal fluid from contaminated pastures decreased propionate production *in vitro*. Engle and Spears (2000a) reported no response in pH, IVDMD, or total VFA production during a 12-h *in vitro* fermentation of ruminal fluid collected from Angus steers fed 0, 10, or 20 mg/kg supplemental Cu. Ruminal fluid from steers fed 0 mg/kg supplemental Cu had decreased soluble Cu in comparison to steers fed 10 and 20 mg/kg supplemental Cu. In contrast, Vazquez-Armijo et al. (2011) reported greater 96-h *in vitro* gas production, IVDMD, ME, and VFA in ruminal fluid from goats fed 21.7 mg Cu/kg diet DM compared to goats fed 10.3 mg Cu/kg diet DM. This discrepancy may be due to differences in fermentation duration or species differences. Arthington (2005) observed decreases in digestibilities of NDF and CP and a tendency for decreased ADF digestibility in

calves that were supplemented with a Cu bolus containing 12.5 g of CuO in comparison to calves without boluses. Both treatment groups were consuming *ad libitum* limpograss hay containing 8.65 mg/kg Cu. There was, however, no difference in total tract OM digestibility between the two groups. Arthington (2005) attributed decreases in digestibility to ruminal that may have impacted fiber degradation. In contrast, Zhang et al. (2011) observed a positive effect of Cu supplementation in cashmere goats. Addition of Cu to the diet improved digestion of both NDF and ADF. A study from the same group supplemented 0, 10, 20, or 30 mg/kg Cu for 50 d, with a 10-d metabolism period. Copper did not influence CP, DM, or ADF digestibility, but NDF digestibility was decreased with 30 mg/kg Cu (Zhang et al., 2009). Lopez-Guisa and Satter (1992) observed increased *in situ* DM disappearance of alfalfa hay, corn cobs, and corn stalks from rumens of Holstein cows given 12.22 mg Cu/kg diet DM versus those supplemented with 2.29 mg Cu/kg diet DM.

It is evident from literature that Cu is required for microbial fermentation and digestibility; however, excessive concentrations can cause modification within the rumen that may adversely affect microbial populations, digestibility of nutrients, and products of fermentation.

COPPER REQUIREMENTS

The Nutrient Requirements of Beef Cattle (National Academies of Sciences, Engineering, and Medicine, 2016) recommends 10 mg Cu/kg diet DM, provided that S and Mo concentrations do not exceed 0.25% and 2 mg/kg diet DM, respectively. The Nutrient Requirements of Dairy Cattle (NRC, 2001) recommends between 73 to 313 mg Cu/day, depending on stage of gestation. Based on average feed intake for the stage of life the cow or heifer is in, Cu requirement can vary from 12 to 15.7 mg Cu/kg diet DM (NRC, 2001).

Dietary Cu can be derived from forages and concentrates in the diet or from mineral supplements. Alfalfa hay contains about 7.82 ± 1.89 mg Cu/kg, whereas fresh alfalfa contains approximately 8.31 ± 2.26 mg Cu/kg (National Academies of Sciences, Engineering, and Medicine, 2016); fresh forages typically have greater Cu content than their dried equivalents. Legumes typically contain greater concentrations of Cu, whereas grasses such as brome grass hay contain, on average, 5.96 ± 7.04 mg Cu/kg (National Academies of Sciences, Engineering, and Medicine, 2016). Cereal grains are relatively low in Cu content and byproduct feeds have greater concentrations of Cu. Liver and blood commonly are used to establish Cu status in cattle. Normal liver Cu values are within the range of 100 to 400 mg/kg tissue DM and tissue concentrations less than 20 to 75 mg/kg tissue DM are indicative of critical deficiencies (National Academies of Sciences, Engineering, and Medicine, 2016; Mertz, 1987; McDowell, 2003; Smart el al., 1992). Plasma Cu is a less invasive method to assess Cu status, with a normal range of 0.5 to 1.5 µg/mL (Mertz, 1987; McDowell, 2003) and with deficiencies at less than 0.6 µg/mL (National Academies of Sciences, Engineering, and Medicine, 2016; Smart el al., 1992).

Deficiency

The first published report of Cu deficiency was by Hart et al. (1928). Copper deficiency can occur due to dietary deficiencies, genetic defects (i.e. Wilson's disease), or excesses of dietary antagonists such as Mo and S. Both compounds are more prominent in grazing scenarios. Increased S and Mo in ruminant diets result in formation of ruminal thiomolybdates and sulfides, which negatively impact absorption of Cu (Mason, 1981). Symptoms of Cu deficiency include anemia, ataxia, bone development disorders, cardiovascular disorders, diarrhea, decreased growth performance, and hair pigmentation

abnormalities (Suttle, 2010; National Academies of Sciences, Engineering, and Medicine, 2016). Mills et al. (1976) reported Fresian calves fed a Cu deficient diet (0.8 mg/kg diet DM) for a prolonged period (257 to 259 d) exhibited swelling and abnormal gaits; postmortem conformation abnormalities in their skeletons; changes in hair pigmentation; diarrhea, which was resolved within 12 h of Cu administration (10 mg); weakening of cardiovascular tissue; a decrease in weight gain after 201 d of the experiment and a reduction in feed efficiency. Thornton (1972) investigated impact of Cu deficiency caused by Mo toxicity on growth performance, and reported increases of 15.9 to 29.9% in weight gain after injection of Cu.

Toxicity

Unlike monogastric livestock, ruminants have a lower tolerance for dietary Cu. Excessive accumulation of Cu in the liver can occur before onset of symptoms. Of all ruminant species, sheep are most sensitive to Cu toxicity because of their reduced ability to excrete Cu via and inherent differences in metallothionein regulation. Copper toxicity is not commonly reported in beef or dairy cattle. Copper toxicity rarely occurs in humans and is otherwise known as Wilson’s disease (Cox and Moore, 2002). The maximum tolerable dietary concentration of Cu is established at 40 mg/kg diet DM for beef and dairy cattle (National Academies of Sciences, Engineering, and Medicine, 2016; NRC, 2001) and 15 mg/kg diet DM in sheep (NRC, 2007). Symptoms of Cu toxicity include anorexia, hemoglobinuria, hemoglobinemia, icterus, necrosis, and death (McDowell, 2003; Mertz, 1987). There are three stages of Cu poisoning: 1) Cu accumulation in the liver, 2) increased blood Cu and circulating blood enzymes, and 3) the hemolytic stage, which is the result of rapid release of Cu from the liver into circulation (Church, 1988). Bradley (1993) encountered a 14% mortality of Holstein cows from prolonged feeding of 37.6 and 22.6 mg

Cu/kg diet DM for lactating and dry cows, respectively. In contrast, Felsman et al. (1973) added Cu to diets of growing calves up to 600 and 900 mg/kg diet DM in two separate 98-d experiments. They observed an increase in ADG in calves fed 900 mg Cu/kg diet DM, but no difference was observed in calves fed 600 mg/kg added Cu when compared to 0 mg/kg added Cu. Copper toxicity can be prevented by high l concentrations of Zn supplementation and can be treated by administration of Mo or S (McDowell, 2003).

BIOAVAILABILITY

The definition of bioavailability is "the degree and rate at which a substance is absorbed into a living system or is made available at the site of physiological activity" (Merriam-Webster, 2017). Copper supplements are broadly categorized as inorganic (sulfates, chlorides, oxides, etc.) or organic (chelates, proteinates, polysaccharide complexes, amino acid complexes, etc.; Association of American Feed Control Officials, 2015). Interest in the relative bioavailability of inorganic and organic trace mineral supplements has increased, but scientific literature is variable in terms of the advantages of organic sources compared to inorganic counterparts. Organic mineral products can be chelated or complexed with multiple amino acids, a single amino acid, or hydrolyzed proteins; and the mineral typically is either a metal ion or a soluble metal salt (Spears, 1991). Inorganic Cu sources differ in relative bioavailability, copper sulfate being the most available followed by copper chloride, cupric carbonate, cupric nitrate, and copper oxide (McDowell, 2003).

Brown and Zeringue (1994) observed solubility and structural integrity of two Cu proteinates and two Cu amino acid chelates *in vitro*. They reported almost complete solubility of the organic products, whereas inorganic sources are limited in solubility; and Cu was mostly not bound to amino acids or ligands when analyzed with gel filtration

chromatography. Brown and Zeringue (1994) hypothesized that increased solubility and decreased chelation was indicative of increased bioavailability, and could potentially have positive impact on livestock performance. Contradictory to the work of Brown and Zeringue (1994), Ward and Spears (1993) observed no difference in *in vitro* solubility of Cu when comparing $CuSO_4$ and a Cu-lysine chelated product. The results of Ward and Spears (1993) suggest similar interactions and ruminal bioavailability between $CuSO_4$ and Cu- lysine.

Multiple studies that have compared organic Cu and inorganic Cu sources in growing, finishing, and dairy cattle report no added benefit of organic Cu sources with respect to performance or Cu status (Mullis et al., 2003; Engle and Spears, 2000b). Yost et al. (2002) conducted a study in Holstein heifers comparing $CuSO_4$ and a Cu-AA complex (Availa-Cu, Zinpro), and observed no differences in growing performance, liver Cu, or plasma Cu when heifers were supplemented 15 or 30 mg Cu/kg diet DM as $CuSO_4$ or Availa-Cu. Similarly, Arthington et al. (2003) provided grazing heifers molasses-based block supplements containing either $CuSO_4$ or Availa-Cu, and reported no differences in performance, liver Cu, or plasma Cu. In a second study by Arthington et al. (2003), steers were supplemented with either 10 mg Cu/kg diet DM as Availa-Cu or tri-basic Cu chloride (TBCC, Micronutrients Inc., Indianapolis, IN) incorporated into molasses-based blocks as in the previous study. The authors reported no difference between the organic and inorganic Cu products in performance, liver Cu, or plasma Cu concentrations.

Advantages to feeding organic Cu sources also have been reported. Cattle grazing pastures with excess ammonium sulfate fertilization, causing a deficiency in Cu, tended to have greater liver Cu concentrations when supplementation of 123 mg/d Availa-Cu was compared to heifers supplemented equivalent amounts as $CuSO_4$ (Arthington, et al., 2002).

Similarly, Hansen et al. (2008) fed Angus and Angus-Simmental cross steers excess dietary concentrations of Mo and S (6 mg Mo/kg diet and 0.15% S) for 28 d. During this feeding period they fed Cu as $CuSO_4$ or Cu glycinate. The authors observed greater relative Cu bioavailability from Cu glycinate, as indicated by liver Cu, plasma Cu, and ceruloplasmin concentration. These studies suggest that organic Cu sources may be beneficial when excess Fe, Mo, or S are present in the diet. Rabiansky et al. (1999) compared effects of feeding Cu-lysine or $CuSO_4$ on Cu status in heifers, and though no differences were observed for Cu source, heifers that were defined as having a low Cu status responded to Cu lysine to a greater extent than those supplemented $CuSO_4$. Dorton et al. (2003) observed increases in Cu status in both growing and finishing steers fed 20 mg Cu/kg diet as Availa Cu in comparison to 20 mg Cu/kg diet as $CuSO_4$.

Bioavailability of Cu from various sources is both ambiguous and contradictory in current literature. Organic Cu sources may be beneficial in some scenarios, but may not be beneficial in others. Further research is necessary to determine both *in vivo* and *in vitro* bioavailability of Cu sources.

COPPER SUPPLEMENTATION IN GROWNING AND FINISHING CATTLE

Copper supplementation of both growing, receiving, and finishing cattle has yielded variable results, and has not been extensively studied. This may be due to the narrow range for deficiency and tolerable dietary concentrations established by the Nutrient Requirements of Beef Cattle (National Academies of Sciences, Engineering, and Medicine, 2016). Ward and Spears (1997) conducted a study to determine the effects of low Cu diets during the receiving, growing, and finishing phases of Angus steers. Calves were either injected with 90

mg of Cu (as Cu glycinate) or not injected. Calves that were injected were supplemented with 7.5 mg Cu/kg diet DM and uninjected calved were not supplemented. Calves remained in the same treatment groups during the growing and finishing phase (0 or 5 mg Cu/kg and 0 or 5 mg Mo/kg). Copper had no impact on ADG prior to weaning, or during the receiving and growing phases; however, Cu increased ADG during the finishing phase. Copper supplementation increased DMI during receiving and growing phases, but had no impact on finishing DMI. The responses in ADG and DMI resulted in improvements in efficiency during finishing. Copper also influenced multiple carcass characteristics; steers had less backfat and greater rib eye areas. Beck et al. (2002) observed that cattle supplemented with 171 mg Cu/kg diet DM as Cu proteinate tended to exhibit greater ADG BW during the receiving period compared to those supplemented 21 mg Cu/kg diet DM. Similarly, Arthington et al. (2003) observed a tendency for increased ADG in heifers supplemented Cu through a molasses-based block supplement, regardless of Cu source. Felix et al. (2012) reported improvements in efficiency of growing cattle fed DDGs when supplemented Cu.

In addition to literature reporting positive impacts of Cu supplementation, some literature has shown no effects or even or negative responses to Cu supplementation in multiple phases of beef production. Genfelbach et al. (1994) fed a control diet (no added Cu), a diet high in Fe (600 mg/kg diet DM added Fe), a diet high in Mo (5 mg/kg diet DM added Mo), and a Cu sufficient diet (10 mg/kg diet DM added Cu) in order to observe impacts of Cu deficiency on first calf heifer and calf performance. No weight change differences were detected in heifers; however, calves in the high Mo group exhibited decreased ADG. There were no differences among cattle fed control, high Fe, or Cu diets, indicating Cu deficiency may not have caused the decrease in ADG. In addition, Yost et al. (2002) reported no effect

of feeding 15 or 30 mg/kg Cu as einorganic or organic sources on performance of Holstein heifers, although they did observe a linear effect of Cu on liver Cu concentration. Engle and Spears (2000b) fed a control diet absent of supplemental Cu in addition to 20 mg/kg added Cu as $CuSO_4$, Cu citrate, Cu proteinate, or Cu chloride, as well as a diet containing 40 mg/kg added Cu as $CuSO_4$. Copper, regardless of source and concentration, decreased ADG, DMI, and G:F. The authors speculated that Cu may have negatively impacted ruminal fermentation, but indicated that these results were unexpected and difficult to explain.

CONCLUSION

Copper is an essential trace mineral in the diets of livestock for multiple enzyme cascades as well as for a healthy ruminal environment. The physiological and biochemical necessity of Cu corresponds to healthy bone development, growth, immune function, and tissue development in mammals. Excess Cu is not a major concern in dairy or beef cattle production, however toxicity is of major concern in sheep production due to their reduced tolerance for high concentrations of dietary Cu. Deficiencies in Cu are common in cattle production, especially in grazing ruminants due to dietary antagonists. Deficiencies can result in decreased animal performance, and in extreme conditions, death. When formulating diets for ruminants, precautions should be taken in order to formulate diets based on Fe, S, and Mo concentrations in concentrates or forages within the diet due to the negative impact on Cu binding in the rumen and intestinal absorption.

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Chapter 2 - Effects of variable concentrations and sources of copper on *in vitro* fermentation by mixed ruminal microbes

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ABSTRACT

Three experiments were conducted to evaluate effects of varying concentrations and inorganic Cu sources on *in vitro* batch culture fermentation. Experiments used 90% corn and 10% SBM (DM basis) as substrate and ruminal fluid was collected from ruminally fistulated Jersey steers fed a 50% concentrate 50% roughage diet. In Exp. 1, $CuSO_4$ was included in cultures at concentrations of 0, 100, 200, 300, 400, or 500 mg Cu/kg substrate DM in order to determine a concentration that would decrease fermentation by 50%. This concentration was used in Exp.3 to evaluate relative impact of varying Cu sources on fermentation by ruminal microbes. Cultures were incubated for 24 h, gas production was measured every 15 min, and IVDMD, VFA, and pH were measured after 24 h. Increasing Cu concentration had a negative linear effect and a quadratic effect on gas production after 24 h ($P < 0.01$; 135.4, 117.7, 15.8, 105.9, and 94.0 mL/g substrate, respectively). Linear and quadratic effects also were observed for IVDMD ($P < 0.01$) and VFAs ($P < 0.01$); increasing Cu decreased IVDMD and VFA production. A linear effect of Cu was observed in pH (5.88 vs. 6.18 for 100 and 500, respectively). Experiment 2 used similar methodology, but with 0, 10, 20, 30, 40, 50, 60, or 70 mg Cu/kg substrate DM incorporated into fermentation flasks. Linear and quadratic effects of Cu were observed for gas production ($P < 0.01$), although a negative response was observed at concentrations greater than 50 mg Cu/kg substrate DM. Copper linearly increased acetate and A:P, linearly decreased propionate ($P < 0.01$), and had a quadratic effect on propionate ($P = 0.03$). Copper tended to decrease IVDMD (Linear, $P = 0.08$), but had no impact on total VFA ($P > 0.31$) or pH ($P > 0.28$). Copper sources were evaluated; sources were $CuSO_4 \bullet 5H_2O$ ($CuSO_4$), $CuCl_2 \bullet 2H_2O$ ($CuCl_2$), CuO (CuO), $CuCO_3 \bullet Cu(OH)_2$ ($CuCO_3$), and $Cu_2(OH)_3 \bullet Cl$ (TBCC; Intellibond C, Micronutrients, Indianapolis, IN), and

were incorporated at 100 mg Cu/kg substrate DM, a concentration great enough to elicit adverse effects on fermentation, as demonstrated in Exp. 1. Source had an effect on gas production ($P < 0.01$), total VFAs ($P < 0.01$), IVDMD ($P < 0.01$), and pH ($P < 0.01$). Copper sulfate and $CuCl_2$ resulted in the greatest negative response in fermentation, and CuO resulted in the greatest fermentation. In conclusion, Cu can negatively impact fermentation by ruminal microbes, and inorganic sources of Cu impact ruminal microorganisms differently.

INTRODUCTION

Copper is essential in multiple enzymatic pathways, maintaining structural integrity of both skeleton and connective tissues, and is necessary for proper growth and function in ruminants (McDowell, 2003). Within the rumen, Cu indirectly aids in removal of free radical oxygen by superoxide dismutase. Without this Cu-dependent enzyme, toxic free radicals would accumulate and cause damaging effects on the ruminal environment (Underwood, 1981). Copper, specifically $CuSO_4$, is known to have antimicrobial properties and could potentially impact ruminal fermentation and metabolism. Essig et al. (1972) observed diminished VFA production and protozoa numbers when steers were fed high concentrations of Cu *in vivo*. In agreement, Slyter and Wolin (1967) reported a depression in both VFA and methane production at non-physiological concentrations of Cu *in vitro* as well as differences in Cu sensitivity based upon substrate source. In contrast, Engle and Spears (2000a) reported no effect of Cu on *in vitro* fermentation; however, Cu concentrations were within normal dietary concentrations. Copper also may impact the microbial population, as demonstrated by Forsberg (1978). Both concentration and source of Cu can influence the ruminal environment, as well, and ruminal modification can impact the amount and form that appears

in the lower GI tract for absorption. Literature regarding Cu sources is contradictory when comparing both inorganic and organic Cu sources. Brown and Zeringue (1994) reported increased ruminal solubility of organic versus inorganic Cu; however, Ward and Spears (1993) reported no difference between organic and inorganic Cu sources. There are many *in vivo* comparisons of organic and inorganic Cu sources on performance and Cu status (Engle and Spears, 2000b; Arthington et al., 2003; Dorton et al., 2003; Hansen et al., 2008), however there is limited information comparing inorganic Cu sources using *in vitro* fermentation systems. Therefore, our objective was to determine at what concentration Cu becomes detrimental to ruminal fermentation by ruminal, and to evaluate effects of concentration and sources of Cu with respect to fermentation by ruminal microbes.

MATERIALS AND METHODS

Experiment 1

***In Vitro* Fermentation**

Experiment 1 was a randomized complete block design with 6 treatments and 3 runs of 6 replicates each, providing 18 observations for each treatment. Within a fermentation run, fermentation flasks were allocated to six blocks, and treatments were distributed randomly within blocks. Forty-two flasks were utilized in each run; 36 flasks assigned to treatments and 6 blanks. Treatments consisted of copper sulfate heptahydrate ($CuSO_4$) added to provide 0, 100, 200, 300, 400, or 500 mg added Cu/kg substrate DM. These concentrations were selected in an attempt to determine at what Cu concentration a 50% reduction in fermentative activity could be observed. Results of this experiment were used to create a model in which Cu sources could be evaluated using a similar *in vitro* system. Treatments were prepared by solubilizing $CuSO_4$ into 25 mL of water to appropriate concentrations. Substrate consisted of

90% corn and 10% soybean meal (DM basis) ground through a 1 mm screen. Prior to ruminal fluid collection, 3 g (DM basis) of substrate were added to each of 36 fermentation flasks. No substrate was added to 6 flasks, which served as blanks (1 per replicate) and were used to correct for DM and VFA contributed by ruminal fluid inoculum.

Ruminal fluid was collected from a fistulated Jersey steer approximately 7 h after feeding. Donor animal diet consisted of 50% concentrate and 50% roughage (dry rolled corn, corn silage, ground alfalfa, and supplement). Ruminal fluid was strained through 4 layers of grade 50 cheesecloth (11 × 9.5 cm) into a pre-warmed insulated container and immediately transported 2 km to the Kansas State University Pre-Harvest Food Safety Laboratory. Once at the laboratory, ruminal fluid was strained through an additional 8 layers of grade 50 cheesecloth (11 × 9.5 cm) into a 2000-mL separatory funnel. The funnel was flushed for 2 min with N_2 gas, capped, and incubated at 39ºC for 30 to 40 minutes to allow stratification of ruminal fluid into 3 layers. The bottom layer, rich in protozoa and feed particles, was discarded and the intermediate layer was collected for use as inoculum for *in vitro* cultures. McDougall's buffer (140 mL) was added to flasks, 10 mL of inoculum were added, 1 mL of the appropriate Cu treatment was added, initial pH was recorded using a bench top pH probe (Thermo Orion pH meter model 230 A, Thermo Fisher Scientific Inc., Waltham, MA), bottles were flushed with N_2 gas, capped with Ankom[RF1] (Ankom RF Gas Production System; Ankom Technology, Macedon, NY) modules, and placed into a 39ºC shaking incubator. Bottles incubated for 24 h at 39ºC while being continuously and gently shaken. Gas pressure readings were recorded as cumulative pressure by modules every 15-min during incubation. Final pH was taken at 24 h, immediately after removing the Ankom module from each flask.

Gas pressure was converted to gas production (mL) per g of substrate using the ideal gas law and Avogadro's law.

Ideal gas law: $n = p\left(\frac{V}{R \times T}\right)$

Avogadro's law: Gas production (mL) = n × 25.6 × 1000

Gas production (mL/g substrate) = Gas production / 3

In these formulas, n is moles of gas produced, p is pressure (kPa), V is volume of headspace in the flask (L), R is the gas constant (8.314472 $L \bullet kPa \bullet K^{-1} \bullet mol^{-1}$), T is temperature (K), 25.6 is the volume 1 mole of gas occupies at 39ºC, and 3 is the g of substrate used.

Volatile Fatty Acid Profiles

Four mL of fluid contents from each flask were combined with 1 mL of 25% (w/v) m-phosphoric acid in an 18-mL vial. Vials were frozen at -20ºC for approximately 24 h. Vials were thawed, vortexed, and 2 mL were transferred to microcentrifuge tubes. Tubes centrifuged at 10,000 × *g* for 15 min. Supernatant was transferred into gas chromatography vials and subsequently analyzed on an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA). Supelco Volatile Standard Mix (46975-U, Supelco, Inc., Bellefonte, PA) was used as a standard to calibrate output. A Supelco Nukol column (15 m × 530 μm × 0.5 μm) was used, with an injection temperature of 275ºC and a flame ionization detector temperature of 300ºC. Hydrogen was used as a carrier gas with a flow rate of 45 mL/min. Concentration (mM) output was converted to total VFA production per g substrate.

Total VFA production = ((0.161 / g substrate) × (VFA concentration × 1.25)) – Blank

In the above equation, 0.161 is the volume of liquid (in L) of the system, 1.25 is the correction for dilution by m-phosphoric acid, and blank VFA concentration was subtracted to correct for VFAs contributed by inoculum within the respective block.

In Vitro Dry Matter Disappearance

For IVDMD analysis, contents of fermentation flasks were completely transferred to 19.0 × 12.7-cm aluminum pans and subsequently dried for 48 h at 105ºC. Pans were weighed after they were dried.

In vitro dry matter disappearance analysis was calculated using the following:

$$\frac{\text{final vessel content DM wt.-blank content DM wt.}}{\text{initial substrate wt.}} \times 100$$

Blanks were used to correct for DM from the ruminal fluid within the respective block.

Experiment 2

Experiment 2 was a randomized complete block design with 8 treatments and 3 runs with 5 flasks of each treatment, allowing for 15 observations per treatment. Within a fermentation run, fermentation flasks were allocated to five blocks, and treatments were distributed randomly within blocks. Forty-five flasks were utilized in each run; 40 flasks assigned treatments and 5 blanks. Randomization methodology was as described for Exp. 1. Treatments consisted of copper sulfate heptahydrate ($CuSO_4$) added at 0, 10, 20, 30, 40, 50, 60, or 70 mg Cu/kg substrate DM. All procedures for ruminal fluid collection, *in vitro* fermentation, VFA analysis, pH measurements, and IVDMD were as described for Exp. 1.

Experiment 3

Experiment 3 was a randomized complete block design with 5 treatments with 3 runs of 4 flasks of each treatment, providing 12 observations per treatment. Twenty-four flasks were utilized in each run; 20 flasks assigned treatments and 4 blanks. Treatments consisted of five different inorganic Cu sources: copper sulfate as $CuSO_4 \bullet 5H_2O$ ($CuSO_4$), copper chloride as $CuCl_2 \bullet 2H_2O$ ($CuCl_2$), copper oxide as CuO (CuO), copper carbonate as $CuCO_3 \bullet Cu(OH)_2$ ($CuCO_3$), and tribasic copper chloride as $Cu_2(OH)_3 \bullet Cl$ (TBCC; Intellibond C,

Micronutrients, Indianapolis, IN). Sources were incorporated into culture bottles at 100 mg Cu/kg substrate DM. This value was established as a concentration sufficient to adversely affect fermentation response in Exp 1. Culture bottles were prepared by adding 13 g of substrate, as described for Exp.1, as well as the appropriate Cu source treatment; added as dry ingredients equaling 100 mg Cu/kg substrate. Ruminal fluid collection and McDougall's buffer preparation was as described for Exp. 1. Ruminal fluid and McDougall's buffer were added to each 1000 mL culture bottle in quantities of 50 mL and 600 mL, respectively. Bottles were purged of O_2 with N_2 gas, initial pH recorded, capped quickly with Ankom modules, and placed in a shaking incubator. Culture bottles were gently agitated and incubated at 39ºC for 24 h. Final pH was recorded, and IVDMD, VFA analyses, and gas production analyses were conducted as described for Exp. 1.

Statistical Methodology

Experiments 1 and 2 were analyzed with Statistical Analysis Software (SAS version 9.4; SAS Inst., Inc. Cary, NC). Volatile fatty acid production, IVDMD, and pH were analyzed using the MIXED procedure with fermentation flask as the experimental unit, a random effect of replicate within run, and Cu concentration as a fixed effect. The PDIFF function was used to determine differences between means. Gas production over time was also analyzed using the MIXED procedure of SAS as a repeated measure, subject being the module within run, and fixed effects of Cu concentration, time, and Cu concentration × time. The SLICE option was used to detect treatment differences at predetermined intervals during the incubation period. Linear and quadratic contrasts were used to evaluate effects of Cu. Experiment 3 was analyzed using the MIXED procedure, with fermentation flask as the experimental unit, replicate within run as a random effect, and Cu source as a fixed effect.

The PDIFF function was used to evaluate differences between means, similar to Exp. 1 and 2. Gas production was analyzed similar to experiments 1 and 2 with the SLICE option; the only difference being Cu source, time, and Cu source × time as fixed effects. Differences were defined as follows: $\alpha \leq 0.10$ as a tendency and $\alpha \leq 0.05$ as a significant effect between treatment means.

RESULTS AND DISCUSSION

Experiment 1

Gas production decreased linearly as Cu concentration increased (Figure 2.1, $P <$ 0.01). A quadratic effect of Cu ($P < 0.01$) was also observed. In comparison to 0 mg/kg Cu, a 27%, 36%, 43%, 43%, and 49% reduction in total gas produced was observed for 100, 200, 300, 400, and 500 mg added Cu/ kg substrate, respectively. Interestingly, at 18 h, all treatments were different; however by 24 h of incubation 300 and 400 mg Cu/kg substrate DM began converging. This may be due to the adaptation of microbes to Cu toxicity, or the ability of remaining microbes to replicate and establish a great enough population to increase fermentative activity. Based on visual analysis of the growth curve, it is likely that the lag time was affected by Cu, and the rate of gas produced may have increased over time for 400 mg added Cu/ kg substrate, explaining the convergence of 300 and 400 mg added Cu/ kg substrate. Similarly, IVDMD decreased linearly with increasing added Cu ($P < 0.01$, Figure 2.1). Total VFA, acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate decreased linearly as Cu concentration increased (P < 0.01, Table 2.1). A quadratic effect of Cu was also observed ($P < 0.01$) for VFA production. The A:P ratio also increased linearly as added Cu in the fermentation flasks increased ($P < 0.01$). We observed a linear increase in pH with increasing Cu ($P < 0.01$); likely due to the decrease in VFA production. Slyter and

Wolin (1967) observed a decrease in VFA production in an *in vitro* continuous culture system when 100 mg/kg Cu (as $CuSO_4$) was added in comparison to no added Cu. In further agreement with our results, Essig et al. (1972) fed 44 mg/kg BW (approximately 17.2 mg Cu/kg BW rations to steers and reported a decrease in acetate, propionate, butyrate, and total VFA as well as a decrease in protozoa numbers when ruminal fluid was collected from the same steers. Hubbert et al. (1958) reported a decrease in cellulose digestion when *in vitro* cultures that contained as little as 2.5 mg/kg Cu. In contrast to our results, Solaiman et al. (2007) observed no effect of feeding goats 100 or 200 mg/kg Cu on VFAs, pH, or protozoa in ruminal fluid collected from these animals. This was, however, not an *in vitro* system, therefore differences in methodology as well as the ruminal environment may explain the contradiction with the current study.

Experiment 2

Two replicates were removed from IVDMD statistical analyses, one from 20 mg/kg and one from 30 mg/kg, due to technicalities unrelated to treatment. There were no quadratic effects of Cu observed ($P > 0.20$). A linear decrease was observed for gas production and acetate, propionate, isobutyrate, isovalerate, and valerate production ($P < 0.01$, Figure 2.2, Table 2.2) with increasing Cu concentration. After 24 h of fermentation, an adverse effect on gas production is observed at Cu concentrations greater than 40 mg Cu/kg substrate DM ($P < 0.05$). No linear effect of Cu was observed for pH ($P = 0.27$), total VFA ($P = 0.62$), or butyrate production ($P = 0.56$). A tendency for a linear decrease for IVDMD with increasing Cu concentration was observed ($P = 0.08$). Negative effects on microbial activity can be observed at concentrations exceeding 40 mg Cu/kg substrate DM, as demonstrated by gas production. According to the Nutrient Requirements of Beef Cattle, cattle can tolerate

upwards of 40 mg/kg Cu in their diet (National Academies of Sciences, Engineering, and Medicine, 2016). Our results are in agreement with this benchmark, as noted by the linear decrease in gas production at 50 mg/kg Cu. Forsberg (1978) reported a 50% inhibition of *in vitro* fermentation using gas production as the dependent variable, at 21 μg Cu/mL (as $CuCl_2$). In contrast, Vázquez-Armijo et al. (2011) noted an increase in both the rate and accumulation of gas produced when 21.7 mg/kg Cu was added compared to the control in which no Cu was added. Both studies are in contrast to our results, where no difference was observed in gas production when 10, 20, 30, or 40 mg/kg Cu was added. Differences in donor animal diets, as well methodology could explain these differences. Hubbert et al. (1958) and Martinez and Church (1970) both reported a depression in cellulolytic activity of ruminal microorganisms at 1 and 1.5 mg/kg Cu, respectively. Additionally, McNaught et al. (1950) reported an *in vitro* inhibition of protein synthesis at 10 mg/kg Cu.

Experiment 3

Results for Exp. 3 are presented in Table 2.3 and Figure 2.3. Gas production was greatest for $CuCO_3$, CuO, and TBCC at 6 h ($P < 0.01$) in comparison to $CuSO_4$ and $CuCl_2$, which were not different ($P = 0.83$). By 12 h of incubation CuO exhibited greater cumulative gas production than IBCC, $CuSO_4$, and $CuCl_2$ ($P < 0.05$), whereas $CuCO_3$ and TBCC were not different ($P = 0.53$). After 24 h of incubation, gas production was decreased by 2.9%, 4.1%, 40.9%, and 43.3% for $CuCO_3$, TBCC, $CuSO_4$, and $CuCl_2$, respectively, compared to CuO. The concentration of Cu (100 mg/kg) was great enough to result in a reduction in microbial activity; therefore decreased gas production indicates greater impact on ruminal microorganisms. *In vitro* dry matter disappearance, VFA production, and pH was not different between $CuSO_4$ and $CuCl_2$ ($P > 0.10$). Volatile fatty acid production was not

different between CuO, $CuCO_3$, and TBCC ($P > 0.10$), however, CuO decreased pH and increased IVDMD compared to $CuCO_3$ and TBCC ($P < 0.02$). An increase in pH, and a decrease in IVDMD and VFA production was detected for $CuSO_4$ and $CuCl_2$, compared to CuO, $CuCO_3$, and TBCC.

Based on gas production, IVDMD, and pH results, it would appear that CuO impacts ruminal microorganisms to a lesser extent, followed by $CuCO_3$ and TBCC. Copper sulfate and $CuCl_2$ impacted fermentation by ruminal microbes to the greatest extent. Solubility of trace minerals may play an important role in the ability of minerals to impact ruminal microflora or to bypass the rumen without forming complexes with other elements in the rumen; which commonly render the mineral unavailable. Multiple ruminant and monogastric studies have reported a decrease in Cu availability when it is in the form of Cu oxide (Lassiter and Bell, 1960; Bunch et al., 1961; Ledoux, et al., 1991; Kegley and Spears, 1994). In agreement with the current study, Ledoux et al. (1991) reported Cu carbonate as having a relative bioavailability of 54.3%, 38.6% less than Cu sulfate. Copper sulfate is almost completely dissociated and is nearly 100% soluble in the rumen, making it more ruminally bioavailable than other inorganic sources such as oxide, TBCC, acetate, and carbonate (McDowell, 2003; Ledoux et al., 1991; Kegley and Spears, 1994). Sulfate trace minerals are bound with ionic bonds and weak ionic chemical bonds subject them to complex and bind with antagonists, such as Mo, S, and Fe; although relative bioavailability is increased in the absence of dietary antagonists. The chemical properties of sulfate minerals would explain the decrease in gas production as well as VFA production, IVDMD, and increase in pH in the current study. However, studies in which no differences were detected between $CuSO_4$ and TBCC (Spears et al., 2004), or $CuSO_4$, cupric carbonate, and CuO in swine (Buescher et al.,

1961). This may be indicative of differences in Cu metabolism between monogastric and ruminant animals. It is important to note that concentration of antagonists in the diet appears to play a role in the bioavailability of each individual Cu source. Tribasic Cu chloride is considered an inorganic Cu source, however unlike the sulfate forms, TBCC contains covalent bonds between Cu ions and hydroxyl and chloride groups. It has been suggested that the chemical bonds result in a decrease in solubility in monogastrics, which may translate to a decrease in ruminal solubility (Cromwell et al., 1998). This may explain the increase in gas production of TBCC when compared to copper sulfate and copper chloride. Kim et al. (2016) studied the effects of copper sulfate and TBCC on tissue concentrations in laying hens, and reported no difference in relative bioavailability. Similarly, in a diet void of antagonists such as Mo and S, Spears et al. (2004) reported no difference in bioavailability in growing cattle. There was an increase in Cu bioavailability, however, in cattle consuming diets high in Mo and S when TBCC was fed, compared to copper sulfate. In conclusion, ruminal microorganisms are affected by both copper source and concentration. Furthermore, Cu sources interact differently with ruminal microbes and could impact microbial fermentation and post-ruminal bioavailability.

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Table 2.1 Experiment 1. Effects of high concentrations of supplemental Cu on *in vitro* fermentation characteristics after 24-h of fermentation

	Concentration[1], mg Cu/kg substrate							*P*-value	
Item	**0**	**100**	**200**	**300**	**400**	**500**	**SEM**	**Linear**	**Quadratic**
IVDMD, %	46.70[a]	38.02[b]	34.05[c]	28.39[d]	25.30[d]	20.77[e]	1.571	< 0.01	0.03
Final pH	5.88[a]	6.04[b]	6.00[b]	6.05[b]	6.06[b]	6.18[c]	0.034	< 0.01	0.95
VFA, mMoles/g substrate									
Total VFA	6.20[a]	4.68[b]	3.58[c]	1.72[d,e]	1.36[e]	1.09[e,f]	0.146	< 0.01	< 0.01
Acetate	2.58[a]	1.84[b]	1.58[c]	0.98[d,e]	0.89[e]	0.79[e,f]	0.078	< 0.01	< 0.01
Propionate	2.89[a]	2.37[b]	1.73[c]	0.64[d]	0.39[e]	0.25[e]	0.063	< 0.01	< 0.01
Isobutyrate	0.03[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.002	< 0.01	< 0.01
Butyrate	0.56[a]	0.37[b]	0.26[b]	0.13[d]	0.10[d]	0.07[d]	0.030	< 0.01	< 0.01
Isovalerate	0.04[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.003	< 0.01	< 0.01
Valerate	0.09[a]	0.10[a]	0.04[b]	0.00[c]	0.00[c]	0.00[c]	0.007	< 0.01	< 0.01
A:P	0.89[a]	0.78[a]	0.92[a]	1.61[b]	2.43[b]	3.82[d]	0.194	< 0.01	< 0.01

[1]Cultures contained 0, 100, 200, 300, 400, or 500 mg/kg Cu in the form of $CuSO_4$.
[a-f] Within row, means without a common superscript letter are different ($P < 0.05$).

Table 2.2 Experiment 2. Effects of increasing supplemental Cu on *in vitro* batch culture fermentation by mixed ruminal microbes after 24-h of fermentation

	Concentration[1], mg Cu/kg substrate DM									*P*-value	
Item	**0**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**SEM**	**Linear**	**Quadratic**
IVDMD, %	52.50	52.23	51.33	50.17	51.06	53.40	50.10	49.68	2.479	0.08	0.83
Final pH	5.98	5.92	5.96	5.99	5.97	5.97	5.90	5.94	0.058	0.27	0.23
VFA, mM/g substrate											
Total VFA	6.68	6.93	6.91	6.56	6.52	6.87	6.81	6.97	0.218	0.62	0.31
Acetate	$3.15^{a,b,c}$	3.30^{a}	$3.25^{a,b}$	$3.02^{a,c}$	2.92^{c}	2.95^{c}	$3.00^{b,c}$	$2.97^{b,c}$	0.117	< 0.01	0.55
Propionate	2.55^{a}	2.57^{a}	2.62^{a}	2.56^{a}	2.59^{a}	2.90^{b}	2.87^{b}	3.05^{b}	0.090	< 0.01	0.03
Isobutyrate	0.04^{a}	0.04^{a}	$0.04^{a,b}$	$0.03^{a,b,c}$	$0.03^{a,b,c}$	$0.03^{b,c}$	0.02^{c}	0.02^{c}	0.005	< 0.01	0.56
Butyrate	0.76	0.82	0.81	0.77	0.79	0.81	0.77	0.76	0.059	0.56	0.26
Isovalerate	0.09^{a}	0.11^{a}	0.10^{a}	$0.09^{a,b}$	$0.09^{a,b}$	$0.09^{a,b}$	0.08^{b}	0.07^{b}	0.008	< 0.01	0.23
Valerate	0.09^{a}	0.09^{a}	0.10^{a}	0.09^{a}	0.09^{a}	0.09^{a}	0.08^{b}	$0.09^{a,b}$	0.005	0.04	0.29
A:P	1.29	1.32	1.29	1.23	1.18	1.11	1.13	1.07	0.034	< 0.01	0.56

[1]Cultures contained 0, 10, 20, 30, 40, 50, 60, or 70 mg/kg Cu in the form of $CuSO_4$.

[a-c]Within row, means without a common superscript letter are different ($P < 0.05$).

Table 2.3 Experiment 3. Effect of Cu source *in vitro* batch culture fermentation by mixed ruminal microbes after 24-h of fermentation

	Copper source[1]						
Item	**$CuSO_4$**	**$CuCl_2$**	**CuO**	**$CuCO_3$**	**TBCC**	**SEM**	***P*-value**
IVDMD, %	24.42[a]	23.54[a]	35.23[b]	33.61[c]	33.39[c]	0.545	< 0.01
Final pH	6.39[a]	6.42[a]	6.28[b]	6.33[c]	6.34[c]	0.017	< 0.01
VFA, mMoles/g substrate							
Total VFA	3.17[a]	3.38[a]	5.19[b]	4.77[b]	4.79[b]	0.232	< 0.01
Acetate	1.32[a]	1.41[a]	2.13[b]	1.99[b]	1.99[b]	0.100	< 0.01
Propionate	1.56[a]	1.64[a]	2.27[b]	2.03[b]	2.05[b]	0.142	0.05
Butyrate	0.17[a]	0.20[a]	0.60[b]	0.58[b]	0.57[b]	0.081	< 0.01
Isovalerate	0.00[a]	0.00[a]	0.028[b]	0.027[b]	0.028[b]	0.008	< 0.01
Valerate	0.05	0.06	0.07	0.06	0.06	0.008	0.45
A:P	0.90	0.91	0.96	1.01	0.99	0.065	0.34

[1] Copper sources were added at 100 mg Cu/kg substrate DM as either copper sulfate as $CuSO_4$•$5H_2O$ ($CuSO_4$), copper chloride as $CuCl_2$•$2H_2O$ ($CuCl_2$), copper oxide (CuO), copper carbonate as $CuCO_3$•$Cu(OH)_2$ ($CuCO_3$), and tribasic copper chloride as $Cu_2(OH)_3$•Cl (TBCC; Intellibond C, Micronutrients, Indianapolis, IN).

[a-c] Within row, means without a common superscript letter are different (*P* < 0.05).

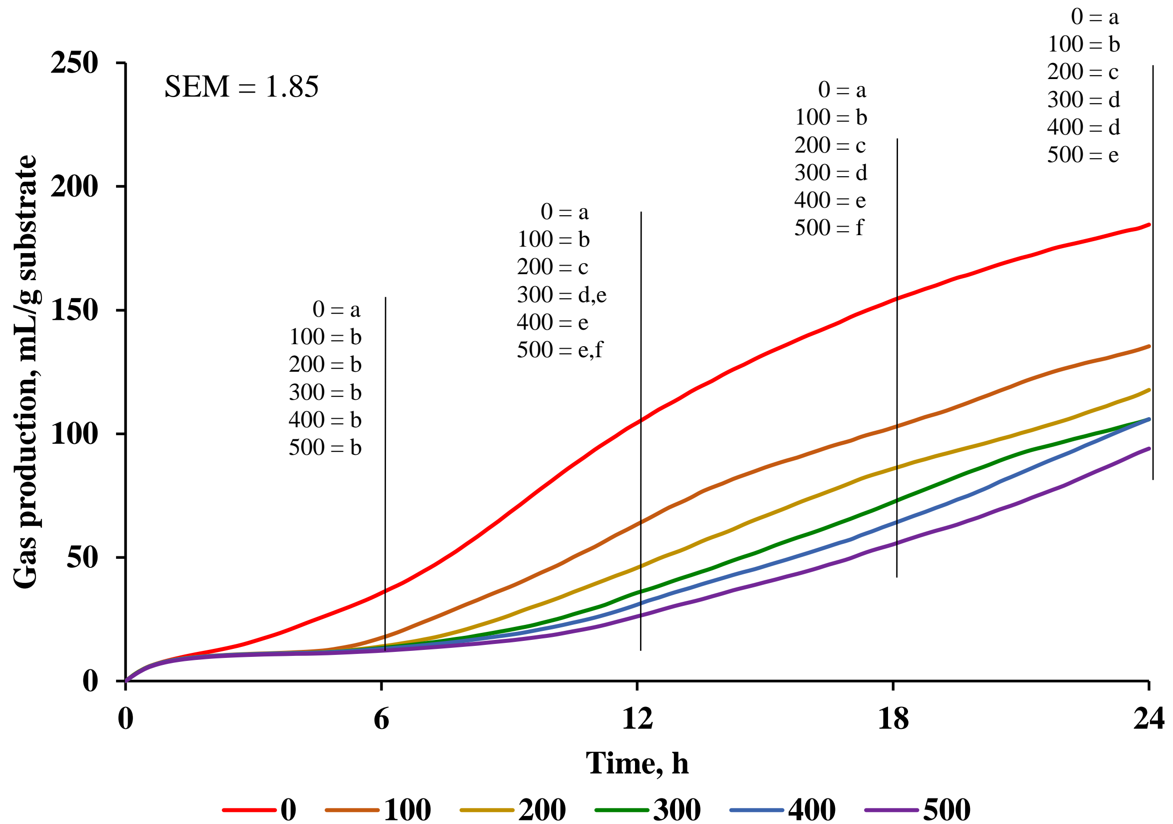

Figure 2.1 Experiment 1. Gas production (mL/g substrate) by mixed ruminal microbes in an *in vitro* batch culture fermentation system over a period of 24 h. Cultures contained 0 (■), 100 (■), 200 (■), 300 (■), 400, or 500 (■) mg Cu/kg substrate DM in the form of $CuSO_4$. Fermentation flasks contained 10 mL ruminal fluid, 140 mL of McDougall's buffer, and 3 g of substrate. There was a copper × time interaction ($P < 0.01$), and both linear and quadratic effects of copper ($P < 0.01$).

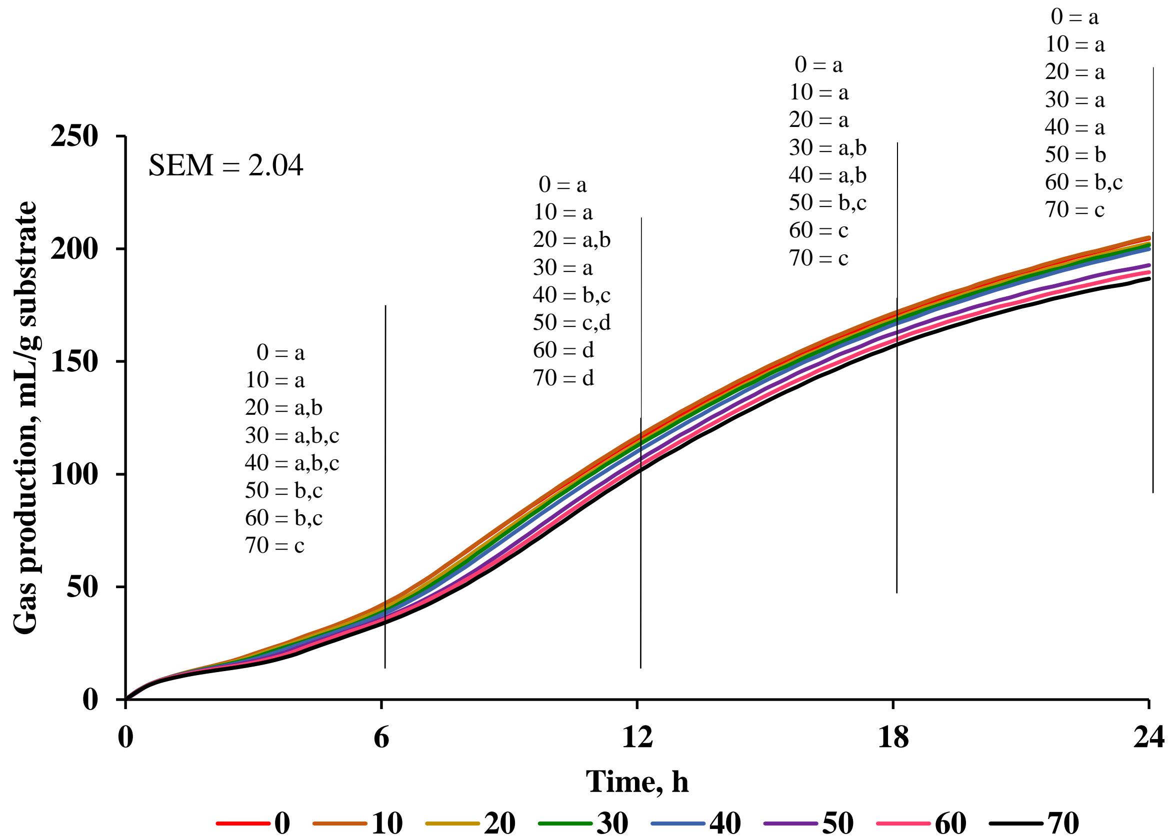

Figure 2.2 Experiment 2. Gas production (mL/g substrate) by mixed ruminal microbes in an *in vitro* batch culture fermentation system over a period of 24 h. Cultures contained 0 (■), 10 (■), 20 (■), 30 (■), 40 (■), 50 (■), 60 (■), or 70 (■) mg Cu/kg substrate DM in the form of $CuSO_4$. Fermentation flasks contained 10 mL ruminal fluid, 140 mL of McDougall's buffer, and 3 g of substrate. There was a copper × time interaction ($P < 0.01$), and both linear and quadratic effects of copper ($P < 0.01$).

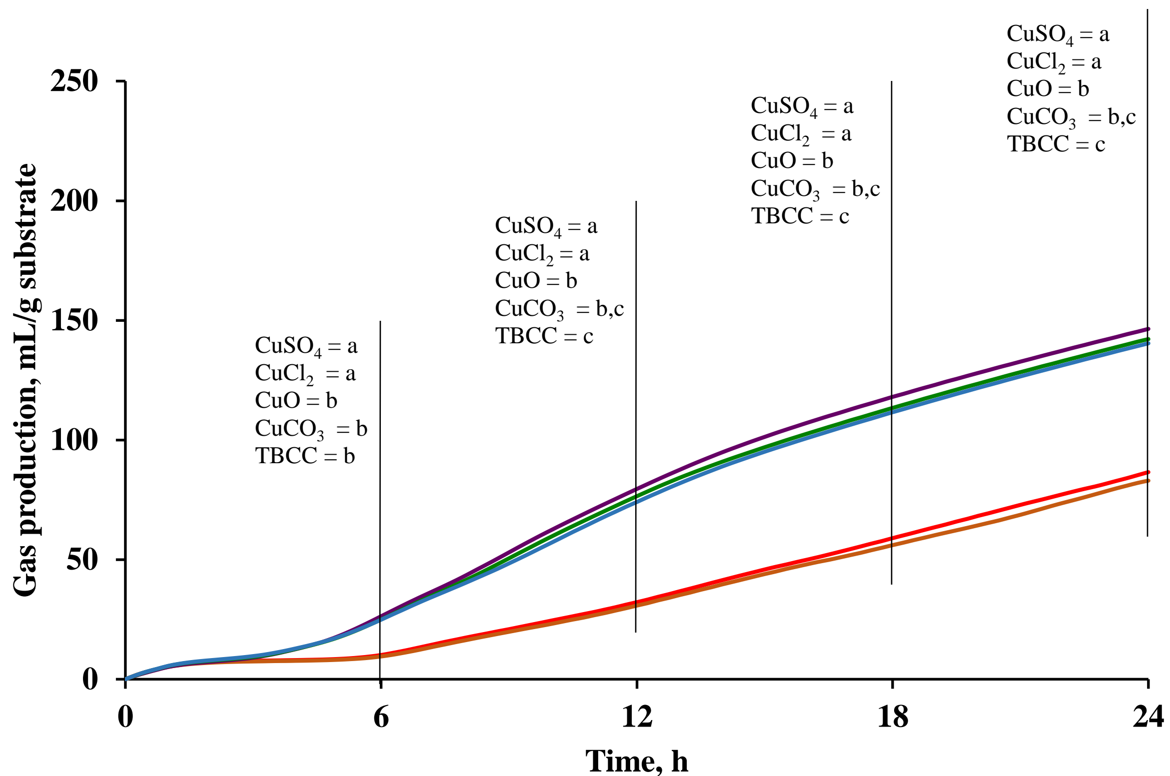

Figure 2.3 Experiment 3. Gas production (mL/g substrate) by mixed ruminal microbes in an *in vitro* batch culture fermentation system over a period of 24 h. Cultures contained 100 mg/kg Cu substrate as either copper sulfate as $CuSO_4 \bullet 5H_2O$ ($CuSO_4$, ■), copper chloride as $CuCl_2 \bullet 2H_2O$ ($CuCl_2$, ■), copper oxide as CuO (CuO, ■), copper carbonate as $CuCO_3 \bullet Cu(OH)_2$ ($CuCO_3$, ■), and tribasic copper chloride as $Cu_2(OH)_3 \bullet Cl$ (TBCC; ■, Intellibond C, Micronutrients, Indianapolis, IN). There was a source × time interaction ($P < 0.01$) and effects of source ($P < 0.01$) and time ($P < 0.01$)

Chapter 3 - Effects of low-moisture molasses block supplements on tissue concentrations of mineral elements and growth performance of forage-fed beef cattle

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ABSTRACT

Feed manufacturing processes, particularly those involving extremes of heat or pH, can alter bioavailability of nutrients. Similarly, form of supplements (pellets, loose minerals, liquids, or blocks) can influence supplement consumption patterns, which may, in turn, impact nutrient utilization. This study was conducted to evaluate changes in tissue concentrations of Cu, P, and Zn in forage-fed heifers in response to supplementation with plain salt (S), a free-choice mineral supplement (M), or a low-moisture process block supplement (B). Heifers (n=360, 253.9 ± 6.7 kg) were weighed, blocked by initial BW, and randomly allocated to treatments consisting of *ad libitum* access to salt (S); loose mineral (M); or low-moisture molasses block plus salt (B). The M and B treatments were manufactured using the identical mineral mixture, adding salt as a diluent to make M, or by incorporating the mineral mixture into a hot, pliable evaporated molasses/oil mixture to make B. Heifers were housed in 24 dirt-surfaced pens (15 animals/pen, 8 pens/treatment) and fed a basal diet consisting (DM basis) of 70% ground brome hay and 30% corn silage for 100 d. Blood and liver biopsy samples were collected on d 0 and 70 from 4 randomly selected heifers/pen to determine changes in concentrations of Cu in liver and Zn and P in plasma. No treatment differences were detected for DMI or G:F ($P > 0.1$), but ADG was greater for M than for S or B ($P = 0.03$). No differences were detected for changes in plasma P or Zn ($P > 0.10$). Liver Cu concentrations were different among treatments ($P < 0.05$), where M had the greatest liver Cu, B was intermediate, S had the least liver Cu (114, 428, and 266 mg/kg tissue DM for S, M, and B, respectively). The percent of heifers identified as having sufficient tissue Cu (i.e., > 87.5 mg/kg tissue DM) was less for S than for M and B (59.4, 100, and 100%, respectively; $P < 0.05$). In

conclusion, tissue mineral content was proportionate to mineral intake, but differences in mineral availability between loose mineral and block supplements were not evident.

Key words: copper, forage, phosphorus, mineral status, zinc

INTRODUCTION

Trace mineral supplementation in forage-fed cattle takes many forms, but frequently is offered as free-choice supplements, including molasses-based blocks. Bioavailability of trace elements in ruminants can be modified during ruminal fermentation, potentially impacting post-ruminal absorption and storage of these elements (McDowell, 2003). Bioavailability of trace minerals is lower in ruminants in comparison to non-ruminants due to the ruminal environment (Spears, 2003), and this difference is most pronounced for forage-fed cattle (Kabija and Smith, 1988). Phosphorus is stored primarily in bone, but free forms are found in blood and other tissues. Phosphorus is the most common mineral found to be deficient in forage-fed cattle (McDowell, 2003). Copper also is deficient under many circumstances, and is an essential cofactor for enzymes such as lysil oxidase, cytochrome C oxidase, and ceruloplasmin. Copper accumulates in liver tissue when fed in excess, and liver concentrations frequently are used to assess Cu status of cattle. Zinc is a component or activator of many enzyme systems and is important in immune function (Hambidge et al., 1986). Little is known of the comparative bioavailability of these mineral elements when offered to cattle as components of different types of supplements. Additionally, consumption patterns and cattle behavior are influenced by delivery method, which could alter mineral status. Consequently, our objective was to compare differences in free-choice intake and tissue concentrations of Cu, P, and Zn in forage-fed cattle supplemented with different types of

supplements (salt, free-choice mineral mixture, or a mineralized molasses block supplement).

MATERIALS AND METHODS:

Animals and Sampling

Procedures were approved by the Kansas State University Institutional Animal Care and Use Committee and were conducted at the Kansas State University Beef Cattle Research Center in Manhattan, Kansas.

Experimental Design

Three hundred sixty crossbred heifers (initial BW = 253.9 ± 6.7 kg) were used in a randomized complete block design with 3 treatments. Heifers were weighed and randomly assigned to experimental pens based on initial BW. Treatments (Table 1) consisted of free choice plain salt (S), a free-choice dry mineral supplement containing salt (M), or a low-moisture process molasses block supplement with separate access to salt (B). Cattle in all treatments were fed the same forage-based diet for 86 days, and then switched to a common total mixed ration for the final 16 days of the study (Table 2) in an attempt to equalize gastrointestinal tract fill among treatments. Heifers were housed in 24 dirt-surfaced pens (15 animals/pen, 8 pens/treatment) and fed the basal diet once daily *ad libitum*.

Animal Processing, Handling, and Housing

Prior to the start of the study heifers were given *ad libitum* access to ground brome hay and water. Heifers were processed 24 to 48 h after arrival at the research center. Initial processing procedure included identification with uniquely numbered eartags, vaccination with Bovishield Gold 5 (Zoetis, Parsippany, NJ) and Ultrabac

7/Somubac (Zoetis), administration of a prophylactic subcutaneous dose of tilmicosin (Micotil, Elanco Animal Health, Greenfield, IN), implanting with Component TE-IH with Tylan (Elanco Animal Health), and treatment for internal and external parasites using Dectomax Pour-On (Zoetis). Before study initiation, heifers were weighed and initial body weights were recorded. These weights were used for initial blocking and heifers were then sorted into experimental pens. Dirt-surfaced drylot pens provided approximately 22 m^2 of pen surface area/animal. Pens were equipped with automatic waterers (shared between two adjacent pens) and fence-line concrete feed bunks. Initial blood samples were drawn via jugular venipuncture from 4 heifers randomly selected from each pen prior to the start of the study (32/treatment), for analysis of plasma Zn and plasma P. Blood was collected into two 6-mL trace element grade EDTA Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and immediately placed on ice. Blood was centrifuged within 15 min of sampling at 2,550 × g for 10 min. Plasma was transferred into plastic tubes, transported to the Kansas State University Ruminant Nutrition Laboratory, and stored at -20°C until further analysis. Liver biopsies were performed on the same subset of cattle using the method described by Engle and Spears (2000). Liver biopsies were transferred into storage tubes, placed on ice, and transferred to the Kansas State University Ruminant Nutrition Laboratory where they were stored at -20°C until subsequent analysis of Cu concentration. Liver Cu was analyzed using inductively coupled plasma mass spectroscopy (Utah State Veterinary Diagnostic Lab, Logan, Utah). The same blood and liver collection procedures were repeated on d 70 of the study to establish final mineral concentrations for the same animals.

Diet Preparation

Heifers were fed a basal diet consisting of 30% corn silage and 70% ground brome hay on a DM basis for the first 86 d. Cattle were then switched to a common total mixed ration for the last 16 d on study to equalize gastrointestinal tract fill among treatment groups on BW (Table 2). Diets were mixed once daily in a truck-mounted feed mixer and fed at approximately 1300 h. Intakes were monitored daily, with adjustments being made as needed to ensure *ad libitum* intake by adjusting feed calls using a computerized feed management system (Micro Beef Technologies; Amarillo, TX). To determine DMI during each 28-d period, excess feed was removed from bunks and dried on a weekly basis or as needed to quantify refused feed. Samples were dried for 48 h in a forced-air oven at 55°C to determine DM content. Salt was provided *ad libitum* to heifers in S and B pens using plastic feeders secured within automotive tires to minimize spillage losses. The dry mineral mixture was offered in the same manner. Blocks were packaged in plastic feeders and offered *ad libitum* in conjunction with *ad libitum* access to loose salt. Mineral was offered free-choice. Mineral, salt, and block feeders were checked daily, filled or replaced as needed, and consumption was recorded. Weekly consumption of supplement and individual minerals was determined by weighing unconsumed product, drying a subsample, and subtracting this amount from the total amount offered. Analysis of the basal diet was conducted by collecting weekly feed samples, compositing samples, and analyzed for DM, CP, Ca, NDF, P, Cu, and Zn (SDK Laboratories, Hutchinson, KS). Supplement was analyzed for P, Cu, and Zn (SDK Laboratories). Dietary intake of individual minerals included both the basal diet and supplement combined.

Analytical Procedures

Zinc concentration of plasma was analyzed using atomic absorption spectrometry (Perkin Elmer Atomic Absorption Spectrometer 3110, PerkinElmer Waltham, MA). Plasma samples for Zn analysis were diluted with 1 mL plasma to 3 mL deionized water before spectrometric analysis, and absorbance was determined at 214 nm. Plasma P samples initially were diluted and proteins precipitated out with 0.25 mL of sample and 1 mL of trichloroacetic acid. Samples were analyzed in duplicate with spectrophotometry (Genesys 20, Spectronics Corporation Westbury, NY) using methodology previously described by Fiske and Subbarow (1925) at 660 nm. Liver biopsy samples were analyzed for Cu concentration (DM basis) using inductively coupled plasma mass spectrometry (Utah Veterinary Diagnostic Laboratory, Logan, UT).

Statistical Methodology

Statistical analyses were performed using the Statistical Analysis System (SAS version 9.4; SAS Inst, Inc. Cary, NC) with the experimental unit being pen and random effect of block. Growth performance (DMI, gain:feed, and ADG) was analyzed with the MIXED procedure of SAS, using treatment as the fixed effect. Supplement intake and intakes of Cu, P, and Zn were analyzed using the MIXED procedure of SAS as repeated measures, with fixed effects of treatment, week, and treatment × week. The random effect was block. Coefficient of variation for supplement intake was calculated using the standard deviation and LSMeans and was analyzed using the MIXED procedure. Fixed effects of treatment and a repeated measure of week were used. Concentrations of plasma P, plasma Zn, and liver Cu were analyzed as repeated measures with the MIXED procedure. Fixed effects were treatment, day, and treatment × day and block was used as

the random effect. Percentages of animals classified as having deficient, marginal, or sufficient liver Cu, and plasma P and Zn were analyzed using the GLIMIX procedure of SAS. Fixed effects included treatment, day, and treatment × day and random effect was block. A tendency for a difference among means was defined as $\alpha \leq 0.10$ and differences of $\alpha \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Three heifers were removed from the study due to either death or chronic toe abscesses unrelated to treatment.

Cattle Performance

Cattle performance is summarized in Table 3. There were no treatment differences for DMI or gain:feed ($P > 0.10$); however, cattle fed M exhibited greater ADG ($P < 0.05$) compared to cattle fed S or B. Additionally, M had a greater final bodyweight compared to S ($P < 0.01$); however, B was similar to M and S ($P > 0.09$) Increased ADG can be attributed to a numerical increase in DMI. Performance is limited by protein and energy supply, and is a function of forage intake, forage quality, and additional supplementation (Holloway et al., 1991; Poppi and McLennan, 1995). The basal diet that was fed for the initial 86 d was designed to emulate moderate quality pasture, with relatively low concentrations of Cu, P, and Zn; however the brome hay had a lower CP than typical, resulting in a diet containing 8% CP. Diets were analyzed using the Beef Cattle Nutrient Requirements Model of the basal diet (BCNRM; National Academies of Sciences, Engineering, and Medicine, 2016). The empirical level model was used and inputs included, Angus-type heifers, 9 months in age, with an initial body weight of 253.9 kg and a final body weight of 309.3 kg. Environmental factors included

minimum and maximum temperatures of 23°C and 35°C, a wind speed of 1.609 km/h, and hide coat and hide factors of 1. Model inputs were corrected using observed performance data, forage intake, supplement intake, and analyzed chemical compositions of feed ingredients and supplements. According to the BCNRM, S was deficient in Ca, P, S, Co, Cu, I, Fe, Se, and Zn, had an MP allowable gain of 0.318 kg/d, ME allowable gain of 0.554 kg/d, and had 98 g/d of MP available for growth; however, required MP for growth was 171 g/d, 43% more than what was supplied. Dietary protein provided 414 and 161 g/d of RDP and RUP, respectively. Although S was deficient in most minerals, the 1st limiting nutrient in the diet was most likely protein; however, heifers exceeded the MP allowable gain by 0.23 kg/d and was similar to the ME allowable gain. Both B and M were sufficient in most minerals, the exceptions being S, Ca, and P. Dry mineral and B had ME allowable gains of 0.573 and 0.561 kg/d, respectively, and MP allowable gains of 0.334 and 0.323 kg/d, respectively. Dietary protein provided 416 g/d of RDP and 161 g/d of RUP in M. Conversely, M provided greater dietary protein (423 g/d of RDP and 163 g/d of RUP). Average daily gain was limited by both protein and energy in S, M, and B; however, protein was likely 1st limiting. In contrast to treatment diets, the TMR fed to equalize gut fill in the final 14 was sufficient in minerals and had MP and ME allowable gains of 1.03 and 1.23 kg/d, respectively. Average daily gain during the initial 86 d was 0.44 kg/d for all treatments; however, ADG increased to 1.17, 1.42, and 1.21 kd/d for S, M, and B, respectively. Increased ADG was likely due to increased protein and energy supply of the diet. In agreement, decreased ADG has been reported in cattle not supplemented with an additional protein or concentrate source when grazing poor quality pastures low in CP when compared to supplemented cattle (Moore et al., 1999).

Titgemeyer et al. (2004) evaluated effects of increasing dietary CP with either cooked molasses blocks or alfalfa hay on performance of heifers fed low quality prairie hay (5.2% CP). They observed increased ADG in heifers supplemented alfalfa hay (16.6% CP) and cooked molasses blocks (27.5% CP) compared to heifers consuming *ad libitum* prairie hay alone. In agreement, Köster et al. (1996) reported an improvement in digestibility of low quality prairie hay when degradable intake protein was increased. Despite treatment differences in ADG, protein and energy likely limited growth to a greater extent than mineral consumption.

Supplement Intake

There was a treatment × week interaction ($P < 0.01$) and an effect of week ($P < 0.01$) detected for supplement intake (Figures 3.1, 3.2, and 3.3). Free choice salt intake was monitored for S as well as B; M had salt mixed into the supplement. Salt intake did not differ between S and B ($P = 0.23$; 95.4 and 100.6 g•animal^{-1}•d^{-1}, respectively); however salt intake was decreased in M compared to S and B ($P < 0.01$; 27.8 g•animal^{-1}•d^{-1}). Cattle require 0.08% dietary Na (National Academies of Sciences, Engineering, and Medicine, 2016), and 0.20% NaCl (NRC, 1984). Salt intake was greater than our expected intake of 15 to 28 g•animal^{-1}•d^{-1}. Grazing cattle can consume 14 to 16 g/d of salt in order to maintain adequate Na status (McDowell, 2003); however, cattle can tolerate up to 1 g salt/kg bodyweight without observing adverse effects on feed intake (NRC, 2005). Stocking density has been reported to impact intake behavior (Stricklin and Kautz-Scanavy, 1984) and limited pen space may have driven supplement intakes. Heifers in this study were allowed 22 m^2 per animal; however, in a grazing scenario yearling cattle have more than 8000 m^2 per animal. In addition to stocking density, salt

hunger may have driven supplement intakes. Cattle have a taste for salt and crave it especially when deficient. Characteristics of this craving are licking of salty objects as well as pica (McDowell, 2003; NRC, 2005). Salt craving has been reported among a wide range of ruminant species (Singh et al., 2000; Villalba et al., 2008; Caecero et al., 2009). Supplement intake differed week by week within the study period and was dependent on treatment. Block intake was greatest during week 1 ($P < 0.01$, Figure 3.3) and proceeded to plateau through the remainder of the study. Novelty of the supplement in pens may explain greater intakes. Supplement intake during week 8 was depressed among all treatments, and is likely due to high temperatures during this particular week in August. Week to week variability in supplement intake was compared using supplement intake CV (Figure 3.4). Within a treatment, weekly intake CV was calculated. Coefficient of variation for supplement intake was 28% for S, 41% for M, and 30% for B. Intake variability was greatest for M ($P < 0.04$), whereas S alone and M were similar ($P = 0.78$). Bailey and Welling (2007) reported a greater attraction to molasses block supplements when compared to dry mineral supplements in cows among various terrains. Maintaining consistent supplement intake is important in order to provide adequate minerals to cattle, as well as for determining supplementation protocols. A review by Bowman and Sowell (1997) of ruminants in various conditions indicated that variation in supplement consumption was greatest for block or liquid supplements, with a CV of 71%, in contrast to 38% for dry supplement; however, our results would suggest an increase in variability for dry mineral supplements. Differences in housing environments may have impacted CV as well. Stricklin and Kautz-Scanavy (1984) reported greater intakes and increased activity when stocking density was increased. Variation in intake may have been due the

number of heifers that visited supplement feeders in a given day. Bailey and Welling (2007) observed an increased percentage of cows that visited molasses block supplements compared to dry mineral supplements in moderate terrain in Montana (74% vs. 56%, respectively). We suspect that delivery method, supplement type (salt-based or molasses based), and stocking density may have influenced supplement consumption.

There was a treatment × week interaction for Cu intake ($P < 0.01$), Zn intake ($P < 0.01$) and P intake ($P < 0.01$) and an effect of week for all Cu, Zn, and P intakes ($P < 0.01$). Nutrient intakes differed among all treatments and nutrients ($P < 0.01$). Copper consumption was less for cattle fed S (i.e. salt) ($P < 0.05$; Figure 3.5) compared to cattle fed M ($P < 0.05$), and cattle fed B were intermediate ($P < 0.05$). Consumption of Zn (Figure 3.6) and P (Figure 3.7) followed a similar trend with differences seen among all treatments ($P < 0.01$). Bailey and Welling (2007) noted higher intakes of Cu, P, and Zn in a commercial dry mineral mix when compared to a low-moisture molasses mineral block.

Plasma Phosphorus

No treatment differences were observed for plasma P ($P > 0.1$; Figure 3.8), however plasma P was decreased by d 70 compared to d 0 ($P = 0.01$). Treatment means for S, M, and B were 6.95, 7.41, and 7.25 mg/dL, respectively. These values are within the normal range of 6 to 8 mg/dL for young animals, and well above a critical deficiency of 4.5 mg/dL (McDowell, 2003). Deficient, marginal, and sufficient status was defined as plasma P concentrations of < 4.5 mg/dL; ≥ 4.5 and < 6 mg/dL; and ≥ 6 mg/dL, respectively. No heifers were classified as deficient in P, and there were no treatment differences or effect of day for marginal or sufficient P status ($P > 0.3$). Kirk and Davis (1970) observed average plasma P concentrations of 5.2 mg/dL in Florida range cattle,

with only 17% of the cattle below 4.0 mg/dL. Geisert et al. (2010) observed an average plasma P of 7.07 mg/dL for finishing cattle with intakes of 14.1 to 29.6 g/d of P, which was lower than our observed P intakes. Within the body, 80 to 85% of P is stored within bone, minimizing blood P, while excess P is excreted via feces and urine (McDowell, 2003). Plasma P is therefore not optimal as a method for determining P status. Data from our study suggest that sampled heifers were sufficient in P, and any excess may have been deposited in bone or excreted, making treatment differences difficult to detect.

Plasma Zinc

Plasma Zn was not different among treatments ($P > 0.1$; Figure 3.9) despite differences in Zn intake. Mean plasma Zn values were within the normal range, 0.6 to 1.2 mg/mL (NRC, 2001 and McDowell, 2003). An effect of day was observed in plasma Zn ($P < 0.01$). Plasma Zn was greater on d 70 compared to d 0 ($P < 0.01$). Zinc status was characterized as deficient, marginal, or sufficient based on plasma Zn concentrations of < 0.6 mg/L; ≥ 0.6 and < 0.8 mg/L; and ≥ 0.8 mg/L, respectively. The proportion of animals classified as sufficient in Zn was not different among treatments ($P > 0.5$, Figure 3.10). Literature is ambiguous with respect to varying concentrations of Zn supplementation and the response in plasma Zn. Malcolm-Callis et al. (2000), Spears and Kegley (2002), and Nunnery et al. (2007), observed no differences in plasma or serum Zn at different intakes or with different sources of Zn. In contrast to our results, however, Van Bibber-Krueger (2016) observed a concentration-dependent response in plasma Zn when zinc sulfate was added to total mixed rations at different concentrations for finishing heifers. This inconsistency may be due to the lack of a storage mechanism for Zn in the body

(McDowell, 2003), or differences in diets. Due to these characteristics, much of the Zn supplemented is excreted in the feces.

Copper Status

Final concentrations of Cu in the liver were different among all treatments (P < 0.01, Figure 3.11). Concentrations of Cu in livers from heifers were lowest for S in comparison to M and B (P < 0.01). Concentration of Cu in livers from heifers supplemented M were greater than liver Cu concentration from heifers supplemented S or B (P < 0.01). McDowell (2003) established normal liver Cu concentrations varied from 100 to 400 mg/kg DM. Deficient, marginal, and sufficient status was defined as liver Cu concentrations of < 35 mg/kg DM, between 35 and 87.5 mg/kg DM, and > 87.5 mg/kg DM, respectively (Smart et al., 1992). The percentage of heifers classified as sufficient in Cu status was decreased to 59.4% (P < 0.01; Figure 3.12) for heifers supplemented S compared to M and B, whereas 100% of the heifers consuming M and B were sufficient in Cu status (P = 0.45). This response is likely reflective of Cu intake among treatments, S having less Cu intake and M having the greatest intake. Liver Cu concentrations suggest that Cu accumulated in tissue as intake increased, which is similar to observations of Engle and Spears (2000). Dorton et al. (2003) observed an increase in liver Cu at 20 mg/kg added Cu in comparison to 10 mg/kg added Cu, and saw an effect of Cu among all time periods in growing and finishing steers.

In summary, tissue mineral status was dependent on mineral intake, as exhibited by liver Cu concentrations. A majority of the heifers within this study were sufficient in both Zn and P, as plasma concentrations of both minerals were within the normal range;

however, differences in mineral bioavailability were not evident between the dry mineral supplement and the low-moisture molasses block supplement.

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Table 3.1 Nutrient composition of supplement provided to heifers on a forage-based diet for 86 d

Nutrient	Dry mineral	Block
Salt, %	23.0	-
Calcium, %	10.5	6.0
Phosphorus, %	7.8	3.6
Potassium, %	0.2	2.5
Magnesium, %	3.6	3.0
Cobalt, mg/kg	21.3	10.0
Copper, mg/kg	2330	1407
Iodine, mg/kg	87.7	42.0
Manganese, mg/kg	7161	3400
Selenium, mg/kg	27.5	13.2
Zinc, mg/kg	8235	3829
Vitamin A, IU/kg	550304	264000
Vitamin D, IU/kg	55030	26400
Vitamin E, IU/kg	550	264

Table 3.2 Composition of basal diets for heifers fed a forage-based diet for 86 d (basal diet) and a total mixed ration fed in an attempt to equalize gut fill (post-treatment diet) for an additional 16 d

Item	Basal diet	Post-treatment diet
Ingredient, % DM		
Steam-flaked corn	-	17.9
Wet corn gluten feed	-	30.0
Ground brome hay	70.0	20.0
Corn silage	30.0	30.0
Vitamin/mineral premix[1]	-	0.47
Limestone	-	1.41
Salt	-	0.25
Nutrient composition[2]		
CP, %	8.00	14.01
Ca, %	0.29	0.70
P, %	0.20	0.49
NDF, %	57.9	35.5
Cu, mg/kg	9.1	10.0
Zn, mg/kg	16.9	30.0
NE_m[3], Mcal/kg	1.19	1.81
NE_g[3], Mcal/kg	0.63	1.18

[1]Premix formulated to provide added levels of the following nutrients: 0.15 mg/kg Co, 10 mg/kg Cu, 0.50 mg/kg I, 20 mg/kg Mn, 0.10 mg/kg Se, 30 mg/kg Zn, 30 g/ton monensin, 2,200 IU/kg vitamin A, and 22 IU/kg vitamin E.
[2]Analysis of the basal diet was conducted by collecting weekly feed samples, and compositing samples for analysis (SDK Laboratories, Hutchinson, KS)
[3]Calculated using the Beef Cattle Nutrient Requirements Model (National Academies of Sciences, Engineering, and Medicine, 2016)

Table 3.3 Growing performance of heifers fed salt, dry mineral, or block supplements for 86 d and a total mixed ration for an additional 14 d[1]

Item	Salt	Dry mineral	Block	SEM	P-value
Body weight, kg	307[b]	312[a]	309[a,b]	1.3	0.02
Average daily gain, kg	0.55[b]	0.58[a]	0.55[b]	0.01	0.03
Dry matter intake[2], kg/d	6.94	7.06	6.92	0.20	0.54
Gain:feed	0.0792	0.0820	0.0790	0.0018	0.11

[1]Heifers were fed a forage-based basal diet as well as supplements for 86 d and switched to a TMR for 14 d in an attempt to equalize gut fill.

[2]Dry matter intake includes only the basal diet and TMR for 100 d and does not include supplement intake.

[a,b] Within row, means without a common superscript letter are different ($P < 0.05$).

Table 3.4 Total dietary intake of minerals for heifers fed a basal diet and salt, dry mineral, or block supplements for 86 d

Item	Salt	Dry mineral	Block	SEM	*P*-value
Copper, mg/d	60.5[c]	350.1[a]	284.0[b]	9.85	< 0.01
Phosphorus, g/d	13.2[c]	23.1[a]	18.8[b]	1.42	< 0.01
Zinc, mg/d	111.7[c]	1134.8[a]	720.5[b]	34.22	< 0.01

[a,b,c] Within row, means without a common superscript letter are different ($P < 0.05$).

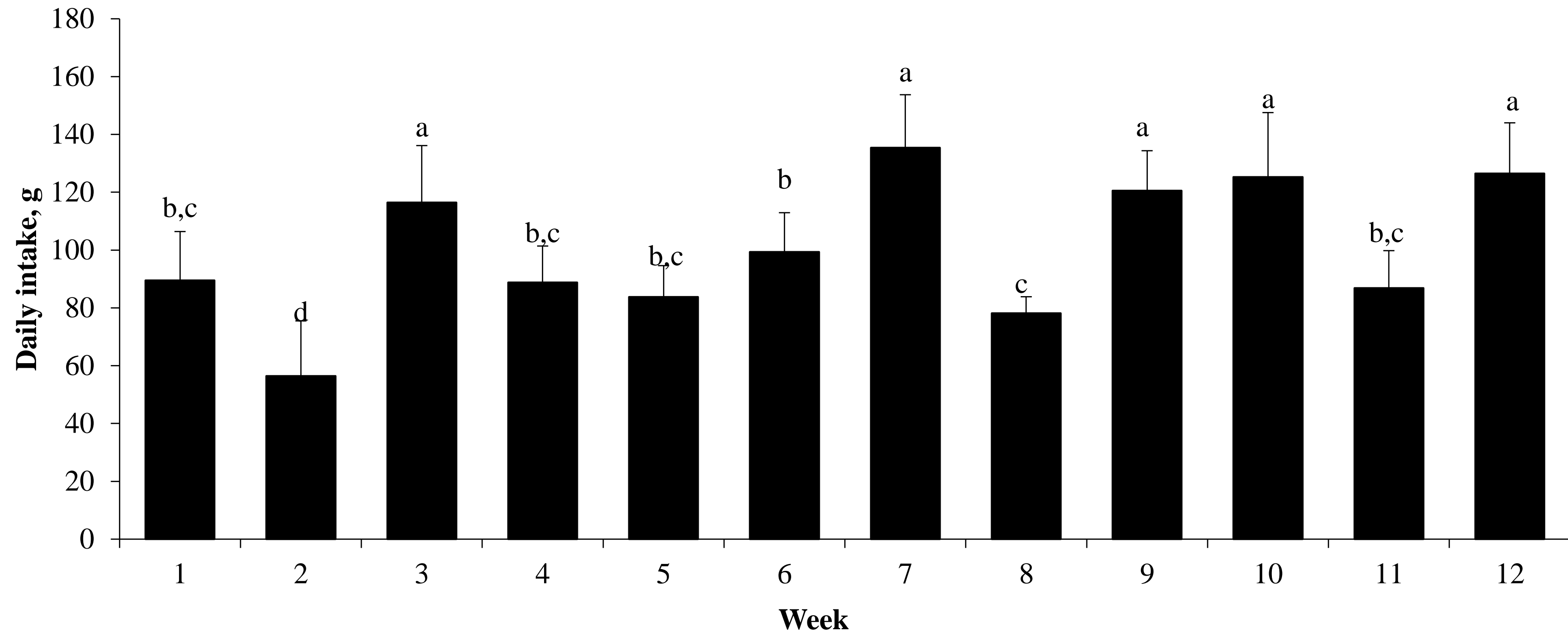

Figure 3.1 Supplement intake of heifers fed salt for 86 d. Supplements were weighed weekly and intakes are expressed as daily consumption per animal. Intakes were recorded on an as-fed basis and converted to DM basis by analyzing weekly DM content of the supplement. $^{a\text{-}d}$ Bars without a common superscript letter are different (P < 0.05). There was a treatment × week interaction, treatment effect, and a week effect (P < 0.01).

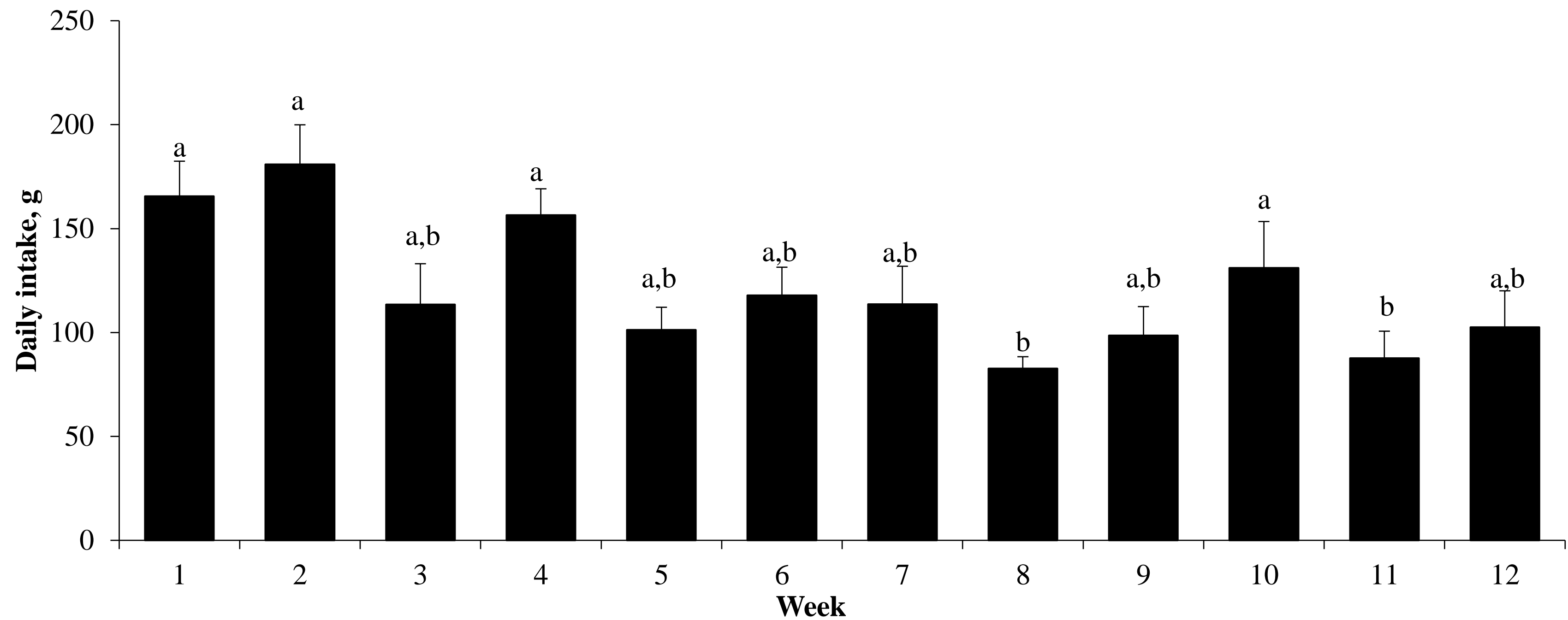

Figure 3.2 Supplement intake of heifers fed dry mineral for 86 d. Supplements were weighed weekly and intakes are expressed as daily consumption per animal. Intakes were recorded on an as-fed basis and converted to DM basis by analyzing weekly DM content of the supplement. [a,b] Bars without a common superscript letter are different (*P* < 0.05).There was a treatment × week interaction, treatment effect, and a week effect (*P* < 0.01).

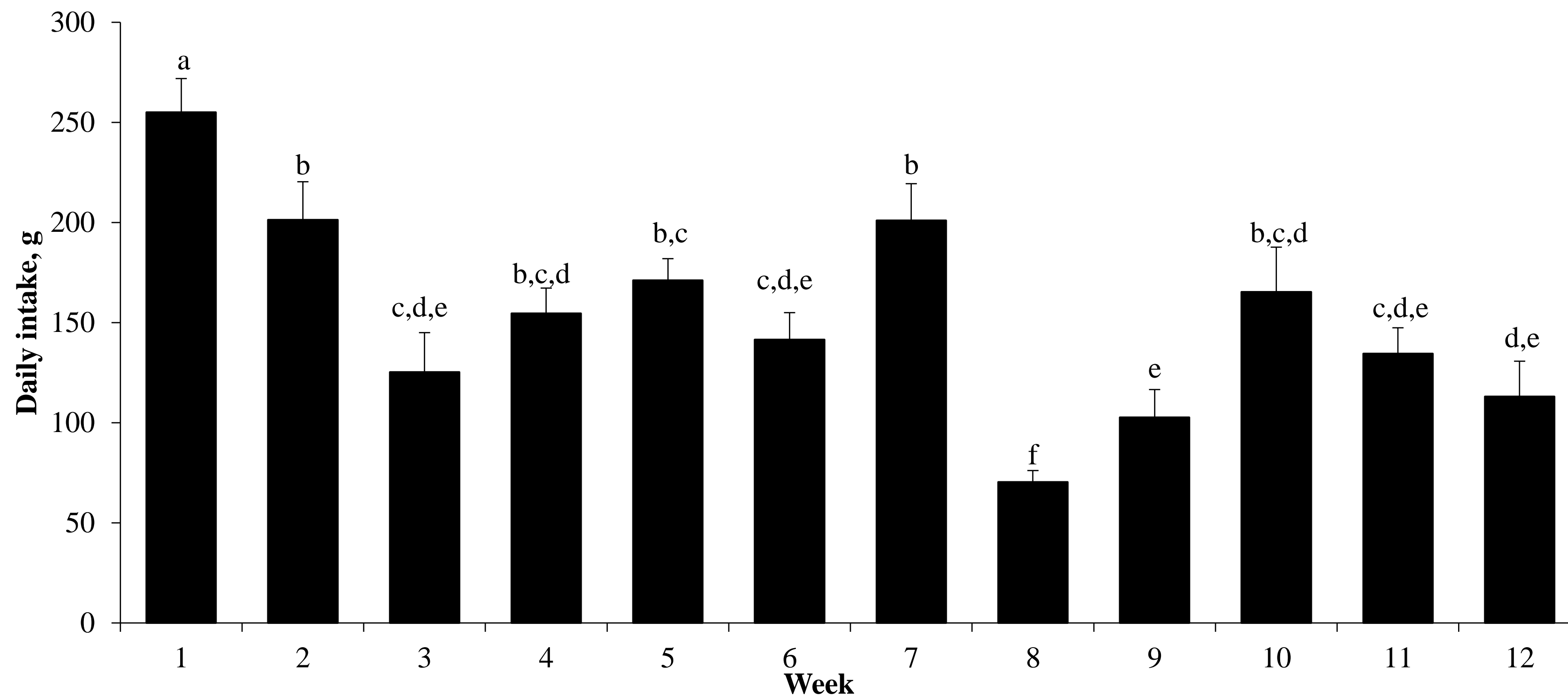

Figure 3.3 Supplement intake of heifers fed a block supplement for 86 d. Supplements were weighed weekly and intakes are expressed as daily consumption per animal. Intakes were recorded on an as-fed basis and converted to DM basis by analyzing weekly DM content of the supplement. [a-f] Bars without a common superscript letter are different ($P < 0.05$). There was a treatment × week interaction, treatment effect, and a week effect ($P < 0.01$).

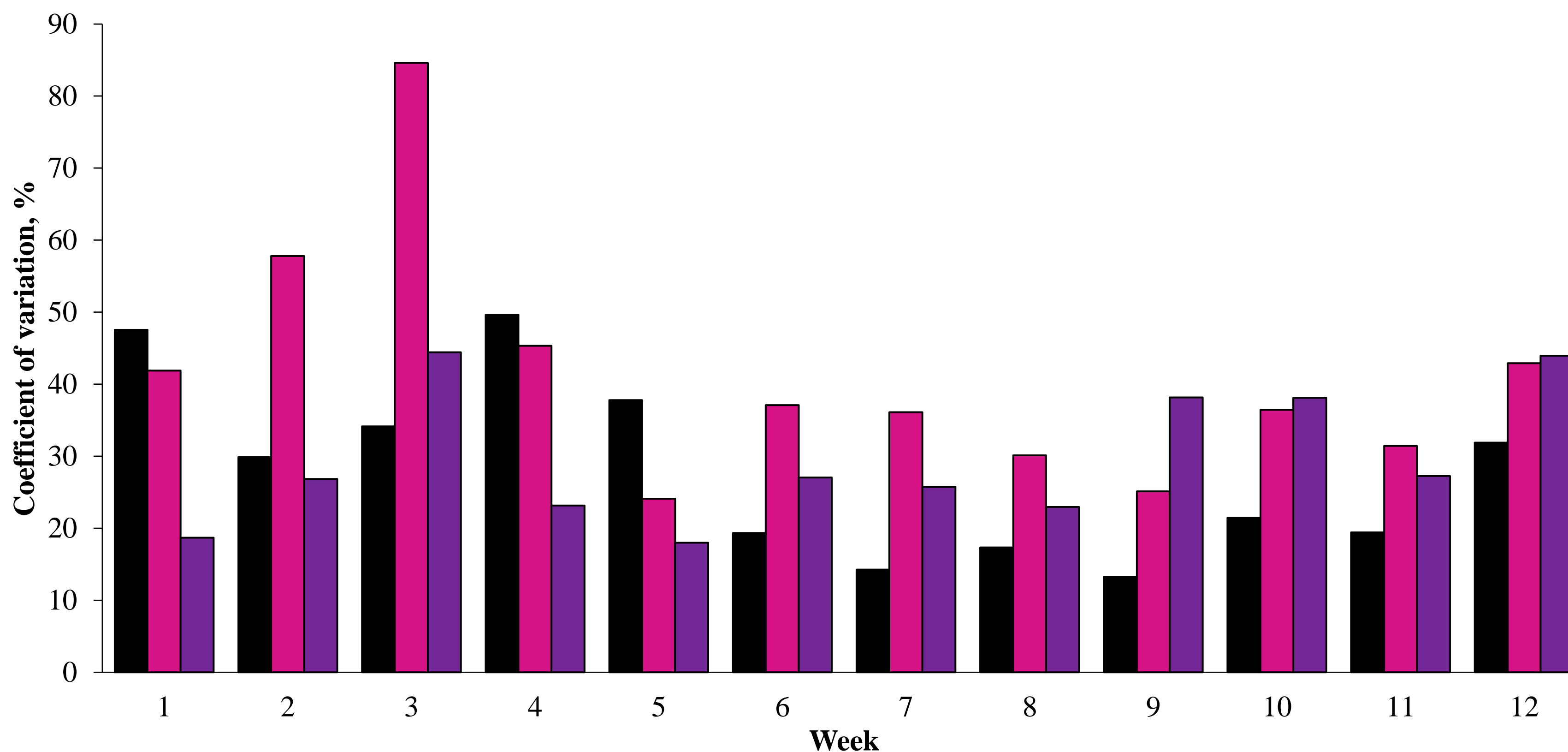

Figure 3.4 Coefficients of variation for supplement intake by heifers fed salt ■, dry mineral ■, or a low-moisture block supplement ■ for 86 d. Supplements were offered free-choice. Salt was offered *ad libitum* to block and salt treatments and was 23% of the dry mineral mix. Forage intakes were recorded daily and averaged by week, and supplement intakes were measured weekly. Effect of treatment (*P* = 0.04).

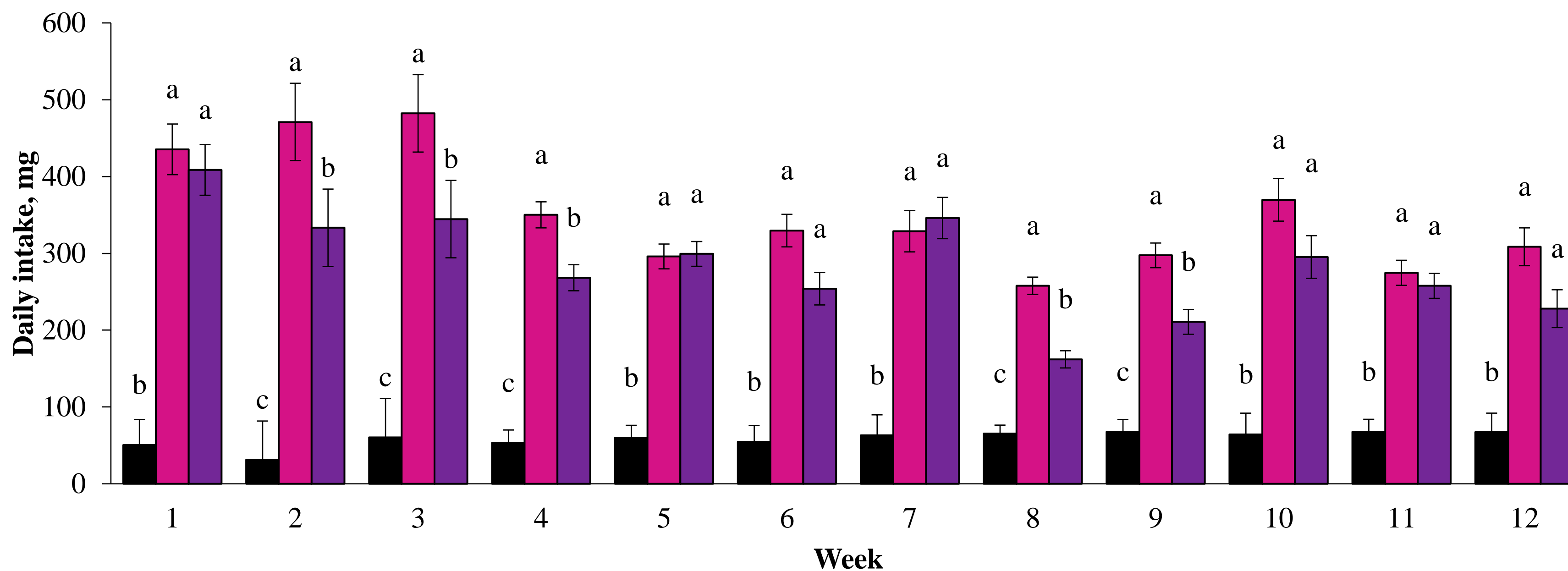

Figure 3.5 Dietary intakes of Cu by heifers fed salt ■, dry mineral ■, or a low-moisture block supplement ■ for 86 d. Supplements were offered free-choice. Salt was offered *ad libitum* to block and salt treatments and was 23% of the dry mineral mix. Forage intakes were recorded daily and averaged by week, and supplement intakes were measured weekly. [a,b,c] Within week, bars without a common superscript letter are different ($P < 0.05$). There was an effect of treatment, week, and a treatment × week interaction ($P < 0.01$).

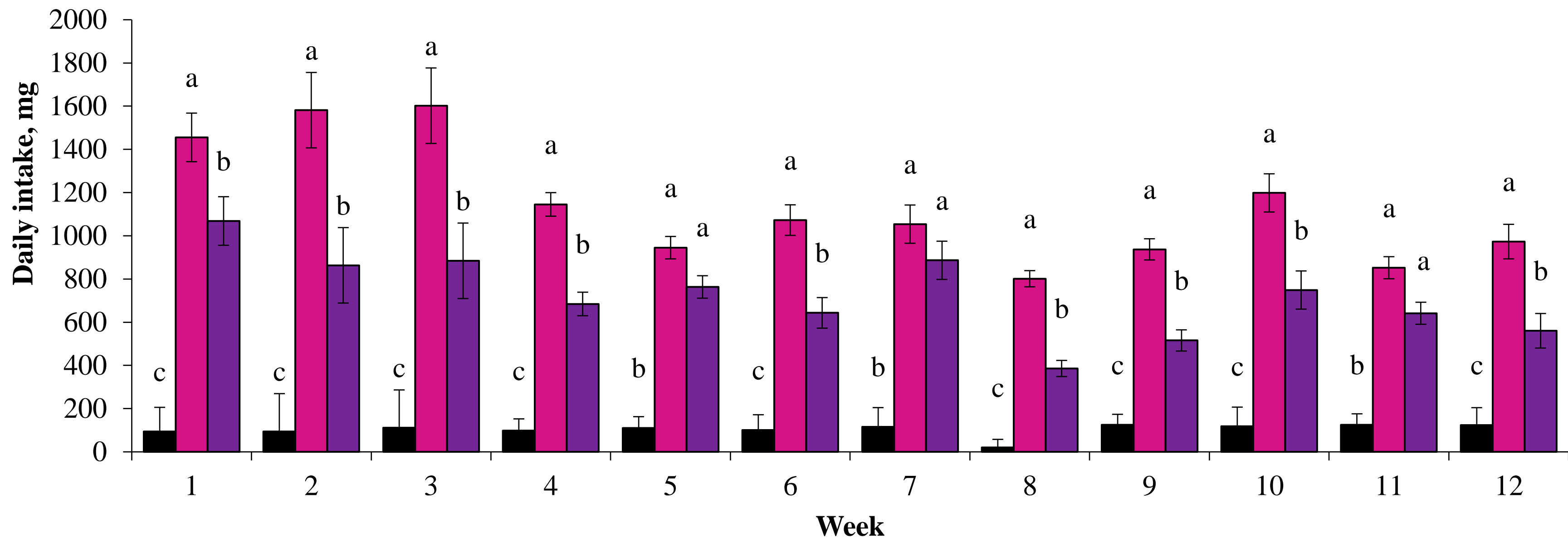

Figure 3.6 Dietary intakes of Zn by heifers fed salt ■, dry mineral ■, or a low-moisture block supplement ■ for 86 d. Supplements were offered free-choice. Salt was offered *ad libitum* to block and salt treatments and was 23% of the dry mineral mix. Forage intakes were recorded daily and averaged by week, and supplement intakes were measured weekly. [a,b,c] Within week, bars without a common superscript letter are different ($P < 0.05$). There was an effect of treatment, week, and a treatment × week interaction ($P < 0.01$).

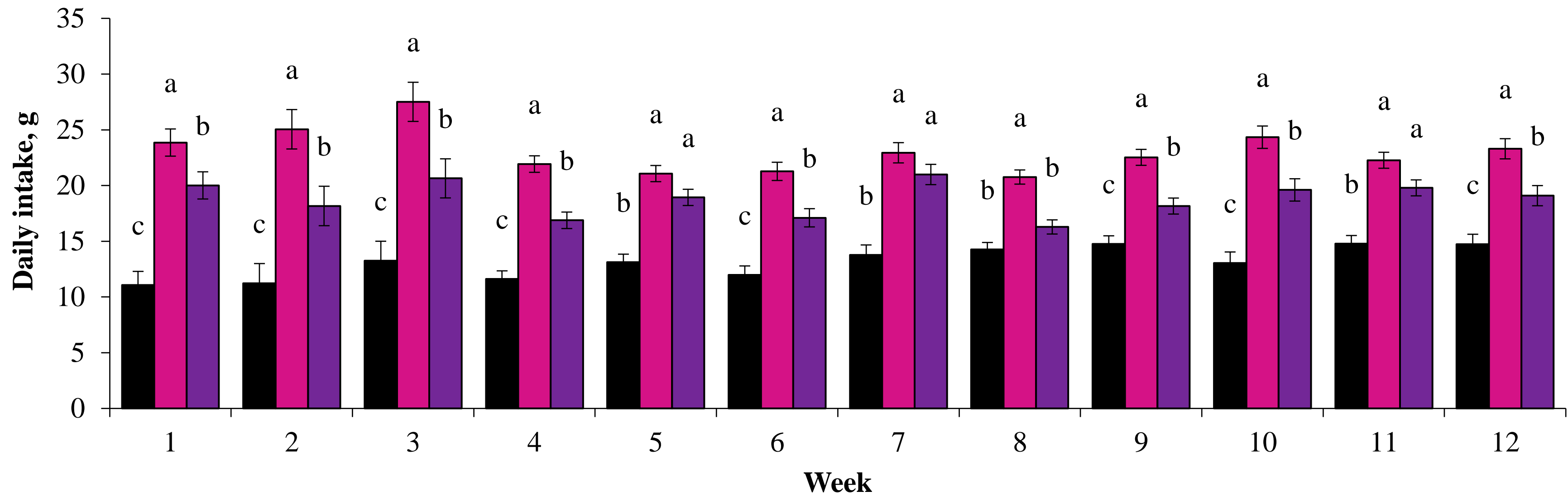

Figure 3.7 Dietary intakes of P by heifers fed salt ■, dry mineral ■, or a low-moisture block supplement ■ for 86 d. Supplements were offered free-choice. Salt was offered *ad libitum* to block and salt treatments and was 23% of the dry mineral mix. Forage intakes were recorded daily and averaged by week, and supplement intakes were measured weekly. [a,b,c] Within week, bars without a common superscript letter are different ($P < 0.05$). There was a treatment × week interaction ($P < 0.01$) as well as effects of treatment and week ($P < 0.01$).

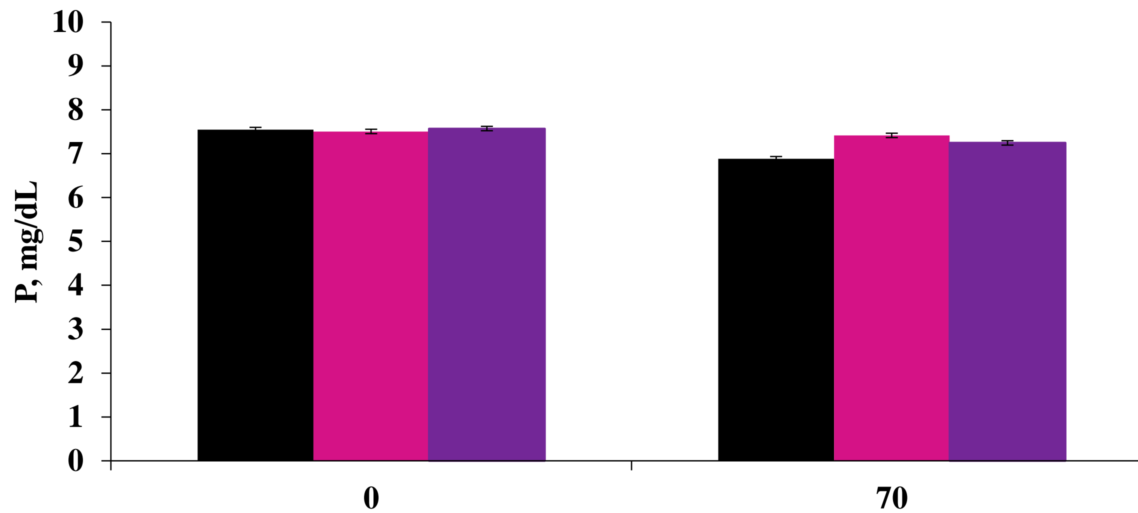

Figure 3.8 Concentration of plasma P in heifers fed salt ■, dry mineral ■, or a low-moisture block supplement ■ for 86 d. Plasma samples were obtained from 32 heifers within each treatment and analyzed using a colorimetric assay. No treatment × day interaction was detected (P = 0.23). There was no effect of treatment (P = 0.32). Plasma P was greater at d 0 compared to d 70 (P < 0.01).

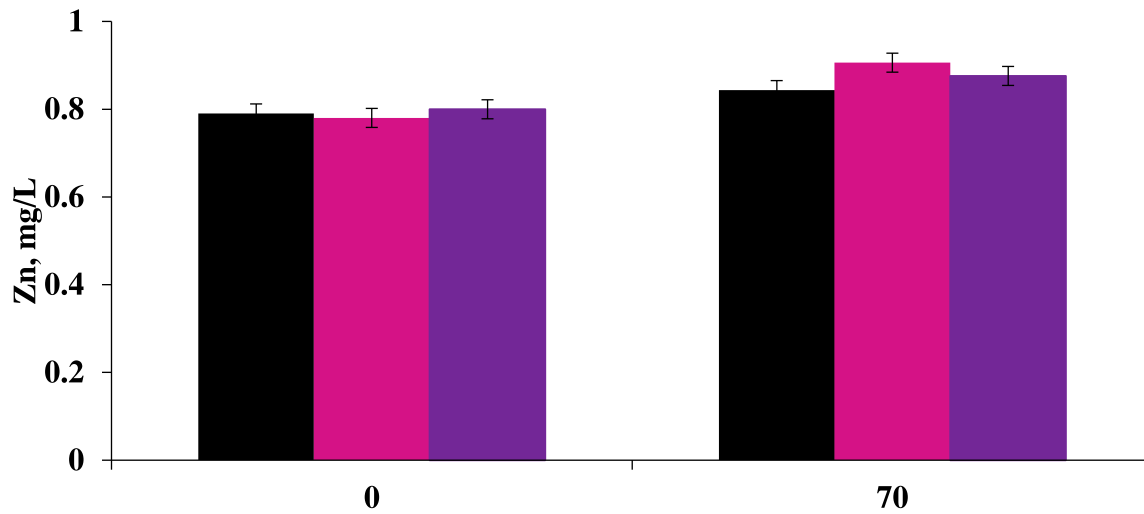

Figure 3.9 Concentration of plasma Zn in heifers fed salt ■, dry mineral ■, or a low-moisture block supplement ■ for 86 d. Plasma samples were obtained from 32 heifers within each treatment, diluted, and analyzed for Zn concentration using atomic absorption spectrometry. No treatment × day interaction was detected (*P* = 0.23). There was no effect of treatment (*P* = 0.32). Day 70 had a greater plasma Zn concentration compared to plasma Zn on d 0 (*P* < 0.01).

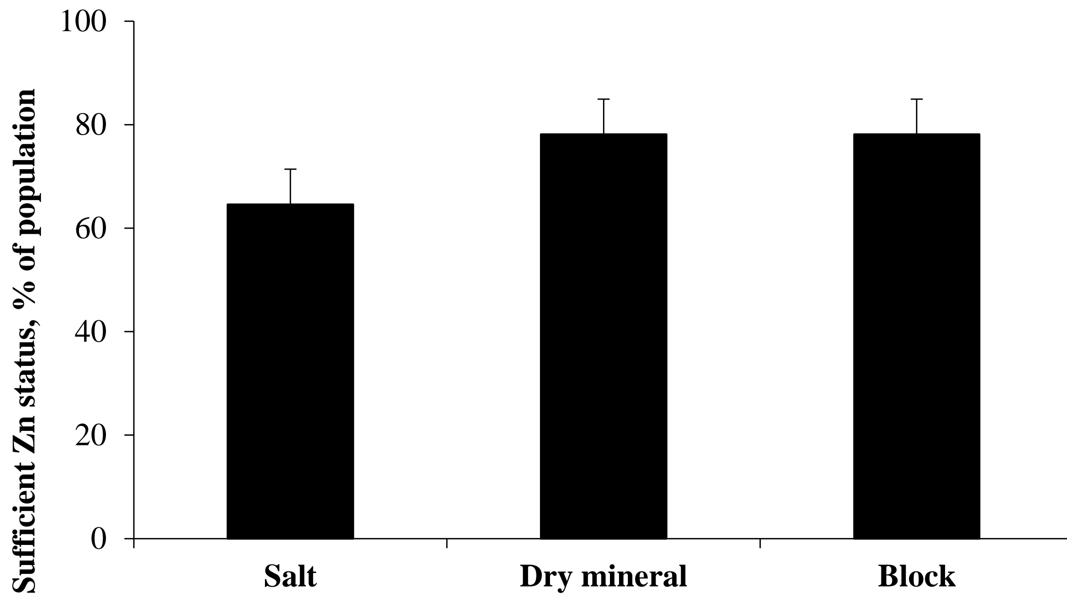

Figure 3.10 Heifers classified as sufficient with respect to plasma Zn status when fed salt, dry mineral, or a low-moisture block supplement for 86 d. Plasma samples were obtained from 32 heifers within each treatment. Supplements were offered free-choice. Salt was offered *ad libitum* to the block and salt treatments and was 23% of the dry mineral mix. Sufficient defined as plasma Zn concentrations of ≥ 0.8 mg/L. Heifers classified as sufficient in Zn were not different (*P* = 0.57).

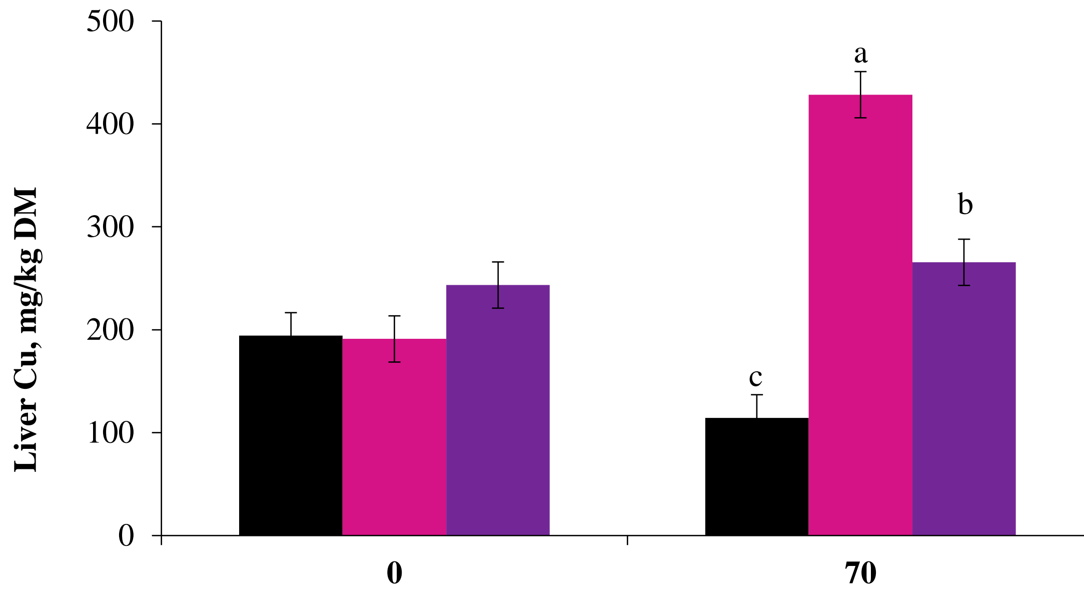

Figure 3.11 Liver Cu concentration of heifers fed salt ■, dry mineral ■, or a low-moisture block supplement ■ for 86 d. Supplements were offered free-choice. Salt was offered *ad libitum* to the block and salt treatments and was 23% of the dry mineral mix. Liver biopsies were sampled from 32 heifers per treatment. Samples were analyzed using ICP for Cu concentration. Treatment × day, $P < 0.01$, treatment effect, $P < 0.01$, effect of day, $P < 0.01$. [a,b,c] Bars without a common superscript letter are different ($P < 0.05$).

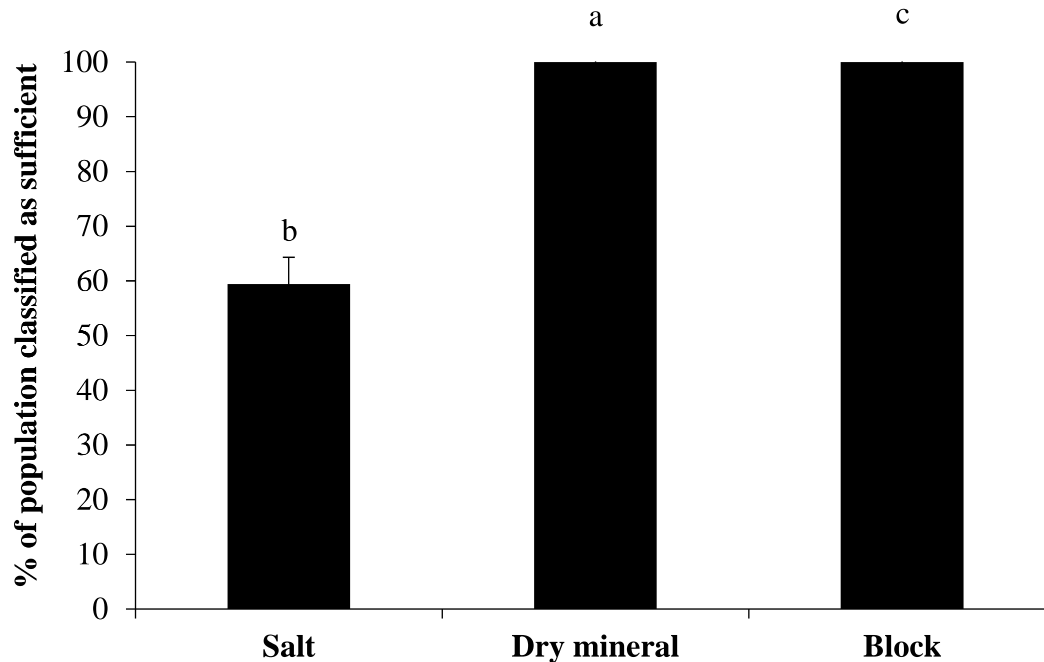

Figure 3.12 Heifers classified as sufficient with respect to liver Cu status fed salt, dry mineral, or a low-moisture block supplement. Supplements were offered free-choice. Salt was offered *ad libitum* to the block and salt treatments and was 23% of the dry mineral mix. Liver biopsies were sampled from 32 heifers per treatment. Sufficient defined as liver Cu concentrations ≥ 87.5 mg Cu/kg tissue dry matter. Treatment effect, $P < 0.01$. [a,b] Bars without a common superscript letter are different ($P < 0.05$).

Chapter 4 - Effects of mineral source and supplement type on *in vitro* fermentation by mixed ruminal microorganisms

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// ABSTRACT

Manufacturing processes involved in the production of mineral supplements could impact chemical structure of trace elements as well as influence interactions between trace elements and organic ingredients in supplements, possibly influencing bioavailability. This study was conducted in order to assess availability of cooked molasses block supplements made with varying mineral concentrations using a batch culture *in vitro* system. This study was a 3 x 4 factorial design with source and concentration of mineral being the factors. Mineral source included: 1) a dry mineral premix consisting of copper sulfate heptahydrate, zinc sulfate monohydrate, manganese sulfate monohydrate, and limestone as a carrier 2) a dry mineral premix and a dehydrated mixture of molasses and fat added separately into fermentation flasks and 3) a mineralized block, which was manufactured by heating the molasses block and incorporating the dry mineral premix into the mixture while it was still hot. Concentrations were 0×, 1×, 5×, and 10×; where 1× was defined as 10 mg Cu/kg, 30 mg Zn/kg, and 40 mg Mn/kg. Impact of supplement type and mineral concentration was analyzed using gas production, IVDMD, pH, and VFA production in a batch culture *in vitro* system after 48 h of incubation. There was no source × concentration effect for gas production ($P = 0.99$). Gas production decreased with increasing trace mineral concentration ($P < 0.01$), and both sources containing molasses blocks resulted in greater gas production than dry mineral alone ($P < 0.01$); however, the molasses block containing sources were not different at any point during the incubation period ($P = 0.39$). *In vitro* dry matter disappearance decreased with increasing trace mineral concentration ($P < 0.01$), and source tended to affect IVDMD ($P = 0.09$). There were effects of concentration on all major VFAs ($P < 0.01$), as concentration increased, VFA production decreased. Treatments containing

molasses blocks had greater total VFA, propionate, butyrate, and valerate production when compared with the negative control ($P < 0.05$); however, there was no effect of source on acetate production ($P = 0.29$). In conclusion, the manufacturing process involved in cooked molasses blocks does not affect mineral availability, and increasing trace mineral concentrations adversely affects fermentation by ruminal microbes.

INTRODUCTION

Manufacturing processes for mineral supplements may impact nutrient bioavailability in livestock. Low-moisture molasses blocks are manufactured by heating a molasses and fat based mixture to high temperatures, cooking the mixture, using a vacuum to remove the water in the molasses, then allowing the mixture to cool slightly, after which a trace mineral premix and other components are added before packaging. Unlike vitamins, minerals cannot be destroyed by heat. However, high-heat processes may impact mineral form or minerals may react with other nutrients in products, potentially influencing bioavailability. In human nutrition, cooking and heating food has been reported to decrease relative bioavailability and has been extensively studied in Fe nutrition (Hallberg, 1981; Kapandis and Lee, 1996). Some heat processes result in Maillard reaction products, which may bind with trace elements, especially Zn (Johnson, 1991; Reddy and Love, 1999). Conversely, cooking methods have been reported to increase bioavailability of trace minerals, by decreasing anti-nutritional factors and increases in solubility (Hurrell et al, 1989; Kapandas and Lee, 1995). Both increasing select dietary trace mineral concentrations fed to ruminants, and increased trace mineral concentrations incorporated into *in vitro* cultures has negatively impacted fermentation (McNaught et al., 1950; Essig et al., 1972; Forsberg, 1978; Katulski and Drouillard, unpublished data). Therefore, our objective was to use an *in vitro* system to

determine relative bioavailability as well as any interactions with source and concentration using supplemental trace minerals (Cu, Zn, and Mn) incorporated into molasses-based block supplements at increasing concentrations of Cu, Mn, and Zn as a trace mineral premix.

MATERIALS AND METHODS

Study Design

The study was conducted as a randomized complete block design, using a 3 × 4 factorial design with 4 runs of 2 replications each, providing 8 observations per treatment. Fermentation flasks were distributed into two blocks, with donor animal being the block. Treatments were then randomly assigned within block. Twenty-six flasks were utilized in each run; 24 flasks assigned treatments and 5 blanks.

In Vitro Fermentation

Treatments for the study consisted of 3 mineral sources and 4 concentrations, for a total of 12 treatment combinations. Mineral source included: 1) a dry mineral premix consisting of copper sulfate heptahydrate, zinc sulfate monohydrate, manganese sulfate monohydrate, and limestone as a carrier (Table 4.1); 2) a dry mineral premix and a dehydrated mixture of molasses and fat added separately into fermentation flasks and, 3) a mineralized block. Mineralized blocks were made by combining 90 g of a dehydrated mixture of molasses and fat that was heated until fluid in consistency (approximately 95°C), and 10 g of a dry mineral premix were added, homogenized, and frozen. Frozen blocks were ground to a powder-like consistency. Molasses blocks were manufactured using an identical trace mineral premix used in the dry mineral alone treatment. Trace mineral premix concentrations were: 1) 0 mg Cu/kg, 0 mg Mn/kg, and 0 mg Zn/kg (0×); 2) 10 mg Cu/kg, 40 mg Mn/kg, and 30 mg Zn/kg (1×); 3) 50 mg Cu/kg, 200 mg Mn/kg, and 150 mg Zn/kg (5×);

and 4) 100 mg Cu/kg, 400 mg Mn/kg, and 300 mg Zn/kg (10×). Prior to ruminal fluid collection, 25 g (10 g alfalfa + 15 g prairie hay) of substrate were added to 24 fermentation flasks. Two blank bottles (1 per replicate) received no substrate for baseline measurements of VFA profiles and substrate contributions of ruminal fluid for IVDMD. Treatment was applied after substrate was added by adding either 1 g of the appropriate mineralized block (0×, 1×, 5×, or 10×), 0.1 g of mineral (0×, 1×, 5×, or 10×), or 0.9 g molasses block + 0.1 g mineral at appropriate mineral concentration (0×, 1×, 5×, or 10×).

Ruminal fluid was collected from two Jersey steers with ruminal cannulas fed a diet that was 14.1% steam-flaked corn, 25% corn gluten feed, 30% ground brome hay, 30% ground alfalfa hay, and 0.9% supplement. Ruminal fluid was filtered through 4 layers of grade 50 cheesecloth (11 × 9.5 cm) as it was transferred to a pre-warmed insulated container. Inoculum was transported immediately to the Kansas State University Pre-Harvest Food Safety Laboratory, approximately 2 km. Upon arrival to the laboratory, ruminal fluid was strained through an additional 8 layers of grade 50 cheesecloth (11 × 9.5 cm) into large separatory funnels. Funnels were gassed with N_2 for 2 min and incubated at 39ºC for 30 to 40 minutes, allowing for stratification into layers consisting of a top mat layer, a bottom layer consisting of hydrated feed particles and protozoa, and an intermediate fluid layer. The bottom sediment fraction was discarded and the clarified liquid layer was collected for use as inoculant.

McDougall's buffer (700 mL) and ruminal inoculum (50 mL) were added to the 1000-mL fermentation vessels, initial pH was recorded with a Thermo Orion pH meter (model 230 A, Thermo Fisher Scientific Inc., Waltham, MA), vessels were gassed briefly with N_2 gas, capped with a Ankom[RF1] (Ankom RF Gas Production System; Ankom

Technology, Macedon, NY) module and placed into a 39ºC shaking incubator (New Brunswick Scientific Inc., New Brunswick, NJ). Fermentation bottles were gently agitated for 48 h. Gas pressure within each vessel was recorded hourly using Ankom computer software. After 48 h of incubation, final pH was recorded.

Using the ideal gas law, gas pressure was converted to moles of gas (n), and subsequently converted to volume of gas produced (mL) using Avogadro's law (Ankom Technology, 2014).

Ideal gas law: $n = p\left(\frac{V}{R \times T}\right)$

Avogadro's law: Gas production (mL) = $n \times 25.6 \times 1000$

In the ideal gas law, p is pressure (kPa), V is the volume of headspace in the fermentation vessels (L), R is the gas constant (8.314472 $L \cdot kPa \cdot K^{-1} \cdot mol^{-1}$), and T is temperature (K). In Avogadro's law, 25.6 is the volume that one mole of gas occupies at 39ºC. Gas production per g of substrate was calculated by:

Gas production per g substrate = Gas production / 25

Where 25 is the g of substrate incorporated into the fermentation vessel.

Volatile Fatty Acid Analyses

After final pH was recorded, 4 mL of fluid contents from each vessel were added to a vial containing 1 mL of 25% (w/v) m-phosphoric acid and frozen. After being frozen for approximately 24 h, vials were thawed, mixed thoroughly, transferred into microcentrifuge tubes, centrifuged at 10,000 × *g* for 15 min, and 2 mL of supernatant were transferred into 12 × 75 gas chromatography vials. Volatile fatty acid analysis was conducted using an Agilent 7890A Gas Chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a Supelco Nukol column (15 m × 530 µm × 0.5 µm, Supelco, Inc., Bellefonte, PA) and flame

ionization detector. Hydrogen was used as a carrier gas (40 mL/min) and inlet and detector temperatures of 275 and 300ºC, respectively. A standard was used (Supelco Volatile Standard Mix (46975-U) to calibrate peaks for individual volatile fatty acids. Production of VFA was calculated using the following formula:

$$\text{Total VFA production} = ((0.7 / 25) \times (\text{VFA concentration} \times 1.25)) - \text{Blank}$$

In this formula, 0.7 is the volume of liquid in the system, 25 is the g of substrate used, VFA concentration is the output from the gas chromatograph, 1.25 corrects for the dilution by m-phosphoric acid, and total VFA production from the blank is subtracted from each respective flask within replicates.

In Vitro Dry Matter Disappearance

After pH and sampling for VFA analysis, the remaining contents in each of the fermentation vessels was transferred into pre-weighed 19.0 × 12.7-cm aluminum pans and placed in a forced air oven and dried at 105ºC until dry (approximately 72 h). Pans were removed from the oven and weighed. The following formula was used to calculate IVDMD and subtracted DM from each blank within respective replicate:

$$\frac{\text{final vessel content DM wt.-blank content DM wt.}}{\text{initial substrate wt.+ mineral wt.}} \times 100$$

Statistical Methodology

Statistical analyses were evaluated using the MIXED procedure of Statistical Analysis System (SAS version 9.4; SAS Inst, Inc. Cary, NC). The experimental unit was fermentation flask and the random effect was replicate within run. *In vitro* dry matter disappearance, VFA production, and pH had fixed effects of mineral source, concentration, and source × concentration. The PDIFF function was used to determine differences between means. Gas production was a repeated measures analysis, using module within run as the

subject and fixed effects of mineral source, concentration, time, and all interactions. The SLICE option, slicing by time, was used to determine at which time differences between treatments could be detected. A tendency for a difference among means was defined as $\alpha \leq 0.10$ and differences of $\alpha \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Fermentation characteristics are presented in Table 4.2. There was no interaction between source and concentration for IVDMD, therefore only main effects will be discussed. *In vitro* dry matter disappearance was affected by mineral concentration ($P < 0.01$) and there was a tendency for source of mineral to affect IVDMD ($P = 0.09$). As mineral concentration increased, IVDMD decreased linearly ($P < 0.01$) by 3.2, 10.8, and 26.1% for 1×, 5×, and 10× respectively, when compared to no added mineral. This is likely due to adverse effects of trace minerals on microbial fermentation. The mineralized block tended to have a greater IVDMD than both dry mineral and block added separately and dry mineral alone ($P > 0.10$).

Initial pH was not different ($P > 0.38$); however, there was both a source ($P < 0.01$) and concentration ($P < 0.01$) effect on final pH recorded at 48 h. Concentration had a linear effect on final pH ($P < 0.01$); as mineral concentration increased in the fermentation vessels, pH increased. This is due to a decrease in organic acid production, as observed in the linear decrease ($P < 0.01$) in total VFA as concentration increased. Volatile fatty acid production was reduced by 38% from 0× to 10×. Dry mineral premix had a greater final pH compared to other mineral forms ($P < 0.03$). This may be due to dry minerals and block added separately and mineralized blocks having greater total VFAs than dry mineral ($P < 0.01$). An interaction between source and concentration was detected for propionate and valerate

production ($P < 0.05$). Similar to total VFA production, a concentration effect was observed for individual VFA production ($P < 0.01$) and A:P ($P < 0.01$). An increase in mineral concentration resulted in a linear decrease in individual VFA production and an increase in A:P ($P < 0.01$). Additionally, there was a quadratic effect of concentration for total VFA production, acetate, propionate, butyrate, isovalerate, and valerate ($P < 0.05$). In general, the quadratic effect was expressed as a plateau in VFA production between 0× and 1× or 5× and 10×, depending on the organic acid. The quadratic effect for total VFA and propionate production is apparent in a plateau in production between 0× and 1× before a depression is observed. However, acetate production was not different between 5× and 10× ($P = 0.23$), causing a quadratic effect for acetate. Butyrate production was not different among 0×, 1×, or 5× ($P > 0.61$); however at 10× a depression is observed ($P < 0.01$). In addition to total VFA production, there was an effect of source for propionate ($P < 0.01$), isobutyrate ($P = 0.04$), butyrate ($P = 0.02$), and valerate ($P < 0.01$) production. Propionate, butyrate, and valerate were not different between dry mineral and block added separately and mineralized blocks ($P > 0.3$); however, dry mineral reduced propionate, butyrate, and valerate production by 11.4, 14.6, and 23.4%, respectively. Isobutyrate production was decreased by mineralized blocks ($P = 0.02$) and tended to decrease by dry mineral and block ($P = 0.07$), when compared to dry mineral alone.

Gas production is presented in Figure 4.1. No three-way interactions were detected ($P = 1.0$). There were source × time ($P < 0.01$) and concentration × time interactions ($P < 0.01$). The interaction indicate that the response in gas production for both source and concentration was dependent upon incubation time; it is likely that the rate of gas production changed over time as well as differences in lag times in microbial growth. There was no

interaction between source and concentration for gas production ($P = 0.99$), therefore only main effects will be discussed. There was an effect of source, concentration, and time ($P < 0.01$). Gas production increased over time, resulting in 48 h of incubation having the greatest amount of gas produced. Gas production decreased as concentration increased ($P < 0.01$). Treatment 0× had the greatest gas production after 48 h of incubation, and 10 × had the least amount of gas produced. Both treatments containing molasses blocks had greater total gas produced when compared to dry mineral alone ($P < 0.01$).

There is limited information on the effects of manufacturing processes in block mineral supplements on mineral bioavailability and fermentation by ruminal microorganisms, and none have been published with respect to ruminal fermentation or impacts on ruminal microorganisms. Based on our results, the manufacturing process of cooked molasses block supplements does not appear to affect *in vitro* fermentation of trace minerals by ruminal microorganisms. The addition of molasses, regardless of source, increased gas production and many organic acids. Sugar cane molasses is 29% sucrose, 12% glucose, and 13% fructose (Damon and Pettitt, 1980) and may cause increased fermentation by ruminal microbes in treatments containing molasses. Molasses is characterized as being rapidly fermentable, and is commonly used as an additional energy source in cattle receiving low quality forage diets; in addition to being a common method of delivery for mineral supplements. Sugar cane molasses blocks reduced ruminal pH, and although sugar cane molasses blocks did not increase total VFA, both beet molasses and concentrated separator by-product blocks increased ruminal VFA (Greenwood et al., 2000). Similarly, Shellito et al. (2006) reported an increase in total VFA concentrations in cattle supplemented with a desugared molasses byproduct. Ferraro et al. (2009) reported a greater *in vitro* rate of gas

production for molasses compared to alfalfa, likely due to the presence of rapidly fermentable carbohydrates such as sucrose. Literature in human nutrition is available in regards to cooking methods and mineral bioavailability; however, it is difficult to directly relate these data to ruminants and ruminal fermentation. A majority of work in food processing and mineral availability is on Fe, although some studies have been published using Zn. Methods involving heat have been reported to reduce (Hemalatha et al., 2007) or have no effect (Johnson, 1991) on Zn bioavailability in humans. Johnson (1991) also reported no difference in bioavailability of Cu in foods under extreme heat. There is no published data on the effects of heat treatment on Cu, Zn, or Mn bioavailability in ruminants or impacts on ruminal microorgansims. Our results would suggest there is no impact of extreme heat on the minerals in question. The effect of trace mineral concentration on ruminal fermentation and microorganisms is variable and depends on individual minerals. In agreement with our results, increasing Cu concentration has been reported to negatively impact fermentation both *in vitro* and *in vivo* (Hubbert et al., 1958; Slyter and Wolin, 1967; Essig et al., 1972). Literature regarding effects of Mn supplementation on fermentation by ruminal microorganisms is sparse, although it has been reported that the ruminal environment can tolerate concentrations around 100 mg Mn/kg DM without negative impacts (Martinez and Church, 1970; Hilal et al., 2016). Arelovich et al. (2000) reported an increase in IVDMD as Mn concentration increased. Zinc has been reported to increase protein synthesis and urea utilization when added at concentrations up to 250 mg Zn/kg DM (Arelovich et al., 2000). In the same study; however, a linear decrease in IVDMD was reported with increasing Zn concentration (Arelovich et al., 2000). Similarly Eryavuz et al. (2009) reported an inhibition of *in vitro* cellulolytic activity at concentrations ranging from

50 to 150 mg/kg Zn, and Martinez and Church (1970) reported concentrations as low as 20 mg Zn/kg inhibit cellulolytic activity. However, the negative effect of Zn on cellulolytic activity has been reported to diminish after extended periods of fermentation (Eryavuz et al., 2009). In conclusion, there appears to be no differences in *in vitro* fermentation by ruminal microbes due to the manufacturing process of cooked molasses block supplements. The addition of molasses increases microbial activity regardless of molasses block processing. In addition, increasing trace minerals adversely affects fermentation by mixed ruminal microorganisms.

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1

Table 4.1 Composition of mineral premixes

	Concentration[1]			
Item	0×	1×	5×	10×
$CuSO_4 \cdot 5H_2O$, g	0.0000	0.0982	0.4912	0.9823
$MnSO_4 \cdot H_2O$, g	0.0000	0.3076	1.5382	3.0765
$ZnSO_4 \cdot H_2O$, g	0.0000	0.2059	1.0293	2.0586
Limestone, g	10.0000	9.3883	6.9413	3.8826

[1]Formulated to have 0 mg Cu/kg, 0 mg Zn/kg, and 0 mg Mn/kg (0×); 10 mg Cu/kg, 30 mg Zn/kg, and 40 mg Mn/kg (1×); 50 mg Cu/kg, 200 mg Mn/kg, and 150 mg Zn/kg (5×); and 100 mg Cu/kg, 400 mg Mn/kg, and 300 mg Zn/kg (10×) added minerals. Mineral premix was added separately in dry mineral treatment and dry mineral + molasses block treatment (0.1 g/fermentation flask), and was incorporated into a block (10 g/block) in the mineralized block treatment

Table 4.2 Effects of mineral source and concentrations[1] on characteristics of *in vitro* batch cultures of mixed ruminal microbes fed forage-based[2] substrate.

	Dry mineral				Dry mineral + molasses block				Mineralized block					*P*-value		
Item	**0×**	**1×**	**5×**	**10×**	**0×**	**1×**	**5×**	**10×**	**0×**	**1×**	**5×**	**10×**	**SEM**	**Source**	**Concentration**	**S × C**
IVDMD, %	27.40	26.50	23.95	19.24	28.29	26.21	25.23	21.29	27.84	28.13	25.29	21.16	0.015	0.09	< 0.01	0.77
Final pH	6.38	6.36	6.52	6.60	6.29	6.32	6.45	6.61	6.26	6.28	6.43	6.60	0.035	< 0.01	< 0.01	0.58
VFA, mmoles/g substrate																
Total VFA	2.26	1.96	1.74	1.45	2.46	2.39	1.85	1.39	2.37	2.28	1.82	1.54	0.099	< 0.01	< 0.01	0.08
Acetate	1.13	0.97	0.98	0.90	1.20	1.14	0.94	0.88	1.14	1.11	0.93	0.94	0.058	0.29	< 0.01	0.13
Propionate	0.87	0.75	0.57	0.43	0.97	0.95	0.64	0.37	0.95	0.90	0.62	0.48	0.042	< 0.01	< 0.01	0.04
Isobutyrate	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.002	0.04	< 0.01	0.92
Butyrate	0.17	0.16	0.14	0.08	0.19	0.19	0.19	0.10	0.18	0.18	0.19	0.09	0.020	0.02	< 0.01	0.90
Isovalerate	0.02	0.02	0.01	0.01	0.02	0.02	0.01	0.01	0.02	0.02	0.01	0.01	0.002	0.69	< 0.01	0.95
Valerate	0.06	0.05	0.03	0.01	0.06	0.07	0.06	0.02	0.06	0.06	0.06	0.01	0.005	< 0.01	< 0.01	0.03
A:P	1.31	1.29	1.79	2.16	1.23	1.20	1.51	2.61	1.20	1.22	1.57	1.99	0.144	0.21	< 0.01	0.08

[1]Factor 1, concentration, was formulated to have 0 mg Cu/kg, 0 mg Zn/kg, and 0 mg Mn/kg (0×); 10 mg Cu/kg, 30 mg Zn/kg, and 40 mg Mn/kg (1×); 50 mg Cu/kg, 200 mg Mn/kg, and 150 mg Zn/kg (5×); and 100 mg Cu/kg, 400 mg Mn/kg, and 300 mg Zn/kg (10×) added minerals. Mineral premix was added separately in dry mineral treatment and dry mineral + molasses block treatments, and was incorporated into blocks of differing concentration in the mineralized block treatment.
[2]Substrate used was 15 g of ground prairie hay and 10 g of ground alfalfa.

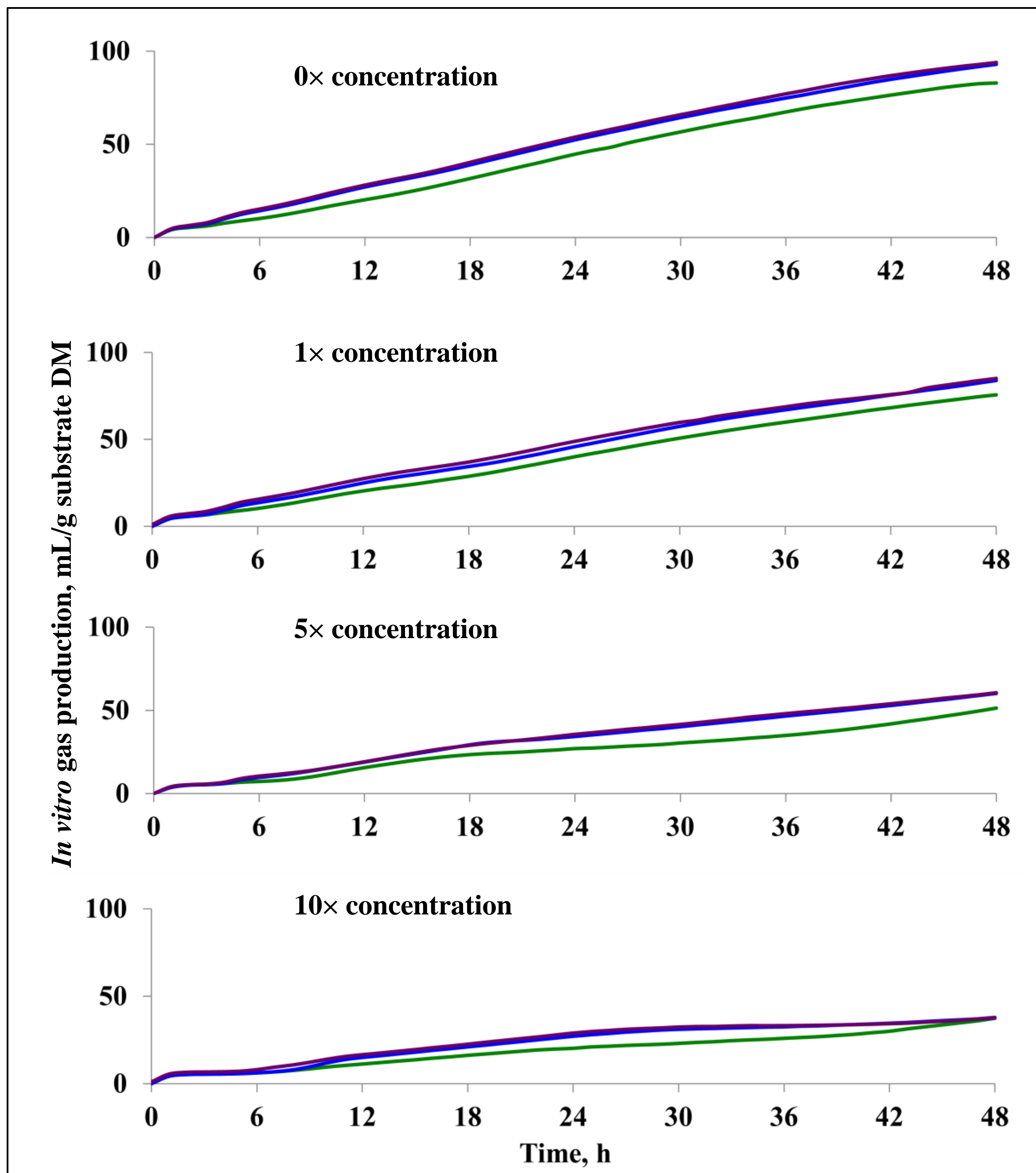

Figure 4.1 ***In vitro*** **gas production (mL/g substrate DM) of mixed ruminal microbes in a forage-based (15 g and 10 g of ground prairie alfalfa hay, respectively)** ***in vitro*** **batch culture fermentation system over 48 h. Two factors were analyzed, source and concentration. Sources were: dry mineral (■) dry mineral and molasses blocks added separately (■), and a mineralized molasses block (■). Concentrations were: 0 mg Cu/kg, 0 mg Zn/kg, and 0 mg Mn/kg (0×); 10 mg Cu/kg, 30 mg Zn/kg, and 40 mg Mn/kg (1×); 50 mg Cu/kg, 200 mg Mn/kg, and 150 mg Zn/kg (5×); and 100 mg Cu/kg, 400 mg Mn/kg, and 300 mg Zn/kg added mineral (10×). There was no source × concentration × time (P = 1.0). There was a source × time (P < 0.01), concentration × time (P < 0.01), and an effect of source (P < 0.01), concentration (P < 0.01), and time (P < 0.01**