

**THE BIOAVAILABILITY OF ZINC AND COPPER IN HOLSTEIN STEERS**

by

Laura Mary Nemec

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by  
Laura Mary Nemec

Approved: \_\_\_\_\_  
Tanya F. Gressley, Ph.D.  
Professor in charge of thesis on behalf of the Advisory Committee

Approved: \_\_\_\_\_  
Jack Gelb, Jr., Ph.D.  
Chair of the Department of Animal and Food Sciences

Approved: \_\_\_\_\_  
Robin Morgan, Ph.D.  
Dean of the College of Agriculture and Natural Resources

Approved: \_\_\_\_\_  
Debra Hess Norris, M.S.  
Vice Provost for Graduate and Professional Education

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## ABSTRACT

This study consisted of three experiments to evaluate the bioavailability of mineral supplements. The aim of Experiment 1 was to determine the bioavailability of the organic forms of copper (2-hydroxy-4-methyl-thiobutyrate; HMTBa Cu) and zinc (HMTBa Zn) when administered abomasally. Experiment 2 was performed to determine the bioavailability of two organic forms and one inorganic form of Zn administered abomasally, and measure neutrophil phagocytic ability and *L-selectin* mRNA. Experiment 3 determined the bioavailability of the same forms of organic zinc and ZnSO<sub>4</sub> administered via ruminal infusion. In all experiments, 4 ruminally cannulated Holstein steers, were assigned to a 4 × 4 Latin square design where each period consisted of 6 d rest and 1 d of treatment. Diets consisted of only alfalfa silage and corn silage to minimize dietary Cu and Zn. Jugular catheters were inserted 24 h prior to treatment and blood samples were taken from 0 to 12 h post treatment (Exp. 1) and 0 to 24 hours post treatment (Exp. 2 and 3). Additional blood samples were taken at 0 and 9 h in Experiment 2 for neutrophil measurements. In Experiment 1, a pulse dose of 4 g HMTBa Cu, raised plasma Cu from 0.5 – 1.5 h following infusion but decreased dry matter intake (DMI). A pulse dose of 8 g HMTBa Zn increased plasma Zn from 1 – 12 h but also decreased DMI whereas 4 g caused an increase in plasma Zn with no side effects. In Experiment 2, both ZnSO<sub>4</sub> and HMTBa Zn increased plasma Zn at 1 – 8 h and 1.5 – 7 h respectively. There were no differences found in neutrophil phagocytosis or *L-selectin* mRNA level. The organic forms of HMTBa Zn and AA-Zn increased plasma Zn at 8, 9, 12, - 16 and 24 h and 8, 9, 12 – 16 and 24 h respectively over the control in Experiment 3. The ZnSO<sub>4</sub> increased plasma levels at 9, 13, 16 and 24 h. The results of

these experiments provide evidence to support an increase the respective plasma concentrations with Cu or Zn supplementation and an equal bioavailability of both organic and inorganic Zn.

## **Chapter 1**

### **INTRODUCTION**

The trace minerals copper and zinc are essential to dairy cows for a wide variety of physiological processes. Inorganic trace minerals (**ITM**) such as sulfates and oxides have been the traditional method of dietary trace mineral supplementation. However, ITM are prone to antagonisms within the digestive tract that limit absorption and consequent mineral bioavailability. These antagonisms occur because minerals dissociate in the neutral or slightly acidic conditions of the cow's rumen and then bind to other minerals or non-nutritive components of digesta, such as fiber, rendering them unavailable in the small intestine. Organic trace minerals (**OTM**) are mineral supplements that bind the mineral to organic ligands. This binding reduces mineral dissociation in the rumen. Research has shown increased absorption and bioavailability of OTM compared to ITM, but responses vary among trials. Some of this variation is the result of different OTM chemistry which includes complexes, chelates, and proteinates that differ in their bioavailability.

## Chapter 2

### LITERATURE REVIEW

#### The Minerals Zinc and Copper

The importance of minerals to the daily maintenance of the bovine at both whole animal and cellular levels has been well documented. Trace minerals, or microminerals, are called such because they are necessary to the cow only in parts per million (ppm). They include Co, Cu, I, Fe, Mn, Se and Zn that are fed to cows in ranges from 0.1 to 80 ppm of the diet DM. Activities of hundreds of different enzymes are dependent upon one or more of these minerals. These enzymes are involved in the regulation of physiological processes including lactational performance, claw tissue formation and bone integrity (Siciliano-Jones et al., 2008). Specifically, the effects of the trace minerals Cu and Zn on cellular functions, animal production and animal health will be the focus of this review.

#### *Zinc: Function*

Zinc is found throughout the body and is essential for a variety of functions ranging from cellular metabolism to the ratio of lean tissue to fatty tissue deposition. After iron, Zn is the second most abundant transition metal in organisms (Broadley et al., 2007). Zinc has a wide variety of structural, catalytic and regulatory functions in cells. Adema disease in cattle and acrodermatitis enteropathica in humans are diseases that result in inadequate Zn absorption from the gut and consequently lead to Zn deficiency

(Machen et al., 1996). These diseases provide valuable information on the functions of Zn. For example, calves with Adema disease are born with normal plasma Zn concentrations that gradually decline. The progression of clinical signs includes skin lesions followed by diarrhea, parakeratosis, growth retardation and ultimately death (Machen et al., 1996).

The importance of Zn in growth and tissue synthesis has been extensively studied as it relates to animal agriculture. It is one of the most widespread minerals throughout the body of cattle, with concentrations tending to be greatest in tissues that are growing or high in protein. In calves, the highest concentration of Zn was found in rib and tibia because of greater rates of tissue synthesis than other tissues examined (Stake et al., 1975). Zinc is also important for synthesis of intramuscular adipose tissue, a characteristic that is monitored closely in beef cattle because of its impact on the grade and price of meat (Smith et al., 2008). For example, studies have shown that exposing bovine preadipocytes to Zn can promote adipogenesis *in vitro* (Smith et al., 2008). In another study, beef cattle fed a growing and finishing diet that met all nutrient and mineral requirements except for Zn requirements were compared to those that were supplemented with Zn (Spears et al., 2002). Control cattle were kept at 33 and 26 mg/kg Zn for growing and finishing diets respectively, whereas supplemented cattle received 58 and 51 mg/kg Zn in their growing and finishing diets. Quality grade was increased because marbling and back fat were increased by Zn supplementation (Spears et al., 2002).

Besides tissue synthesis and growth, Zn is essential to the functioning of a wide variety of enzyme systems, and is the only metal that appears in all six classes of

enzymes. Many metalloenzymes such as copper-zinc superoxide dismutase (Cu/Zn SOD), carbonic anhydrase, alcohol dehydrogenase, carboxypeptidase, and alkaline phosphatase include Zn as an integral component (NRC, 2001). Cu/Zn SOD is in the oxidoreductase enzyme class and the 2 subunits, each consisting of a Zn- and Cu-containing polypeptide, are joined by hydrophobic and electrostatic interactions (Marikovsky et al., 2003). Both carboxypeptidase A and B are Zn-containing enzymes that cleave a single amino acid at a time from the C-terminus of peptides or proteins (Vallee et al., 1960). Likewise, alkaline phosphatase, is involved in the dephosphorylation of many kinds of molecules including nucleotides and proteins, contains Zn. Additional enzymes use Zn as an integral component and still others are activated in the presence of Zn. Zinc functions at the activation site in both carbonic anhydrase and alcohol dehydrogenase (Vallee et al., 1960). Carbonic anhydrase, which catalyzes the conversion of  $\text{CO}_2$  to bicarbonate, uses a Zn atom in a Zn prosthetic group. During catalysis this group serves as an attachment site for one molecule of water which becomes a hydroxide ion and then serves to attack the carbon dioxide, forming bicarbonate (Vallee et al., 1960). Alcohol dehydrogenase facilitates the conversion of alcohols to aldehydes or ketones with the reduction of  $\text{NAD}^+$  to NADH. At this active site, Zn serves to bind the incoming alcohol and hold it as it is converted to an aldehyde or ketone. The examples above are just a few of the many enzymes containing Zn. As a consequence, Zn deficiency decreases function of a wide variety of enzymes and leads to the broad array of Zn deficiency symptoms (Vallee et al., 1960).

### *Zinc: Absorption and Transport*

Because Zn is critical to so many aspects of animal function, Zn homeostasis is tightly regulated, and regulatory mechanisms operate primarily at the levels of absorption and excretion. The small intestine serves as the main site of Zn absorption as it passes through the digestive tract. Zinc absorption occurs to the greatest extent and most efficiently in the beginning of the intestine, specifically in the duodenum. When calves were injected with 600  $\mu\text{Ci}$  radio-labeled  $^{65}\text{Zn}$  into the proximal part of the intestine, blood Zn was found to peak 1 h later while injection into the distal end resulted in a peak 2 or more h later (Hampton et al., 1976). Zinc absorption in the small intestine is regulated by a homeostatic mechanism in response to both physiological need and dietary intake. Absorption rate reflects tissue Zn requirements and is greater for calves than adults and for lactating than non-lactating cows (Miller et al., 1970). For example, radio labeled  $^{65}\text{Zn}$  was absorbed at a greater rate in lactating cows versus non-lactating cows (Stake et al., 1975). The extent of Zn absorption also responds to dietary Zn concentration. Two experiments done by Miller et al. (1991) examined Zn uptake and metabolism in both control and Zn deficient calves administered either IV or oral  $^{65}\text{Zn}$ . Zinc deficient calves that were dosed either orally or by IV had increased concentrations of labeled Zn in intestinal tissue samples for up to 10 weeks following the dose when compared to calves fed requirement levels of Zn (Miller et al., 1991).

Fecal excretion is the most prominent route of Zn excretion. Fecal Zn includes both unabsorbed dietary Zn as well as absorbed Zn that has been secreted in bile or pancreatic fluids. Unabsorbed dietary Zn includes both unavailable dietary Zn (described in greater detail later in this literature review) and available dietary Zn that was not

absorbed in response to the homeostatic mechanisms described above. When Zn absorption is greater than Zn requirements, additional Zn above requirements is typically secreted in the bile or pancreatic fluids attached to various molecules such as glutathione (GSH; Dijkstra et al., 1993). Zinc attached to these molecules can then be excreted in feces along with unabsorbed dietary Zn. The National Research Council (NRC, 2001) estimates that 85% of Zn consumed by dairy cows fed at Zn requirements is excreted in the feces. However, this percentage will vary in response to Zn requirements. One study found that daily fecal Zn excretion averaged 62% of Zn intake for growing calves (5 – 12 months) with relatively high Zn requirements compared to 86% for lactating cows with lower Zn requirements (Miller et al., 1965). In that trial urinary excretion of Zn was insignificant and was less than 0.2% of dietary intake (Miller et al., 1965).

A variety of Zn carrier and transporter proteins are involved in Zn homeostasis, both within the body and within an individual cell. These proteins include albumin, metallothionein (MT), Zip proteins, and Zn transporter (ZnT) proteins. Other high molecular weight proteins between 45-55 kDa are also involved, though their roles are poorly understood and will not be discussed in this review (Wapnir, 1990). Albumin is the primary extracellular Zn transport protein, and it is important for the transport of Zn from intestinal epithelial cells to the liver in portal blood as well as from the liver to the body in systemic blood (Kinkaid et al., 1979). Approximately 70% of plasma Zn is bound to albumin and the other 30% is attached to an  $\alpha_2$ -macroglobulin complex (Rowe et al., 2000). However, the  $\alpha_2$ -macroglobulin complex is not readily available to be exchanged with cells. Therefore, the albumin-bound Zn is believed to represent 99% of the total exchangeable Zn in blood plasma (Rowe et al., 2000).

Zip proteins and ZnT proteins are transmembrane Zn transport proteins that are primarily involved in the transport of Zn into or out of a cell and into or out of cellular compartments. The mammalian family of Zip proteins contains 14 members, Zip 1 to Zip 14, and ZnT proteins consist of 10 members, ZnT1 through ZnT10 (Cousins et al., 2006). Zip proteins increase Zn in the cytoplasm by transporting Zn from extracellular fluid or from intracellular compartments (Cousins et al., 2006). Zinc transporter proteins generally lower intracellular Zn by mediating Zn transport from the cytoplasm to extracellular fluid or from cytoplasm to intracellular vesicles (Cousins et al., 2006). The precise mechanisms of Zn transport are not well understood for these transport proteins, but the different proteins are believed to be important in regulating Zn metabolism based on cell type and function (Cousins et al., 2006). For example, ZnT1 is highly expressed in the duodenum and jejunum and is believed to be important for transfer of Zn from intestinal epithelial cells to the portal blood (Cousins et al., 2006). ZnT2 is found in pancreatic cells and is down-regulated in Zn deficiency, presumably to reduce Zn loss to the feces via pancreatic secretions (Cousins et al., 2006). ZnT3 and ZnT4 are much less researched but ZnT3 seems to be confined to the brain and testis, whereas ZnT4 has been found in the mammary glands as well as the brain (McMahon et al., 1998).

While Zip and ZnT proteins are important for transport of Zn into and out of the cytoplasm, MT appears to be important for storage and transport of Zn within a cell. Thionein is a strong Zn acceptor due a high concentration of sulfate groups on cysteine residues, and thionein becomes MT when bound to Zn (Claus et al., 1998). Metallothionein has both  $\alpha$ - and  $\beta$ -domains and there are a total of 20 cysteines that allow for the high Zn binding capability (Cousins et al., 2006). In the  $\beta$ -domain, MT

binds 3 Zn atoms via interactions with 9 cysteines whereas in the  $\alpha$ -domain MT binds 4 Zn because of interactions with 2 cysteines (Cousins et al., 2006). Zinc bound to MT can be carried to another part of the cell where MT donates Zn to the organelle or protein that needs it (Claus et al., 1998). Metallothionein is present throughout the body, and is found in highest concentrations in parenchymal cells of the intestine, pancreas, kidney and liver. Ultra structural localization used in the liver of male Wistar rats demonstrated that MT is present in both the nuclear and cytoplasmic compartments of the cell (Sorland et al., 1993). The ratio of thionein to MT is believed to be a key regulator of intracellular Zn homeostasis (Cousins et al., 2006).

#### *Copper: Function*

Copper is another trace mineral that is found widely throughout the body and is used in a variety of processes affecting maintenance, growth and lactation. It is just slightly less abundant than Zn in organisms, but is widely distributed throughout the body with greatest concentrations found in the liver, brain, skeletal muscle and bone. A deficiency in Cu can cause a loss of hair pigmentation, poor growth rates and the fragility of bones (NRC, 2001). Anemia is also in part caused by a Cu-deficiency, as there is a shortened survival rate for Cu-deficient red blood cells in these patients. Additionally, a study found that nutritionally anemic calves could be restored to health through Cu supplementation (Knoop et al., 1935).

Copper is required for the function of a variety of enzymes and proteins. These include cytochrome oxidase, Cu/Zn SOD and MT (Linder M.C. 1996). Cytochrome oxidase is the final enzyme in the respiratory electron transport chain of mitochondria

and it completes the reduction of oxygen to water (Buse et al., 1999). Within cytochrome oxidase there are 2 Cu containing centers, Cu<sub>A</sub> and Cu<sub>B</sub>, both of which participate in oxygen reduction (Buse et al., 1999). Copper/Zn SOD is important in the antioxidant system, helping to neutralize the superoxide radical (Fridovich, 1978). Copper/Zn SOD consists of 2 subunits each containing a Zn and Cu, with the enzymatic activity dependent on the presence of Cu (Marikovsky et al., 2003). Metallothioneine is regulated by the concentration of Cu present in the plasma and can either increase or decrease Cu absorption to maintain Cu homeostasis (Suzuki et al., 2002).

#### *Copper: Absorption and Transport*

As with Zn, Cu homeostasis is tightly maintained, in particular through regulation of liver Cu. Copper can be absorbed from both the rumen and the upper portion of the small intestine, especially the duodenum (Linder, 1996). However, only a small fraction of Cu is sufficiently solubilized in the rumen and absorption there is nutritionally insignificant (Wapnir, 1998). Copper that is absorbed in the small intestine is transferred across the basolateral membrane of the intestinal epithelial cells and into the portal blood (Linder, 1996). The liver is the central organ of Cu homeostasis with a tremendous capacity for both storage and secretion of this mineral. Hepatocytes can sense cytoplasmic Cu concentration and regulate whether the Cu is released into the plasma for transport to other tissues, stored in the liver parenchymal cells for future use, or secreted into the bile (Luza et al., 1996). If an animal consumes inadequate dietary Cu, the liver can release stored Cu into the plasma; however if the liver releases large amounts of Cu, quick onset of Cu toxicity can occur (Luza et al., 1996).

As with Zn, fecal excretion is the primary mechanism for the excretion of Cu. Liver Cu homeostasis is primarily regulated by biliary Cu excretion, and the amount of Cu appearing in the bile is directly proportional to the size of the hepatic Cu pool (Arrese et al., 1998). Once secreted into the bile, Cu re-absorption in the small intestine is limited because Cu in bile is primarily in non-absorbable chelated forms (Luza et al., 1996). It has been shown that at least half of the dietary Cu that is absorbed by the small intestine reappears in the bile as stable chelates and is excreted in the feces (Wapnir, 1998). Similarly to Zn, the urinary excretion of Cu is low and accounts for less than 3% of Cu intake (Luza et al., 1996).

The primary proteins involved in Cu transport and storage are albumin, transcuperin, ceruloplasmin, GSH, and Cu chaperone proteins. Albumin, transcuperin, and ceruloplasmin are important for blood and extracellular Cu transport. Albumin binds Cu at a Cu-specific binding site in its amino-terminal (Linder, 1996). Transcuperin is a large plasma protein that has a higher affinity for ionic Cu than either albumin or histidine (Linder, 1996). Absorption and transfer of Cu from the intestinal epithelial cell into the bloodstream occur in two distinct phases that incorporate different carrier proteins. During the first stage when the Cu is transported from the portal blood to the liver, albumin and transcuperin are the main Cu carriers. These transport proteins appear to have somewhat overlapping roles. For example, transcuperin is able to exchange Cu directly with albumin and vice versa as seen when <sup>67</sup>Cu labeled albumin was mixed with unlabeled transcuperin *in vitro*, and the radiolabel was found on both proteins (Linder, 1996). The second stage of Cu transport involves the transfer of Cu from the liver to blood and surrounding tissues. Ceruloplasmin is the main carrier protein during this stage.

Ceruloplasmin is synthesized within the hepatocytes and is secreted from the liver as the holoprotein with six to seven atoms of Cu incorporated (Linder, 1996). In the general circulation, ceruloplasmin contains approximately 75% of plasma Cu while albumin contains roughly 5% (Luza et al. 1996).

Ceruloplasmin, albumin and histidine are all capable of exchanging bound copper with the surface of cells (Harris, 2000). Glutathione and Cu chaperone proteins are important for Cu transfer within cells. For example, GSH transfers Cu to the Cu binding sites in Cu/Zn SOD and ceruloplasmin (Harris, 2000). The family of Cu chaperones includes at least three proteins that transport Cu within the cell to Cu binding sites. Copper chaperones appear to have more specific targets than GSH and they are capable of transferring Cu throughout the cell, including across intracellular membranes (Harris, 2000).

### **Zinc and the Immune System**

The first line of defense of the immune system is the ability to deny the pathogen access to the body. This is done through physical barriers such as skin and mucous membranes within the respiratory and gastrointestinal tracts. If physical barriers are penetrated, innate immunity is the second line of defense against pathogens, and consists of rapidly responding chemical and cellular defenses. For the innate immune system, each invading pathogen is treated identically, and the intensity and duration of the response remains similar for different pathogens. The acquired immune system is the third defense system and has the ability to recognize and remember invaders (Tizard,

2008). This in turn allows for an antigen-specific response and is the reason vaccines are used in many animals, including cows. A decrease in any of these aspects of immune defense in dairy cows can lead to increased disease incidence and result in a loss of milk, cows and income to the farmer.

Many nutrients are essential for maintaining a healthy immune system to prevent disease and aid in disease recovery. Zinc is one such nutrient because of its general role in many physiological functions as well as its specific role in the antioxidant system. For example, Zn deficiency has been shown to reduce WBC function including innate immune cells like neutrophils and natural killer (NK) cells, as well as acquired immune cells such as lymphocytes (Fernandes et al., 1979). The remainder of this section describes the role of Zn in the three lines of defense of the immune system.

### *Physical Barriers*

The first barrier of the immune system is the integrity of the skin. Though not an actual immune system tissue, the skin is an extremely effective barrier to microbial infection (Tizard, 2008). Zinc and Cu work together to build and crosslink collagen and keratin fibers to increase strength of the skin. Zinc is required for the synthesis of two key structural proteins, collagen and elastin, while Cu-dependent lysyl oxidase, a cross-linking enzyme, enhances the strength of the skin. This is especially important in the teat ends of lactating cattle because the skin and keratin prevent bacteria from traveling up into the mammary gland (Sordillo, 1997). Another primary defense barrier in cows is the mucosal lining of their GI tract which serves as the first line of defense against any ingested pathogens. It has been found that the gut mucosa has one of the highest protein turnover rates of all the tissues in the body, making Zn very important in sustaining the

integrity of this barrier (Scott et al., 2000). For example, parasites were better able to survive in Zn-deficient human hosts as compared to well-nourished hosts in a study done by Scott et al. (2000).

Keratin proteins comprise a major portion of the protective matrix of the skin, hair, horn and hoof of mammals. The process of keratinization transforms functional epidermal cells into cornified, dead and structurally stable cells (Tomlinson et al., 2004). Zinc plays a key role in the process of keratinization as part of the catalytic, formation and regulatory processes. Specifically, keratinization requires the use of Zn-fingers for establishment of protein–protein interactions, specifically the formation of stable disulfide bonds between cysteine residues, which enable the bending of keratin. When there is a decrease in the Zn-fingers present in the cell there is a decrease in the development of keratin filaments needed for the keratinocyte to form (Tomlinson et al., 2004). Further evidence comes from the observation that there are lower Zn concentrations in the claws of lame cows than those of healthy cows with no history of lameness (Tomlinson et al., 2004).

### *Innate Immunity*

White blood cells or leukocytes of the innate immune system include phagocytes, dendritic cells, mast cells, eosinophils, basophils and NK cells (Tizard, 2008). Phagocytes include macrophages and neutrophils, both of which have the ability to phagocytize or engulf the invading microbe and destroy it. Dendritic cells also engulf foreign molecules but only to process and bring their antigen markers to the surface to be recognized by other immune cells. Mast cells, eosinophils and basophils respond to chemical signals of infection and migrate to an inflammatory site. Basophils stimulate vasodilation and

increase the blood flow to the infected area whereas the former degranulate to release cytokines and ROS that help combat infection. Lastly, NK cells are large cytotoxic cells that use multiple receptors to distinguish between normal and abnormal cells causing damage and ultimately killing abnormal cells. These innate lymphocytes have additional functions as well that include the release of cytokines to attract more immune cells to an infection, the release of interleukin (IL) factors which activate other immune cells and the direct activation of other WBC. For instance, activated NK cells secrete INF- $\gamma$  which promote T-cell production (Tizard, 2008).

Zinc has been shown to affect several aspects of innate immune function. For example, a Zn deficiency decreased phagocytosis by macrophages and cytotoxicity in neutrophils and NK cells (Ibs and Rink, 2003). Keen and Gershwin (1990) also showed that the generation of the oxidative burst by neutrophils, which is the release of ROS to destroy foreign cells, is impaired by decreased plasma Zn concentrations *in vivo*. On the other hand, high Zn levels have been shown to increase the activation of macrophages and neutrophils (Rink et al., 2000). Natural killer cells have an inhibitory receptor on their surface that requires Zn for activation. Activation signals on NK cells are not dependent on Zn, so although a decrease in Zn will suppress cytotoxicity, high levels of Zn can suppress their direct killing activity as well (Rajagopalan et al., 1995).

### *Adaptive Immunity*

Adaptive or acquired immunity refers to the antigen-specific defense mechanisms that take several days to respond to a challenge and are designed to react with and remove a specific antigen. Unlike the innate immune system, the acquired immune system is

antigen dependent and specific, meaning it requires a certain antigen to become activated and once activated it seeks and destroys only that particular antigen. Additionally, the adaptive immune system has the ability for immunological memory which allows the system to be activated immediately upon the return of the same or similar antigen.

B-cells and T-cells are the two types of lymphocytes that are the main components of the adaptive system. B-cells or B-lymphocytes are part of humoral immunity which is antibody mediated. In mammals, B-cells are produced in the bone marrow and then migrate to the spleen where they are “educated” to recognize a certain antigen and are then released as mature B-cells (Tizard, 2008). T-cells or T-lymphocytes play a central role in cell-mediated immunity. T-cells differentiate into several different subtypes upon antigen stimulation, and helper and cytotoxic T-cells are the most prominent. Helper T-cells can either stimulate macrophages to kill the bacteria they have engulfed and recruit other leukocytes to the site of infection, or secrete IL factors to stimulate antibody production by B-cells. Cytotoxic T-cells have the ability to destroy infected cells as well as tumor cells (Tizard, 2008).

A deficiency in Zn can adversely affect both the B and T-cells of the adaptive immune system. In both cytotoxic and helper T-cells, a deficiency in Zn results in a decrease in normal function and an increase in autoreactivity (Ibs and Rink, 2003). Autoreactivity is the process of mounting an inappropriate immune response against one’s own body. Specifically in the helper T-cells, TH1 and TH2, Zn depletion decreases TH1 cytokine production but does not affect the TH2 cells, leading to impaired T-cell function, including a decreased proliferation of T lymphocytes (Mehrzaad and Zhao,

2008). Furthermore, calves with Zn deficiency due to Adema disease were shown to have decreased lymphocyte replication in response to the mitogens phytohemagglutinin, concanavalin A and pokeweed mitogen (Perryman et al., 1989). One study found that a Zn deficiency in lactating cows led to the atrophy of the thymus, irregular serum concentrations of antibodies and WBC and an increased somatic cell count (SCC; Sordillo, 1997). Zinc deficient mice have shown a reduced antibody response following immunization (Ibs and Rink, 2003). Additionally, Zn deficiency in humans has also resulted in a decreased antibody production and the inability of the body to respond to neoantigens (Scott et al., 2000).

#### *Disease Susceptibility*

There is some evidence that Zn may be important in recovery from mastitis and periparturient diseases. Mastitis is inflammation of udder tissue most often caused by bacterial infection and is the most common and costly disease of dairy cattle (Bar et al., 2008). Mastitis costs about \$179 per cow per year with much of this loss being associated with decreased milk production (Bar et al., 2008). Some studies have demonstrated a depression in plasma Zn concentration following mastitis and have suggested that Zn may be important in mediating mastitis recovery. For example, a rapid decrease of plasma Zn concentration followed acute mastitis induced by both *Staphylococcus aureus* (Middleton et al., 2004) and *Escherichia coli* (Erskine and Bartlett, 1993). In the experiment done by Erskine and Bartlett (1993), infusion of 50 colony forming units of *E. coli* into one quarter of 6 lactating Holstein cows reduced plasma Zn by 28%. Additional studies have shown that the numbers of *E. coli* in milk and severity of

systemic clinical signs following an *E. coli* challenge were positively correlated with the magnitude of decrease in plasma Zn (Erskine and Bartlett, 1993).

The cow is most susceptible to infections including mastitis and metritis during the month after parturition (LeBlanc et al., 2006). This corresponds to an acute decrease in plasma Zn concentrations at parturition (Kehoe et al., 2007). This Zn decline is largely due to the production and secretion of colostrum at calving which contains approximately 38 mg/kg Zn (Kehoe et al., 2007). Milk fever, or parturient paresis, is a disease that occurs around calving and is caused by low blood calcium levels. Milk fever is typically caused by the considerable drain on a cow's blood calcium needed to supply the developing calf and as a constituent of colostrum and milk (Goff and Stabel, 1990). Cows with milk fever tend to have a greater decline in plasma Zn than those without milk fever (Goff and Stabel, 1990). The decline in plasma Zn in mastitis, parturition and in association with periparturient diseases has led to some speculation that Zn supplementation during these times may reduce disease susceptibility (Goff and Stabel, 1990).

### **Trace Mineral Supplements**

As detailed above, processes such as overall immune function, antioxidant functions and hoof integrity in the dairy cow are dependent on minerals. Most diets today are supplemented with trace minerals to maintain cow health and production. Inorganic trace minerals (ITM) have traditionally been used for the bulk of mineral supplementation, most often in sulfate or oxide forms. Two traditional forms of ITM Zn

supplementation in the animal feed industry are Zn oxide (ZnO) and Zn sulfate (ZnSO<sub>4</sub>; McDowell, 1992), whereas cupric sulfate (CuSO<sub>4</sub>) is the traditional form of supplemental Cu (Spears, 2003).

The absorption of ITM is relatively low because of their susceptibility to a variety of antagonisms throughout the digestive tract. The common denominator in all of these antagonisms is that the mineral cation is dissociated from the anion and can form insoluble and indigestible compounds. In ITM salts, the mineral and anion are joined by an ionic bond which can dissociate in the aqueous conditions of the gut. The initial dissociation of the cation and anion in the rumen leaves the separated mineral ions unprotected. These ions can then form tight bonds with other minerals or digestive components in the rumen. This re-binding does not necessarily occur between the two ions that were initially dissociated. When the mineral binds other minerals or non-nutritive digesta such as fiber, it is rendered indigestible and is lost via excretion. Out of the total amount of Zn or Cu fed, it is estimated that only 15% and 4% respectively, are actually absorbed (NRC, 2001).

Antagonisms that reduce mineral availability can occur between one mineral and another or a mineral and components of the digesta. Mineral antagonists of Cu include sulfur, molybdenum and iron, while antagonists of Zn consist of calcium, phosphorus and iron (NRC, 2001). Such antagonisms result in the formation of insoluble compounds that cannot be absorbed in the digestive tract (Spears, 2003). A three-way interaction between Cu, Mo and S has been found, resulting in insoluble thiomolybdates (Spears, 2003). Antagonisms can also occur between minerals and insoluble dietary components such as fiber. When the mineral reaches the small intestine it can bind to fiber particles and form

an insoluble compound that is excreted from the animal (McDonald et al., 1996). Mineral to mineral antagonisms can also occur among bioavailable minerals at the transporter level of the intestine. Both Zn and Cu are antagonistic to each other when they are not fed in equilibrium due to competition for transporters. Generally, Zn will interfere with Cu absorption by binding and sequestering MT which also binds Cu (Spears, 2003). In cases of high dietary Cu and marginal Zn, Cu can do the same as well (NRC, 2001).

### *Organic Trace Mineral Supplements*

Organic trace minerals (OTM) are a more recent development in animal nutrition and provide an alternative to supplementing trace minerals as inorganic salts. The proposed benefit of OTM comes from a difference in chemistry which gives the molecule more protection against the formation of insoluble compounds in the rumen. Organic trace minerals are classified according to their chemistry as complexes, chelates, or proteinates (Table 1).

**Table 1:** Classification of organic trace minerals according to their chemistry

<b>Compound Name</b>	<b>Compound Chemistry</b>	<b>Feed Industry Example</b>
<b>Metal Amino Acid Complex</b>	Product resulting from complexing a soluble metal salt with an amino acid(s); consists of one bond Metallic ion + 1 amino acid	Availa Zn
<b>Metal Amino Acid Chelate</b>	Product resulting from the reaction of a metal ion from a soluble metal salt with amino acids to form coordinate covalent bonds Metallic ion + 2 amino acids	Mintrex Zn
<b>Metal Proteinate</b>	Product resulting from the chelation of a soluble salt with amino acids and/or partially hydrolyzed protein Metallic ion + 3+ amino acids	BioPlex Cu

Information from Acda and Chae, 2002.

In all of these forms, the supplemental trace mineral is bound to amino acid(s) or proteins. In complexes, chelates, or proteinates the metal is complexed with an amino acid, with 2 or more amino acids which form a heterocyclic ring with the metal, or bound to a partially hydrolyzed protein, respectively (Spears, 1996). Within the complex, chelate, or proteinate the mineral is relatively inert in aqueous conditions due to the coordinate covalent or ionic bonding by the amino acid ligands (Fairweather-Tait, 1996). The coordinate bonds in OTM keep the mineral stable and less prone to interactions than ITM (Richards, 2006). Thus, OTM provide a greater stability at the neutral to slightly acidic pH of the rumen and are more resistant to antagonisms within the gut (Spears, 1996). The amount of mineral that is available for absorption in the small intestine is then greater for OTM than ITM. The potential benefits of OTM to the dairy industry include

increased milk production, decreased amount of trace mineral supplementation and decreased disease incidence in dairy cows.

Although OTM are less susceptible to gut antagonisms than ITM, their chemistry can inhibit small intestinal absorption. Depending on OTM chemistry, absorption can occur via diffusion or transport-mediated absorption. Diffusion can be hindered if the OTM is too large for passage across the cell membrane, and transport-mediated absorption can be hindered if transporters are too specific, accepting minerals only in their ionic forms and not when bound to an organic carrier (Harmon, 2000). Because of this, intact absorption, or the process of absorbing the mineral and its organic carrier, is not believed to be a significant route of OTM uptake. For example, *in vitro* absorption experiments with a Zn-methionine complex (ZnMet) showed that Zn and methionine were absorbed separately (Richards, 2006). However, different OTM are not equally stable at lower pH and may differ in the increase in bioavailability for a given mineral. In the case of 2-hydroxy-4-methyl-thiobutyrate (HMTBa) Zn, *in vitro* experiments have shown that the mineral dissociated in an acidic aqueous solution (pH = 2), which is analogous to the abomasum. It then can be delivered to the absorptive epithelium of the small intestine in its soluble form. Thionein captures the Zn, which then becomes MT and the HMTBa ligand can be absorbed by a separate diffusion or carrier system (Richards, 2006). Although more research is needed to truly understand the absorption mechanisms, OTM are believed to have a greater protection as they are carried to the intestine and are more available for absorption than ITM.

The fraction of the administered trace mineral that is able to reach systemic circulation or tissues is defined as the bioavailability (Fairweather-Tait, 1996). Because

OTM have a greater ability for absorption in the small intestine than ITM, they are said to have greater bioavailability. Bioavailability of OTM has been quantified through a variety of measures including expression of mineral responsive biomarkers, such as MT for Zn, and mineral levels in the animal's plasma or tissue (Wright and Spears, 2004). Both plasma and tissue measurements have limitations in their ability to reflect mineral bioavailability. This is because the body regulates mineral status and increased mineral absorption may or may not be reflected by an increase in plasma or tissue concentration (Wright and Spears, 2004). For example, certain tissues such as the liver have the capacity to store and release minerals based upon feed mineral bioavailability and animal needs. As a consequence, plasma and tissue mineral concentrations do not always change to reflect changes in dietary mineral availability.

Mineral responsive biomarkers are generally believed to be a more sensitive indicator of mineral status than plasma or tissue concentrations. Unlike tissue or plasma concentrations, they do not represent mineral stores and are therefore more likely to reflect changes in mineral availability when feeding different supplements (Hambidge, 2003). These biomarkers include MT mRNA concentration for Zn, ceruloplasmin mRNA concentration for Cu and Cu/Zn SOD activity for either Cu or Zn. Recently plasma diamine oxidase activity has also been suggested as a possible biomarker for Cu (Legleiter and Spears, 2007).

### *Supplementation Effects*

Studies have compared the effects of ITM versus OTM on blood mineral concentrations with varied results. Steer calves were fed a soybean meal based protein

supplement consisting of either the organic complexes ZnMet and Cu-lysine (CuLys; ZinPro, Bloomington, MN), or the inorganic sulfate forms (Nockles et al., 1993). Calves were fed the supplemented diets for 30 d prior to the start of the experiment through d 5, followed by four periods consisting of a mineral depletion (d 6-8) when the supplemental minerals were removed, an adrenocorticotrophic hormone (ACTH) challenge (d 9-11), a continued mineral depletion (d 12-14) and finally a repletion period (d 15-18). Calves fed the OTM had 53% greater plasma Cu concentrations and decreased Cu excretion during the repletion period when compared to calves fed ITM, though no differences were found in plasma or excreted Zn (Nockles et al., 1993). Another study supplemented lactating cows with Zn, Cu and Se OTM chelates (Bioplex Zn, Bioplex Cu and Sel-Plex; Alltech, Inc., Lexington, KY) compared to ITM (O'Donoghue and Boland, 2002). Copper, Zn and Se were provided at a level of 100 mg, 300 mg and 2 mg/cow/day, respectively, for both treatments. Unlike the calf study, there were no treatment effects on blood mineral concentrations. However there was a significant reduction in the somatic cell count for cows fed OTM compared to ITM (O'Donoghue and Boland, 2002).

Despite the variable effects of OTM on blood mineral concentrations, tissue mineral concentrations and mineral responsive biomarkers are generally increased by OTM compared to ITM. In one study, 40 crossbred wether lambs were fed one of four sources of supplemental Zn: ZnLys, ZnMet, ZnSO<sub>4</sub>, or ZnO and compared to a negative control that was not supplemented with any Zn (Rojas et al., 1995). Concentrations of Zn were determined in serum, liver, pancreas, kidney, bone, bone marrow, hoof, skin, cornea and leg muscle. Metallothionein concentration was determined in liver, pancreas and

kidney. All forms of supplemented Zn increased the serum Zn concentration in comparison to the control, but there was no improvement by OTM compared to ITM. However, OTM did enhance tissue Zn as indicated by increased Zn and MT concentrations in the liver, pancreas and kidney in comparison to the ITM treatments (Rojas et al., 1995). Another study was done to compare the bioavailability of supplemental dietary Zn as ITM or OTM for young calves. Holstein heifer calves were fed one of four diets: a control that consisted of 65 ppm of Zn, two OTM supplemented diets of 150 or 300 ppm Zn from a combination of ZnMet and ZnLys, or an ITM supplemented diet containing 300 ppm Zn from ZnO (Kincaid et al., 1997). Concentrations of Zn in the serum and liver were increased for the 300 ppm Zn from OTM compared to the 300 ppm Zn from the ITM (Kincaid et al., 1997). In a different study, ewes were fed one of two different sources of Cu, either CuSO<sub>4</sub> or Cu-proteinates administered at 10, 20 or 30 mg/kg of feed (Eckert et al., 1999). Organic trace minerals increased kidney Cu concentration compared to ITM, but only at the 20 mg/kg Cu supplementation level. However, ewes fed the OTM had numeric increases in plasma ceruloplasmin concentration at all supplementation levels compared to ITM (Eckert et al., 1999). In summary, tissue mineral concentrations and mineral responsive biomarkers appear to better reflect increases in mineral availability from OTM versus ITM than blood plasma levels.

The majority of health problems in dairy cattle occur at or around the time of calving which is known as the periparturient or transition period. A possible benefit of OTM supplementation includes increased mineral availability throughout the transition period which may help prevent disease. Research has shown that immune functions,

especially those of the neutrophils, are reduced during this time and can contribute to post-calving diseases such as mastitis, retained placenta and metritis (Rajala and Grohn, 1998; LeBlanc et al., 2006). Some researchers have asserted that the increased bioavailability of OTM compared to ITM fed during this period may ease the transition into lactation and improve animal performance and health (Spears, 1996). In one study, 250 multi- and primi-parous cows were supplemented with 360 mg of Zn, 125 mg of Cu, 200 mg of Mn and 12 mg of Co as either ITM sulfates or OTM amino-acid complexed forms from approximately 21 d pre-partum to 245 days in milk (DIM; Scilliano-Jones et al., 2008). Cows fed OTM had greater milk, protein and solids yields as well as a decreased incidence of sole ulcers and interdigital dermatitis compared to ITM supplemented cows (Scilliano-Jones et al., 2008). In another study, 31 pregnant Holstein heifers were fed a diet from 120 d prepartum to 60 DIM that was either not supplemented with Cu or supplemented with 10 ppm Cu from OTM (Cu-protein) or ITM (Cu-sulfate; Harmon et al., 1998). Those fed OTM had higher mean liver and plasma Cu concentrations compared to those fed the ITM, and this difference was greatest at calving. OTM also resulted in a greater number of mammary quarters being confirmed uninfected by coagulase negative *staph.* organisms (Harmon et al., 1998).

Challenges with viruses such as infectious bovine rhinotracheitis virus (IBRV) are another way to test effects of OTM versus ITM supplementation on animal immune response. Chirase et al. (1991) performed a study that consisted of three separate experiments to examine the effects of supplementing ZnMet to feedlot steers challenged with IBRV. Experiments 1 and 2 used 30 crossbred steers fed either a control diet with 31 ppm Zn or a supplemented diet with 90 ppm ZnMet. Steers were fed the diets for 7 days

and then challenged with IBRV on d 8. Measures were then taken 0, 7 and 14 d following the challenge. Supplementation with OTM improved IBRV response as measured by a lower reduction in dry matter intake (DMI) following the challenge compared to un-supplemented steers. The third experiment compared a control diet (96 ppm Zn) to a supplemented diet with either 163 ppm of ZnO or 171 ppm of ZnMet. The OTM supplemented steers had improved response to IBRV as measured by a slower drop in DMI following the challenge, a faster return to pre-challenge DMI and lower temperatures compared with control and ZnO supplemented steers (Chirase et al., 1991). These data suggest that OTM have greater bioavailability than ITM as measured by an increased recovery rate following an IBRV challenge.

### **Summary**

Dietary trace minerals must be supplied to dairy cows to support physiological processes such as lactational performance, claw tissue formation and bone integrity (Siciliano-Jones et al., 2008). Organic forms of trace minerals are believed to undergo fewer negative interactions in the digestive tract of ruminants than inorganic trace minerals and thus provide greater bioavailability. Use of organic in place of inorganic trace mineral supplements may provide the animal with a healthier immune system, greater milk production, and/or allow the producer to reduce dietary supplementation levels. Copper and zinc play a vital role in dairy cow nutrition and health. We conducted the following studies to evaluate the bioavailability of different organic and inorganic Cu and Zn supplements administered ruminally and abomasally to steers.

## Chapter 3

### MATERIALS and METHODS

#### Experiment 1

##### *Objective*

We hypothesize that Cu or Zn administered into the abomasum will increase the respective plasma mineral concentrations. The objective of Experiment 1 was to administer organic forms of the trace minerals zinc or copper via abomasal infusion and to determine their bioavailability by measuring their blood mineral concentrations.

##### *Animals and treatments*

Four ruminally cannulated Holstein steers with an average age of 1½ years and an average weight of 640 kg were used in Experiment 1. Steers were adjusted to the ration (Table 2) for 2 weeks prior to the start of the first period. The experiment was conducted as a 4 × 4 Latin square, with each steer receiving each treatment over 4 periods. Each period lasted a total of 7 days, with 6 days allotted for rest and recovery of the animals and the seventh day used for abomasal infusion treatment and blood sampling. Four abomasal infusion treatments were administered as a single pulse dose, consisting of either 4 g of Cu, 4 g of Zn, 8 g of Zn, or a control containing no Cu or Zn. Copper and zinc were supplied as 2-hydroxy-4-methyl-thiobutyrate (HMTBa) organic

complexes containing 15% Cu and 16% Zn, respectively (Mintrex Cu and Mintrex Zn, Novus International, Inc., St. Louis, MO). The amount of organic mineral administered was 27, 25 and 50 g to supply 4 g Cu, 4 g Zn and 8 g Zn respectively.

Steers were housed in individual  $2.4 \times 6.4$  m pens with ad libitum access to feed and water. Steers were fed a diet containing only alfalfa silage and corn silage (Table 2) at approximately 0800 h each day. Left over feed was removed and weighed just prior to feeding to allow for calculation of intake. Abomasal infusions were completed through a tubing line that was placed in each steer through the ruminal cannula during the adjustment period and remained in place during the entire experiment (Gressley et al, 2006). Treatment was administered by mixing the complexed mineral with 700 ml of tap water and flushing this mixture through the tubing with a 140 mL catheter tip syringe (California Veterinary Supply, Pahrump, NV). The volume of water was the minimum necessary to insure that the entire dose was administered.

Jugular catheters (BD Angiocath 14 ga  $2.1 \times 133$  mm, Franklin Lakes, NJ) were inserted on day 6 of each period and held in place with sutures, Elastikon (Johnson & Johnson, New Brunswick, NJ) and Vet Wrap (3M Animal Care Products, St. Paul, MN). Twenty ml of heparinized saline (10 units/ml, 0.9% sodium chloride) was flushed through the catheters every 12 hours to prevent clotting overnight until the sampling period began the following day. During the majority of the experiment the steers were allowed to move about freely in their pens. However, during day 6 and 7 of each period, steers were tied by halter to the front end of their stalls. This restrained them for jugular bleeding, but still allowed them to access their feed and water and the ability to lie down.

All procedures were approved by the University of Delaware Agricultural Animal Care and Use Committee.

### *Sampling*

Representative ration samples were taken daily and corn silage and alfalfa silage were sampled weekly and stored at -20°C until dry matter and nutrient analysis. At 0700 h on day 7 of each period, blood was taken to serve as a baseline (0 hr) measure. Immediately following this baseline blood sampling, the treatment was abomasally infused and blood samples were taken at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 and 12 h post infusion. For each sampling time, 5 ml of blood was discarded to flush out residual heparin in the catheter line, a 20 ml blood sample was collected, and 10 ml of heparinized saline was flushed through the line. Blood samples were placed in 10 ml BD Vacutainer K<sub>2</sub> EDTA tubes (BD Vacutainer Systems, Franklin Lakes, NJ), shaken, and placed on ice until centrifugation. At the 0 h and 10 h time points, an extra 10 mL of blood was taken from the steers receiving either the 8 g Zn or the control treatments to measure white blood cell metallothionein (MT) mRNA levels. Jugular catheters were removed immediately after the 24 h blood sample, at approximately 0700 h the next day.

### *Feed and Blood Analysis*

Dry matter (DM) analysis was conducted weekly on corn silage and alfalfa silage to adjust the ration for dry matter variation. Ration samples from each day were composited into weekly samples and analyzed for DM and nutrient composition. Dry matter was determined using approximately 100 g samples in a forced air oven at 60°C for 48 h. Nutrient composition was analyzed by wet chemistry (Cumberland Valley Analytical Services, Hagerstown, MD).

Vacutainer tubes were centrifuged at 600 x g for 20 min at 4°C. Four ml of plasma was then transferred via transfer pipettes into each of two plastic vials and frozen at -20°C. Blood collected for the MT analysis was processed immediately using the LeukoLOCK™ Total RNA Isolation System (Ambion Inc LeukoLOCK™ Fractionation and Stabilization Kit, cat # AM1933 Austin, TX). After isolation, RNA was stored in RNA later (Ambion Inc. Austin, TX) at -20°C. At the end of the trial, plasma and RNA samples were mailed to Novus International, Inc. on ice packs for analysis of mineral concentration and MT mRNA level, respectively. Plasma Zn and Cu concentrations were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES; Perkin Elmer, Optima 2100 DV). Plasma was diluted 1:50 in 0.1% HNO<sub>3</sub> prior to analysis. The mRNA levels of MT were measured by qPCR.

#### *Statistical Analysis*

Data were analyzed using Proc Mixed in the Statistical Analysis System (SAS) program. For intake, the model included the fixed effects of period and treatment and the random effect of steer. For plasma minerals, the model also included hour after infusion as a repeated measure. Comparisons were made between each treatment and between treatments at each time point. Significance was declared at  $P \leq 0.05$  and a trend at  $P \leq 0.10$ .

## **Experiment 2**

### *Objective*

We hypothesize that Zn administered into the abomasum as an OTM will be equally as available as Zn administered as an ITM. The objective of Experiment 2 was to

administer two different organic forms and one inorganic form of zinc via abomasal infusion and to determine the bioavailability by measuring its concentration in the blood.

#### *Animals and treatment*

Animals, feeding, housing and period lengths were the same as in Experiment 1. Experimental design was also a  $3 \times 3$  Latin square. Three abomasal infusion treatments were either 4 g of organic Zn, 4 g of inorganic Zn, or a control containing no Zn. Organic zinc was supplied as a HMTBa complex (Mintrex Zn, Novus International, Inc., St. Louis, MO) and inorganic Zn as zinc sulfate. Placement of abomasal infusion lines and jugular catheters were the same as in Experiment 1. Treatment was administered by mixing the mineral with 1200 ml of tap water and dosed abomasally.

#### *Sampling*

Feed sampling was as in Experiment 1. To determine Zn bioavailability, 20 ml of blood was taken to serve as a baseline (0 h) measure at 0700 h. Immediately following this baseline blood sampling, the treatment was abomasally infused and serial blood samples were taken at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, and 24 h post infusion. Additionally, 40 ml of blood was taken at the 0 and 9 h time points and divided into four 10 ml BD Vacutainer ACD Solution A tubes (BD Vacutainer Systems, Franklin Lakes, NJ) for measurement of neutrophil phagocytosis and *L-selectin* mRNA level.

#### *Feed and Blood Analysis*

Feed and plasma analyses were conducted as in Experiment 1. For neutrophil isolation at 0 and 9 h, vacutainer tubes were spun at 1000 x g for 20 min at 4°C. Plasma, lymphocyte layers and 2/3 of the red cell pack were removed. Red cell packs from 2 vacutainer tubes for each animal were combined into one 50 ml conical tube containing

34 ml of 4°C phosphate buffered saline (PBS). The process was repeated for the additional 2 tubes. Ten ml of 1.064 g/ml Percoll (No. P4937-500ml, Sigma-Aldrich Co., St. Louis, MO) was layered underneath the PBS/red cell pack mixture. Tubes were then centrifuged at 400 x g for 40 min at 22°C, after which the supernatant, mononuclear cell layer and Percoll were aseptically aspirated. The erythrocytes were lysed by adding 12 ml 4°C hypotonic lysing solution (10.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM NaH<sub>2</sub>PO<sub>4</sub>) for 90 seconds, during which time the tube was inverted to break up the cell pellet. Isotonicity was then restored by adding 6 ml of 4°C hypertonic restoring solution (10.6 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM NaH<sub>2</sub>PO<sub>4</sub>, 430 mM NaCl) and inverting. Tubes were centrifuged at 800 x g for 5 min at 4°C and the supernatant was poured off. Ten ml of RPMI (No. 017K2310 Sigma-Aldrich, St. Louis, MO) was then added, and the tubes were vortexed and spun at 800 x g for 5 min at 4°C and supernatant was decanted. To clean the pellet, this process was repeated once more, and 2 ml of 4°C PBS was then added. A sterilized pasture pipette was used to break up the neutrophil pellet.

The sample from one 50 ml conical tube was then split between 2 -1.5 ml microcentrifuge tubes. These tubes were then spun at 9400 x g for 10 min at 4°C. The supernatant was removed using a sterilized pasture pipette and 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) was added. Samples were stored at -80°C until RNA was harvested.

#### *Phagocytosis Assay*

The second 50 ml conical tube was used to examine neutrophil engulfment of fluorescent beads (Polysciences Inc. Flouresbrite Carboxylate Microspheres [2.63% Solids-Latex] No. 565702, Warrington, PA). Neutrophils were adjusted to a

concentration of  $1 \times 10^6$  cells/ml in RPMI. Beads were incubated in fetal bovine serum (No.F2442-100 ml, Sigma-Aldrich Co., St. Louis, MO) at room temperature for 45 minutes. To assess neutrophil phagocytosis,  $1 \times 10^6$  neutrophils and  $1 \times 10^7$  beads were added into triplicate polypropylene tubes. Tubes were covered in parafilm and aluminum foil and then placed in a shaker incubator at 37°C for 2 h. Following incubation, tubes were centrifuged for 5 min at 1000 x g at 4°C and decanted. One ml of RPMI was added to each tube and tubes were vortexed. Tubes were again centrifuged for 5 min at 1000 x g at 4°C and decanted. Addition of 1 ml RPMI, centrifugation, and decanting the liquid was repeated once. Two-thousand  $\mu$ l of 4% paraformaldehyde/PBS solution was then added to each tube and the tube was vortexed to mix. Samples were then transferred to sterile filter topped tubes (Ref. 352235 BD Falcon, Franklin Lakes, NJ). Samples were covered with parafilm and aluminum foil and held at 4°C for no more than 2 weeks before flow cytometric analysis.

#### *mRNA Isolation and Quantification*

RNA was isolated from the neutrophils using the PureLink™ Micro to Midi Total RNA Purification System (Invitrogen, Carlsbad, CA). Three-hundred ng of RNA isolated from each sample was DNase treated (New Englan Biolabs, Ipswich, MA) and used to create 48  $\mu$ l cDNA using the Promega Reverse Transcriptase System (A3500, Promega Corporation, Madison, WI). mRNA levels of  $\beta$ -actin and *L-selectin* were quantified using real time RT-PCR. Primers for  $\beta$ -actin were chosen using Primer 3 (<http://frodo.wi.mit.edu/>) and primers for *L-selectin* were as published (Burton et al., 2005). Ten  $\mu$ l of reaction mix containing 5  $\mu$ l of SYBR green master mix (Applied Biosystems, Foster City, CA), 4  $\mu$ l of cDNA, 0.4  $\mu$ l of each of the left and right primers

(10 mM) and 0.2 µl of water was used. mRNA was measured in triplicate wells and each sample was quantified using a standard curve obtained by serial dilution of bovine neutrophil cDNA.

#### *Statistical Analysis*

Intake and plasma minerals were analyzed as in the first trial. mRNA levels of *L-selectin* relative to housekeeping genes were analyzed using the MIXED procedure of SAS. Significance was again declared at  $P \leq 0.05$  and a trend at  $P \leq 0.10$ .

### **Experiment 3:**

#### *Objective*

We hypothesize that Zn administered into the rumen as an OTM will be more bioavailable than Zn administered as an ITM. The objective of Experiment 3 was to administer two organic forms and one inorganic form of zinc via ruminal infusion and to determine its bioavailability by measuring its concentrations in the blood.

#### *Animals and treatment*

Animals, feeding, and housing were the same as in Experiments 1 and 2. Steers were ruminally dosed with either: 4 g of Zn supplied as one of two types of organic Zn, or as one type of inorganic Zn, or a control containing no Zn. Organic Zn was supplied as a HMTBa complex (25 g of Mintrex Zn, Novus International, Inc., St. Louis, MO) or as a zinc amino acid complex (100 g of Availa-Zn 40, Zinpro Corporation, Eden Prairie, MN). Inorganic Zn was supplied as 11 g of zinc sulfate. Latin square design, period length and insertion of jugular catheters remained the same as Experiment 1.

Ruminal infusions were performed at 0700 h on day 7. Three-hundred and fifty-five ml containers were used to administer the mineral into the rumen. Treatments were administered directly into the rumen by removing the cannula plug and dosing the mineral under the rumen mat. A snap lid was used to insure the treatments remained dry and did not leak out until below the rumen mat. Once the container and lid were successfully below the rumen mat the lid was removed and the mineral was allowed to mix with the rumen contents. Any excess mineral was flushed out by inserting a flexible PVC tube (0.64 cm inner diameter) into the rumen and into the cup and then flushing 50 ml of tap water through it twice.

#### *Sampling*

Representative ration samples were taken as in the previous trials. At 0700 h on d 7, 20 ml of blood was taken to serve as a baseline (0 h) measure. Immediately following this baseline blood sampling, the treatment was ruminally infused and serial blood samples were taken at 2, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 20 and 24 h post infusion. Jugular catheters were removed immediately after the 24 h blood sample, at approximately 0700 h the next day.

#### *Blood and Feed Analysis*

Vacutainer tubes were spun at 1000 x g for 20 min at 4°C. Four ml of plasma was then transferred to each of two tubes, frozen and stored as in the previous trials. At the end of the trial, half of these samples were mailed to Novus International Inc, on ice packs, for analysis of methionine. The remaining plasma was analyzed for Zn. Plasma Zn was measured by ICP-MS (Perkin Elmer, Optima 2100 DV). Plasma was diluted 1:50 in 0.1% HNO<sub>3</sub> prior to analysis.

### *Statistical Analysis*

Intake and plasma minerals were analyzed as in the first trial. Significance was again declared at  $P \leq 0.05$  and a trend at  $P \leq 0.10$ .

## Chapter 4

### RESULTS

#### Experiment 1

The dietary ingredients and nutrient composition are shown in Table 2. For growing beef animals, the recommended dietary concentration of Cu is 10 mg/kg and 30 mg Zn/kg on a dry matter basis (NRC, 2001). For this trial the dietary Cu and Zn levels were kept as low as possible by feeding the steers a ration containing only forage without mineral supplementation. Individually, corn silage Cu and Zn for this experiment was 6 and 33 ppm respectively. Alfalfa silage Cu and Zn was 8 and 30 ppm, respectively. Our ration averaged 12.5 mg/kg Cu and 37.5 mg/kg Zn (Table 2), both of which were slightly above requirements.

Diarrhea was noted from those steers dosed with 4 g of Cu and 8 g of Zn in Experiment 1. During periods 1 – 3, steers receiving the Cu treatment consistently had watery diarrhea beginning at 12 h, with return to normal feces by 24 h. Loose stool was noted for the 8 g Zn treatment in period 1 for steer 3 from 10 h to 12 h and in period 3 for steer 5 at 6 h. There was no diarrhea observed during the fourth period. 4 g Cu and 8 g Zn also depressed DMI during day of infusion when compared to the 4 g Zn and control treatments (Figure 1). All steers returned to their pre-treatment intake levels within 1 day following treatment (data not shown).

The only treatment that altered plasma Cu concentration was 4 g of Cu (Figure 2). In the steers treated with 4 g Cu there was a rapid rise in plasma Cu concentration from 1.1 ppm at 0 h to 1.7 ppm at 0.75 h post infusion. Plasma Cu rapidly decreased and was not different from the control from 2 through 6 h after treatment. Additionally, plasma Cu tended to be lower for 4 g Cu than the control at 8 h ( $P = 0.07$ ) and 12 h ( $P = 0.08$ ).

Plasma Zn for the Cu treatment did not differ from the control except that it tended to be lower at 12 h ( $P = 0.09$ ; Figure 3). Both 4 g and 8 g Zn increased plasma Zn concentrations when compared to the control and Cu treatments. For 4 g Zn, plasma Zn concentration tended to be greater than the control at 0.75 h and was above the control for the remaining time points. For 8 g Zn, plasma Zn concentration was greater than the control from 0.5 h through the end of sampling. There were also differences in plasma Zn when comparing the 4 g and 8 g Zn treatments. Plasma Zn was greater for the 8 g Zn than the 4 g Zn treatment at 1 through 12 hours and tended to be greater at 0.5 h ( $P = 0.06$ ) and 0.75 h ( $P = 0.08$ ). It is noteworthy to mention that plasma Zn from neither the high nor low Zn treatments ever returned to baseline within the 12 h sampling period. Peak plasma Zn concentrations were observed at 6 h post-treatment with concentrations of 2.84 ppm and 3.81 ppm for the 4 g and 8 g Zn, respectively, compared to 1.01 ppm for the control.

White blood cell MT mRNA levels were low, suggesting that RNA degradation had occurred (data not shown).

## **Experiment 2**

Based on the negative impacts of the 4 g Cu and 8 g Zn treatments on fecal consistency and DMI observed in Experiment 1, the 4 g Zn treatment was further evaluated in Experiment 2.

In Experiment 2, corn silage Zn was 30 ppm, and alfalfa silage Zn was 32 ppm. No diarrhea was noted on treatment days. Dry matter intake from the day of infusion tended to be lower for HMTBa Zn ( $P = 0.07$ ) and was lower for Zn sulfate ( $P < 0.01$ ) relative to the control (Figure 4). Dry matter intake for HMTBa Zn was greater than for Zn sulfate ( $P = 0.04$ ).

Both HMTBa Zn and Zn sulfate increased plasma Zn of the steers (Figure 5). Plasma Zn for the HMTBa Zn treatment was greater than the control from 1.5 to 7 h ( $P < 0.05$ ) and tended to be above the control at both 1 and 8 h ( $P = 0.07$  and  $P = 0.06$  respectively). For Zn sulfate, plasma Zn was greater than the control from 1 to 8 h and tended to be above the control for 9 and 10 h ( $P = 0.07$  and  $P = 0.10$  respectively). There were no differences in plasma Zn between the HMTBa Zn and Zn sulfate treatments. Peak plasma Zn concentrations occurred at 3 h post-infusion and were 1.98 ppm and 2.39 ppm for HMTBa Zn and Zn sulfate respectively, as compared to the control at 0.55 ppm. The control plasma Zn did not remain consistent over the sampling period and dropped from 1.10 ppm at 0 h to 0.38 ppm at 24 h. Both HMTBa Zn and Zn sulfate plasma Zn concentrations drifted down over time, dropping from an average of 1.03 ppm at 0 h to 0.63 ppm at 24 h. This decrease over time is believed to be due to analytical drift associated with the ICP-OES method.

Neutrophil phagocytosis and mRNA levels of *L*-selectin are presented in Figures 6 and 7, respectively. On average about 30% of neutrophils engulfed at least one fluorescent bead during the 2 h incubation period, with no differences among the treatments. Expression of relative neutrophil mRNA levels of *L-selectin* also did not differ among treatments.

### **Experiment 3**

Corn and alfalfa silage Zn was 20 ppm and 29 ppm in this experiment, respectively. No diarrhea was noted on treatment days. Dry matter intake from the day of infusion tended to be lower for ZnSO<sub>4</sub> in comparison to the control and AA-Zn treatments ( $P=0.10$  and  $P=0.09$  respectively; Figure 8).

Plasma Zn concentrations for the control treated steers remained relatively constant over time and averaged 0.98 ppm. All three Zn treatments increased plasma Zn compared to the control (Figure 9). Plasma Zn for the HMTBa Zn treatment was greater than the control at 8, 9, 12, 13, 14, 16 and 24 h ( $P < 0.05$ ) and tended to be greater at 20 h ( $P = 0.06$ ). For AA-Zn, plasma Zn was greater than the control at 8, 9, 12 – 16 and 24 h ( $P < 0.05$ ) and tended to be above the control at 10 h post infusion ( $P = 0.09$ ). The Zn-sulfate treatment increased plasma Zn above the control at 9, 13, 16 and 24 h ( $P < 0.05$ ) with a tendency to increase plasma Zn at 20 h ( $P=0.09$ ). There were no differences in plasma Zn among the HMTBa Zn, AA-Zn and Zn sulfate treatments.

## Chapter 5

### DISCUSSION

#### Dietary Copper and Zinc

For the studies described in this thesis, dietary Cu and Zn were kept at a minimum by feeding steers only alfalfa silage and corn silage with no added grain (Table 2). However, both Cu and Zn are difficult minerals to feed below requirements when feeding diets that contain traditional feedstuffs. The dietary Cu concentration in Experiment 1 was 12.5 ppm which meets the requirements for beef cattle (10 ppm; NRC, 2000). For dairy cows, the required dietary concentration of Cu is 13.7 ppm for a dry cow and 15.7 ppm for a lactating cow (NRC, 2001), so our diet would have been slightly deficient for a dairy cow. The dietary concentration of Zn in Experiments 1 and 2 was 37.5 and 36.3 ppm, respectively, which meets the requirements for beef cattle (30 ppm; NRC, 2000), and in Experiment 3 was 23.5 ppm, which is slightly lower than requirements (Table 2). For dairy cows the required dietary Zn concentration 22.8 ppm for a dry cow and 63 ppm for a lactating cow (NRC, 2001), so all diets were sufficient for a dry cow but deficient for a lactating cow.

Concentrations of Cu and Zn in forages vary both among and within forage species, and availability of forage minerals is affected by dietary mineral antagonisms. Forages vary greatly in Cu and Zn content depending on mineral availability in the soil and plant maturity. For example, legumes are usually higher in Cu and Zn than grasses

(NRC, 2000). These intra and inter-forage variations account for the reported standard deviations in forage mineral content and the variability in dietary Zn found in these trials. According to the NRC (2001), corn silage contains an average of 6 ppm (SD = 7) of Cu and 23 ppm (SD = 8) of Zn. Legume silage contains 10 ppm (SD = 3) and 29 ppm (SD = 8) of Cu and Zn, respectively. Corn silage Zn ranged from 20 to 33 ppm and alfalfa silage Zn ranged from 29 to 32 ppm throughout the three trials.

The NRC (2001) assumes a 4% availability of Cu in forages and a 15% availability of forage Zn. This, however, is noted to be an estimate that is affected by antagonisms with other dietary minerals, including molybdenum (NRC, 2001). Variable forage mineral concentrations, interactions and antagonisms that can take place in the soil, plant and animal make ration formulation difficult. As a consequence, there is still disagreement among nutritionists on whether to include or not include forage mineral concentrations when formulating dietary rations.

### **Bioavailability and Toxicity of Abomasally Infused Cu or Zn**

In these experiments, plasma concentrations of Cu or Zn were measured to evaluate mineral bioavailability to the animal. Plasma was an acceptable measure of bioavailability because abomasal or ruminal infusion of organic Cu, inorganic Zn, or organic Zn in the 3 experiments consistently increased the respective mineral concentrations in the plasma. Our studies were performed as pulse dose experiments, and we are not aware of similar studies. Pulse dose experiments were conducted to establish an immediate and large magnitude response in the plasma minerals as well as eliminate the possibility of homeostatic regulation that could take place during feeding

experiments. However, studies that have supplemented minerals through feeding have predominately found increased plasma mineral concentrations as well. In previous studies using growing ruminants, dietary supplementation of Cu and Zn in both inorganic and organic forms have been shown to increase plasma mineral concentrations (Kinkaid and Conrath, 1979; Jenkins and Hidioglou, 1991; Rojas et al., 1995; Wright and Spears, 2004). However, trace mineral supplementation of Cu resulted in no change in the plasma mineral concentration in one study using mature ewes (Eckert et al., 1999) and an inconsistent increase in plasma Cu in growing heifers (Rabiansky et al., 1999). Abomasal or ruminal infusion of Cu and Zn increased plasma mineral concentrations in Holstein steers to similar extents, indicating that both inorganic and organic forms of these minerals were bioavailable in both the rumen and intestine.

Growing beef steers have Zn requirements of approximately 0.3 g/d (NRC, 2000). In Experiment 1, the diet provided the control steers with 0.4 g/d Zn. On the day of infusion, the steers were administered 4 or 8 g of Zn directly into their abomasum. The 4 g dose of Zn was 13.3 times greater than the requirement of a steer. The 8 g dose of Zn was 26.8 times greater than the required amount for steers. The doses utilized were well above requirements in order to obtain a substantial and statistically significant increase in plasma Zn concentration. In Experiment 1, we observed that steers abomasally infused with 8 g Zn had a greater increase in plasma Zn concentration than those infused with only 4 g Zn (Figure 3). However, we believe the 8 g pulse dose of Zn elicited a toxicity response as indicated by diarrhea and a decrease in DMI on the day of infusion (Figure 1). Similar results were found in preruminant calves that were fed dietary Zn at 1.3, 6.7, 16.7, 23.3, and 30 times Zn requirements (Jenkins and Hidioglou, 1991). Those fed 23.3

or 30 times the Zn requirements showed toxicity signs, including decreased DMI and diarrhea, while those fed the lower doses were not adversely affected (Jenkins and Hidioglou, 1991). We conclude that dosing the steers with 8 g of Zn caused a toxicity response whereas 4 g of Zn did not. For this reason, only the 4 g Zn dose was further evaluated in Experiments 2 and 3.

Beef cattle require 0.1 g/d dietary Cu (NRC, 2000). The control steers in Experiment 1 consumed 0.28 g/d Cu. However, the 4 g pulse dose of Cu was 40 times the required amount for steers. It has been stated that chronic Cu poisoning can occur when feeding 4-5 fold the Cu requirement (NRC, 2001). Additionally, acute poisoning may follow intakes of 0.02 – 0.1 g/kg body weight Cu in sheep and young calves and of 0.2 – 0.8 g/kg body weight in mature cattle (The Merck Veterinary Manual, 2008). The 4 g pulse dose of Cu used in Exp 1 was well above the g/d requirements of 0.1 g/d (NRC, 2001), but averaging at 0.00625 g/kg was still under the acute and chronic poison level.

Cattle are also very sensitive to high levels of dietary Cu. When cattle consume excessive amounts of Cu, Cu accumulates in their livers. Liver Cu accumulation does not directly affect the animal. However, when liver Cu stores are released, there is a transient rise in red blood cell Cu, followed by a spike of whole blood Cu that ultimately leads to hemolytic crisis (Ammerman, 1969; NRC, 2001). Hemolytic crisis occurs when Cu induces destruction of red blood cells at a faster rate than the body can compensate by producing more red blood cells. This beginning of a Cu toxicity response was reported in a study by Eckert et al. (1999) using ewes. Copper concentrations in the liver of ewes were increased for those fed 30 mg/kg CuSO<sub>4</sub> compared to those fed either 10 or 20 mg/kg CuSO<sub>4</sub>. Those fed 30 mg/kg also had a numerical decrease of red blood cell and

whole blood hemoglobin levels, indicating that liver Cu release was initiating a toxicity response.

In Experiment 1, 4 g of abomasal Cu increased plasma Cu but also caused a toxicity response as evidenced by diarrhea and a 61% decrease in DMI, with steers consuming only an average of 4 kg/d on the day of infusion (Figure 1). Plasma Cu concentration quickly rose and fell within 2 hr following infusion and was numerically though not significantly below baseline from 8 – 12 h (Figure 2). This numeric decrease in plasma Cu concentration at the end of sampling suggests that homeostatic mechanisms were initiated to decrease Cu toxicity. Because of this small tolerable range, it would be hard to dose an amount small enough to avoid a toxicity response but large enough to elicit a measurable effect on plasma Cu concentrations. Additionally, homeostatic regulation, as evidenced by the rapid drop in plasma Cu concentration shortly following infusion, is difficult to control experimentally. We believe that dosing 4 g of Cu directly into the abomasum induced a toxicity response from the steers as indicated by diarrhea, decreased DMI, and rapid homeostatic regulation of plasma Cu concentration. We concluded that a pulse dose of Cu was not a good model to study Cu bioavailability and choose not to include it in further studies.

### **Bioavailability of Abomasally or Ruminally Infused Zn**

The main benefit of organic compared to inorganic trace mineral supplementation in ruminants is believed to be a reduction in negative interactions in the rumen that can render the mineral unavailable. However, some have postulated that the ruminal protection provided by organic trace minerals may limit their absorption in the small

intestine. Inorganic trace minerals are primarily salts that dissociate readily in neutral pH of the rumen whereas OTM are bound covalently and are less apt to dissociate at a neutral pH. For example, when placed in a pH 5 solution, inorganic ZnSO<sub>4</sub> was at 97% soluble whereas the organic forms of Zn were less than 50% soluble (Cao et al., 2000). These results led Cao et al. (2000) to conclude that because the intestine will have a pH greater than 5; organic chelates will be much less soluble and available for absorption than inorganic salts. However, Cao et al. (2000) also found that when placed in an acidic solution (pH = 2), both organic and inorganic forms of Zn were greater than 90% soluble. Richards (2006) concluded that OTM should be absorbed by the small intestine in a similar manner to ITM, rendering both as available to the animal. For example, *in vitro* absorption experiments with a ZnMet showed that Zn and methionine were absorbed separately, indicating that the chelate did not hinder absorption mechanisms (Richards, 2006).

We performed Experiment 2 to compare the bioavailability of HMTBa Zn to ZnSO<sub>4</sub> when infused into the abomasum. This allowed us to directly evaluate whether small intestinal absorption was negatively impacted by the chelate. We found that both ZnSO<sub>4</sub> and HMTBa Zn caused a similar increase in plasma Zn when compared to the control (Figure 5). Although there was no benefit of organic over inorganic Zn, there was no detriment, indicating that absorption of chelated Zn was not compromised. Based on plasma as a measure of bioavailability, HMTBa Zn was equally as available as inorganic Zn in the intestine.

Antagonisms among disassociated minerals in the digestive tract are most likely to occur in the neutral to slightly acidic pH of the rumen. In the case of inorganic salts

such as sulfates, the ionic bonds are weak and more likely to dissociate in the rumen than those covalent bonds that hold the organic chelates together (Richards et al., 2008). Therefore, organic minerals are less available in the rumen than inorganic forms, making them more resistant to antagonisms that yield insoluble and indigestible mineral complexes (Spears, 1996). In Experiment 3, we hypothesized that organic Zn supplementation would increase plasma Zn concentrations more than inorganic Zn supplementation when dosed into the rumen.

Previous *in vivo* feeding studies in ruminants have shown enhanced bioavailability of Zn fed as OTM versus ITM. Organic forms of Zn such as ZnMet and ZnLys have been shown to have greater absorption and retention in young calves than ZnO when measured by Zn concentrations in liver and serum (Kincaid et al., 1997). The two predominant inorganic forms of Zn, ZnSO<sub>4</sub> and ZnO, were previously considered to have similar bioavailability in ruminants (Kincaid, 1979). However, NRC (2001) has stated that ZnO is a less available than ZnSO<sub>4</sub>. Therefore, the differences observed by Kincaid et al. (1997) may have been due to comparing organic Zn to ZnO instead of a general difference between OTM and ITM. In a study done by Nocek et al. (2006), cows were fed 1 of 4 diets over 2 lactations: 100% NRC requirements of Cu, Co, Mn, and Zn as sulfates, amino acid complexes, a mix of the both, or 75% of NRC requirements as amino acid complexes only. They found that milk yield, and fat and protein percent were higher during the second lactation in cows fed at 100% NRC requirements with minerals supplied as amino acid complexes compared to 100% supplied as sulfates. Somatic cell count was lower for the cows fed 100% complexed and the mix of complexes and sulfates than for cows on the other 2 treatments. They additionally found that feeding

75% of requirements as amino acid complexes resulted in the same performance as feeding 100% as sulfates (Nocek et al., 2006). Together, these results suggest that amino acid complexed ITM were more bioavailable than sulfate forms. In support of these findings, liver Zn concentration and fecal Zn concentration were higher in amino acid-complexed Zn than ZnSO<sub>4</sub> supplemented ewes, which indicates that organic Zn was more bioavailable than the inorganic form (Hatfield et al., 2001). However, other studies have shown no effect of supplement type on animals. During an 18 d study by Nockels et al. (1993), no differences in the bioavailability of ZnSO<sub>4</sub> or ZnMet were found as measured by serum Zn or Zn digestibility.

Our data do not support our hypothesis that ruminally infused organic Zn would increase plasma Zn concentration more than inorganic Zn. Ruminal infusion of HMTBa Zn, AA-Zn and ZnSO<sub>4</sub> in Experiment 3 all provided bioavailable Zn, as evidenced by increased plasma Zn concentration (Figure 9), but the organic forms did not improve bioavailability compared to ZnSO<sub>4</sub>. Plasma Zn concentration was increased for AA-Zn at 8, 9, 12, 13, 14, 16, 24 hrs, HMTBa Zn 8, 9, 12, 13, 14, 16, 24 hrs, and ZnSO<sub>4</sub> 9, 13, 16, 24 hrs compared to the control. However, the supplemented steers still had greater plasma Zn concentrations than control steers at the end of the measurement period, indicating that the sampling period was not long enough. During Experiments 1 and 2 we were able to see the plasma mineral concentration peak and then return to baseline. However, because the minerals were infused into the rumen in Experiment 3 instead of the abomasum, a period longer than 24 hrs would have been required to provide adequate time to allow the minerals to reach the absorptive intestinal epithelium and be absorbed into the blood. Plasma Zn concentrations for supplemented steers were not yet declining

at the 24 h blood sampling and we cannot predict how plasma Zn would have continued to change over time. The coordinate bonds in OTM keep the mineral more stable and less prone to interactions in the rumen than ITM, and differences in mineral bioavailability may have been magnified at longer times post-infusion (Richards, 2006). Additionally, the magnitude of the increase in plasma Zn following ruminal Zn infusion in Experiment 3 was lower than in Experiments 1 and 2.

Additional studies with ruminally dosed Zn are needed to further evaluate bioavailability of organic and inorganic forms of Zn. In addition to extending the sampling time, or increasing the dose, it may also be beneficial to extend the deprivation period that the steers experienced. The deprivation period in these trials consisted of the first 6 d of each period where steers were fed only forages. Prior to these studies, the steers had been fed a diet that exceeded their mineral requirements, and this may have decreased the potential for response to the mineral pulse dose. Specifically, tissue and liver Zn stores may have already been adequate to support a brief deprivation period. Hence when the steers were placed on the deprivation, diet homeostatic mechanisms may have released the stored Zn and buffered any changes in plasma Zn concentrations. A future rumen pulse dose study using a longer deprivation period, a longer sampling period, and a larger pulse dose should improve our understanding of Zn bioavailability.

In Experiments 2 and 3, DMI for those steers infused with ZnSO<sub>4</sub> decreased on the day of treatment compared to the control and organic Zn treatments (Figures 4 and 8). A previous study found that 2 g/d of dietary ZnSO<sub>4</sub> negatively affected diet palatability and decreased DMI in steers (Miller et al., 1989). However, the decrease seen in our experiments could not have been caused by an effect on palatability because the minerals

were not fed. We believe that the ZnSO<sub>4</sub> treatment induced a slight toxicity response that did not occur for either of the organic chelates. One reason ZnSO<sub>4</sub> may have induced toxicity is if it had greater availability than its organic counterparts. In Experiment 2, treatments were infused into the abomasum and the increase in plasma Zn concentration for the ZnSO<sub>4</sub> treated steers was numerically higher than for HMTBa Zn, suggesting a greater availability. However, in Experiment 3 the treatments were ruminally infused and there were no numeric or significant differences in plasma Zn concentrations for any of the Zn forms. The decline in intake in response to ruminally or abomasally infused ZnSO<sub>4</sub> was similar, suggesting that the intake effect occurred post-ruminally. It is possible that the chelated organic forms were absorbed more gradually from the small intestine than ZnSO<sub>4</sub>. A slower absorption rate may have decreased any negative effects of the large Zn dose on intake. In summary, the decrease in DMI on the day of Zn infusion in Experiments 2 and 3 occurred only in the steers dosed with ZnSO<sub>4</sub>, possibly due to a slight toxicity reaction that did not occur with the organic Zn.

Plasma minerals are one method of measuring mineral bioavailability. The main benefit of using plasma mineral concentration as a biomarker is that is a very simple and minimally invasive way of acquiring a sample for analysis. Blood samples can be obtained through jugular catheterization as in these trials or simply through jugular venipuncture. However, plasma minerals may not be the optimal biomarker. Some researchers have combined plasma mineral concentrations with tissue concentrations to measure bioavailability. For example, Wright and Spears (2004) measured both plasma and liver mineral concentrations as a marker for bioavailability. In humans, liver Cu concentration is considered to be better than plasma Cu concentration (Olivares et al.,

2008). However, some researchers feel that neither plasma nor tissue measures are ideal biomarkers because of homeostatic mechanisms that maintain plasma levels when intake is inadequate and certain tissues may be more affected by homeostatic regulation than others (Hambidge, 2003). In these cases, researchers have chosen to look at a marker specific for the mineral of interest, such as monocyte MT messenger RNA or red cell membrane Zn for Zn bioavailability (Hambidge, 2003). Metallothionein may also be used as a measurement of Zn bioavailability as shown by Richards and Cousins (1976), where they saw MT in both liver and intestinal mucosal cytosol responded rapidly to an altered dietary Zn level.

Another alternative for evaluating bioavailability of mineral supplements is to measure the actual mineral function. Some human studies have shown erythrocyte Cu/Zn SOD activity is a better biomarker of Cu status than plasma Cu concentration (Milne D.B., 1998). In most experiments, researchers have evaluated the effects of dietary mineral supplements. Our experiments evaluated a pulse dose of minerals which resulted in a more dramatic increase in mineral availability than traditional feeding studies. Plasma Zn concentrations in all three experiments were increased by abomasal or ruminal infusions of organic or inorganic Zn. Plasma Zn concentrations were sensitive enough to detect bioavailability when comparing Zn treatments to the control. Plasma Zn concentrations were also sensitive enough to detect the difference in dosage amount in Exp 1 and resulted in higher plasma concentrations for the 8 g dose when compared to the 4 g dose.

## **Zinc and the Immune System**

Zinc is essential for immune function. In Experiment 2 we looked at *L-selectin* mRNA levels and phagocytosis as measures of neutrophil function. However, we saw no treatment effects on either measure. Because the time between dosing and immune measurements was only 9 hours, it is possible that though this was enough time for the plasma concentration to be altered, it was likely not enough time to alter neutrophil function. This short time period was chosen because the effects resulting from a pulse dose of Zn were expected to likely be transient. Feeding trials that have supplemented Zn for a longer period of time have delivered varying results. One such study by Kinkaid et al. (1997) saw no Zn effect on phagocytic or intracellular killing ability of neutrophils. This lack of treatment effect was also seen in mitogen-induced lymphocyte blastogenesis, interleukin-2 production, and lymphocyte cytotoxicity (Kinkaid et al., 1997). An additional study we performed (Nemec et al., 2010), found that lactating cows supplemented with amino acid chelated Cu, Mn and Zn had a 2.5 fold higher antibody titer to a rabies vaccination than those supplemented with sulfate forms of the minerals. Other studies that have presented animals with an immune challenge have seen benefits of organic Zn compared to inorganic Zn. Supplementation of steers with ZnMet versus ZnO resulted in increased antibody titers in response to a bovine herpesvirus-1 vaccine (Spears, 1996). Additionally, calves fed ZnMet for a period of 7 d recovered faster than those fed ZnO when challenged with IBRV (Chirase et al., 1991). Though Zn is a necessary component of the immune system and has been shown to enhance immune function in feeding trials, our studies showed no short-term enhancement by pulse dose Zn supplementation on neutrophil measures.

## **Summary and Conclusions**

In all 3 experiments, the infusion of Cu or Zn into either the abomasum or rumen increased bioavailable minerals as indicated by an increase in plasma Cu or Zn concentration. In Experiment 1, abomasal infusions of both 4 g of Cu and 8 g of Zn resulted in an increase in plasma concentration of the respective mineral, but also resulted in a toxicity reaction. However, 4 g of Zn did not cause a toxicity reaction and was utilized in the subsequent studies to evaluate Zn bioavailability. Because a relatively large abomasal pulse dose of Cu is required to increase plasma concentrations, we concluded that abomasal Cu infusion is not an appropriate method to quantify Cu bioavailability. Copper bioavailability would be better studied through the portal vein because this would allow measures to be taken before any homeostatic mechanisms came into play. Additionally portal blood measurements would enhance the ability to see significant effects with smaller non-toxic doses. In Experiments 2 and 3, infusion of chelated Zn products did not differ in their bioavailability when compared to ZnSO<sub>4</sub>. This lack of difference was a positive result in Experiment 2, as some have raised concern that chelated minerals may be less available for absorption at the intestinal epithelium than inorganic minerals. We found, that both organic and inorganic Zn had equal bioavailability. Therefore, producers can feed organic Zn in amino acid or HMTBa forms without concern for hindered intestinal availability. Unfortunately, Ruminal infusion of organic forms of Zn in Experiment 3 did not increase plasma Zn concentrations in comparison to the ZnSO<sub>4</sub>. Ruminal infusion of organic Zn was expected to increase plasma Zn concentration compared to inorganic Zn because the rumen is the site of the

majority of antagonisms. However the magnitude of increase in plasma Zn in response to all ruminal Zn supplements was low and plasma Zn concentrations in Zn infused animals were elevated above the control when blood sampling ceased. Additionally, in Experiment 2 an abomasal pulse dose of Zn did not enhance neutrophil phagocytosis or *L-selectin* mRNA between treatments. Though Zn is involved in many neutrophil functions, we conclude that a single dose of Zn is not likely to enhance immune function in the short term. However, further studies would be required to determine whether a pulse dose of Zn may benefit long-term animal health. Future studies including additional blood sampling time points and a larger Zn dose are required to better evaluate potential differences among ruminally infused Zn supplements.

**Table 2.** Ingredient composition of the three experimental diets and analyzed nutrient contents of the experimental diets.

	<b>Experiment 1</b>	<b>Experiment 2</b>	<b>Experiment 3</b>
<b>Ingredient</b>	<b>% of DM</b>		
<b>Alfalfa Silage</b>	47.6	47.6	46.5
<b>Corn Silage</b>	52.4	52.4	53.5
<b>DM%</b>	48.9	38.6	46.6
<b>Nutrient</b>	<b>% of DM</b>		
<b>Crude Protein</b>	13.1	12.7	13.0
<b>ADF</b>	31.3	31.4	30.0
<b>NDF</b>	42.6	43.7	40.3
<b>Ash</b>	8.63	12.1	6.68
<b>Starch</b>	16.4	20.7	17.5
<b>NFC<sup>1</sup></b>	33.8	29.1	38.0
<b>Calcium</b>	0.73	0.51	0.87
<b>Magnesium</b>	0.23	0.22	0.21
<b>Potassium</b>	2.03	2.21	1.64
<b>Sodium</b>	0.04	0.04	0.05
<b>Nutrient</b>	<b>ppm</b>		
<b>Iron</b>	476	1361	360
<b>Manganese</b>	34.3	56.8	26.3
<b>Zinc</b>	37.5	36.3	23.5
<b>Copper</b>	12.5	11.5	7.50

<sup>1</sup>NFC: Non-fiber carbohydrates.

**Table 3.** Treatment values for plasma Cu and plasma Zn in experiment 1

	Treatment <sup>1</sup>					P - values		
	Control	4 g Cu	4 g Zn	8 g Zn	SEM	Treatment	Time	Treatment × Time
Plasma Cu, ppm	1.070 <sup>a</sup>	1.224 <sup>b</sup>	1.009 <sup>a</sup>	0.971 <sup>a</sup>	0.082	0.04	< 0.001	< 0.001
Plasma Zn, ppm	1.002 <sup>a</sup>	0.884 <sup>a</sup>	1.978 <sup>b</sup>	2.753 <sup>c</sup>	0.147	< 0.001	< 0.001	< 0.001

<sup>1</sup> Treatments were abomasal infusions of 2-hydroxy-4-methyl-thiobutyrate (HMTBa) Cu at 4 g of total Cu, HMTBa Zn at either 4 g or 8 g total Zn

<sup>a,b,c</sup> Means in rows with unlike superscripts differ,  $P < 0.05$ .

**Table 4.** Treatment values for plasma Zn in experiments 2 and 3

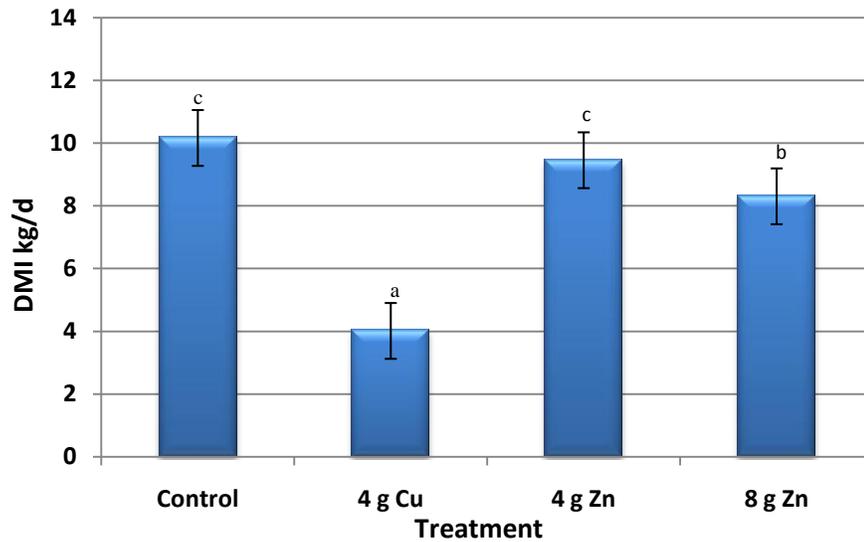
	Treatment <sup>1</sup>					P - values		
	Control	ZnSO <sub>4</sub>	HMTBa Zn	AA-Zn	SEM	Treatment	Time	Treatment × Time
Plasma Zn, Exp 2	0.663 <sup>a</sup>	1.705 <sup>b</sup>	1.469 <sup>b</sup>	ND <sup>2</sup>	0.222	0.026	<0.001	0.399
Plasma Zn, Exp 3	0.975	1.055	1.079	1.064	0.489	0.182	<0.001	0.025

<sup>1</sup> Treatments were 2-hydroxy-4-methyl-thiobutyrate (HMTBa) Zn, ZnSO<sub>4</sub>, or amino acid chelated Zn abomasally infused in Experiment 2 and ruminally infused in Experiment 3

<sup>2</sup> ND = Not determined.

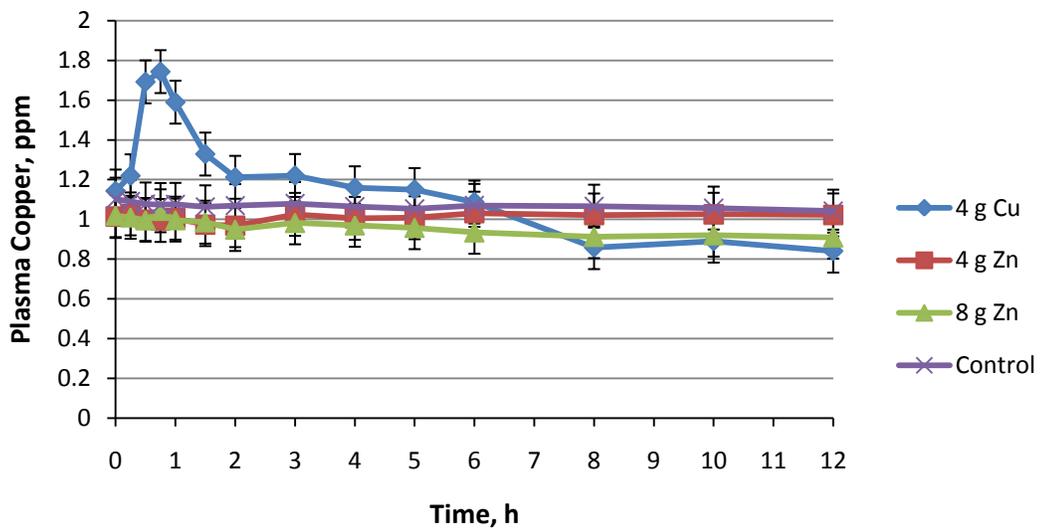
<sup>a,b</sup> Means in rows with unlike superscripts differ,  $P < 0.05$ .

**Figure 1.** Average dry matter intake on day of infusion during Experiment 1. SEM = 1.52.

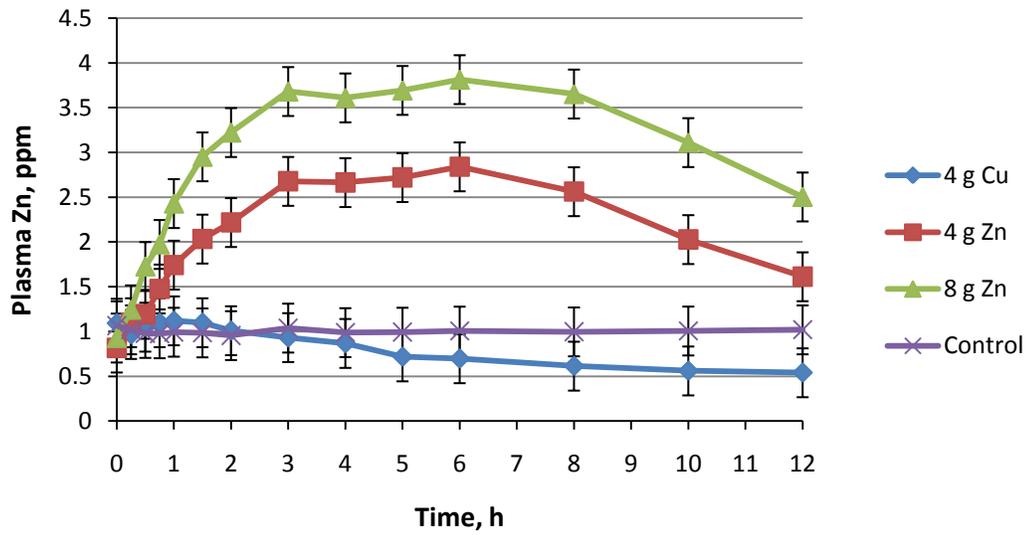


<sup>a,b,c</sup> Bars with unlike superscripts differ,  $P < 0.05$ .

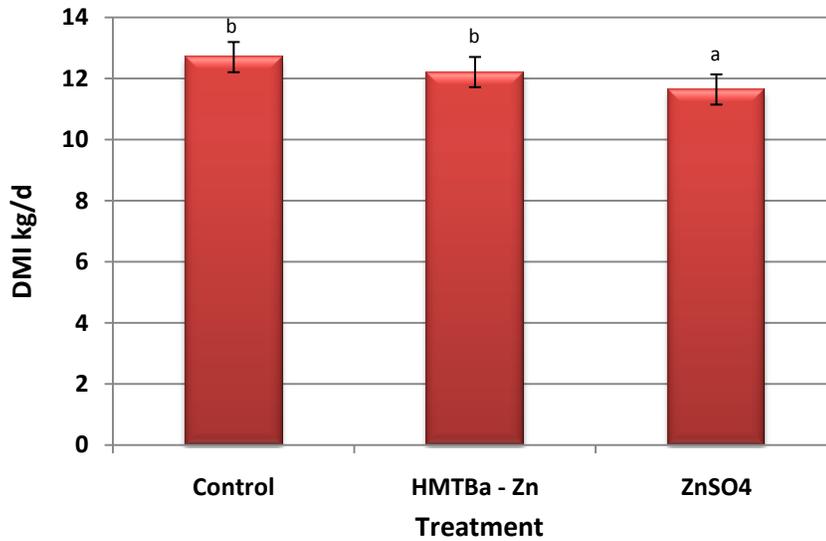
**Figure 2.** Effect of abomasal infusions of 4 or 8 g of Zn from HMTBa Zn or 4 g of Cu from HMTBa Cu on plasma Cu in Experiment 1. SEM = 0.147.



**Figure 3.** Effect of abomasal infusions of 4 or 8 g of Zn from HMTBa Zn or 4 g of Cu from HMTBa Cu on plasma Zn in Experiment 1. SEM = 0.082

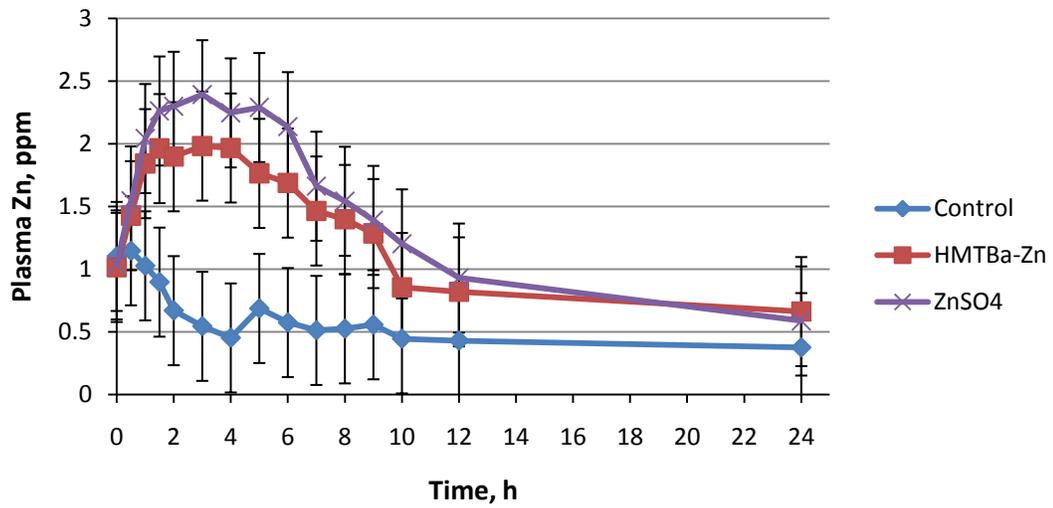


**Figure 4.** Average dry matter intake on day of infusion during Experiment 2. SEM = 1.22

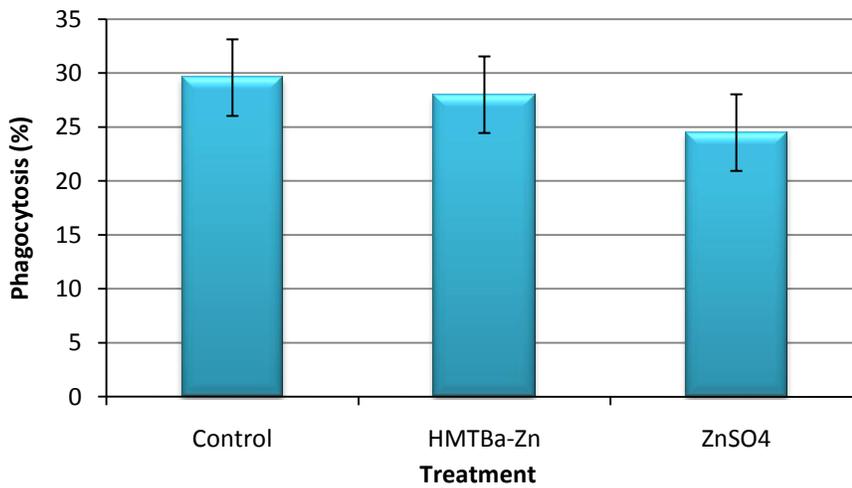


<sup>a,b</sup> Bars with unlike superscripts differ,  $P < 0.05$ .

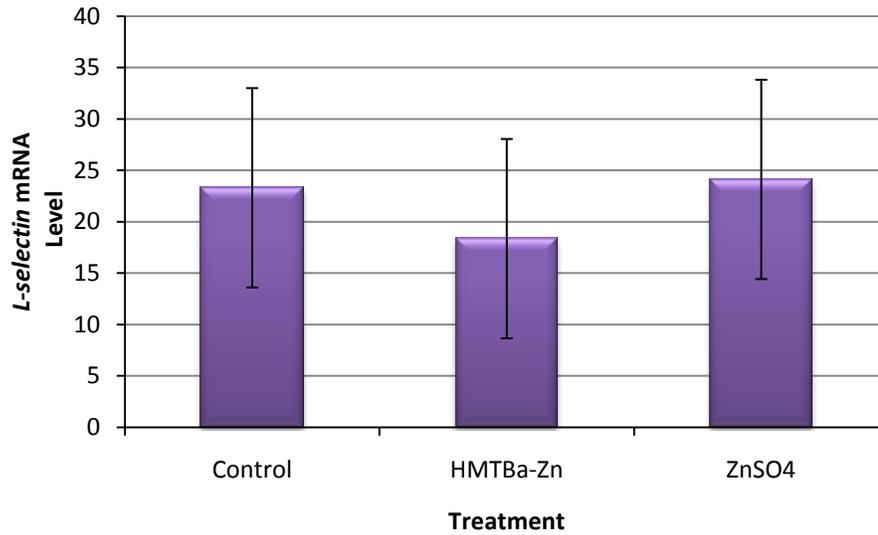
**Figure 5.** Effect of abomasal infusions of 0 or 4 g of Zn from HMTBa Zn or ZnSO<sub>4</sub> on plasma zinc concentrations in Experiment 2. SEM = 0.222



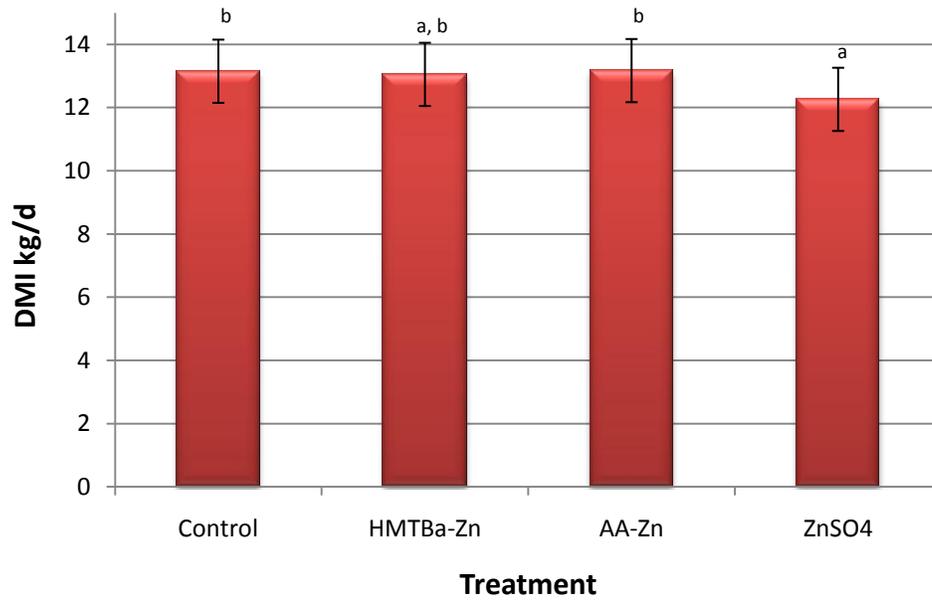
**Figure 6.** The percentage of neutrophils that engulfed at least 1 fluorescent bead during a 2 hr incubation in Experiment 2. There were no treatment differences. SEM = 4.08



**Figure 7.** Neutrophil mRNA levels of *L-selectin* relative to  $\beta$ -actin in Experiment 2. There were no treatment differences. SED = 7.08

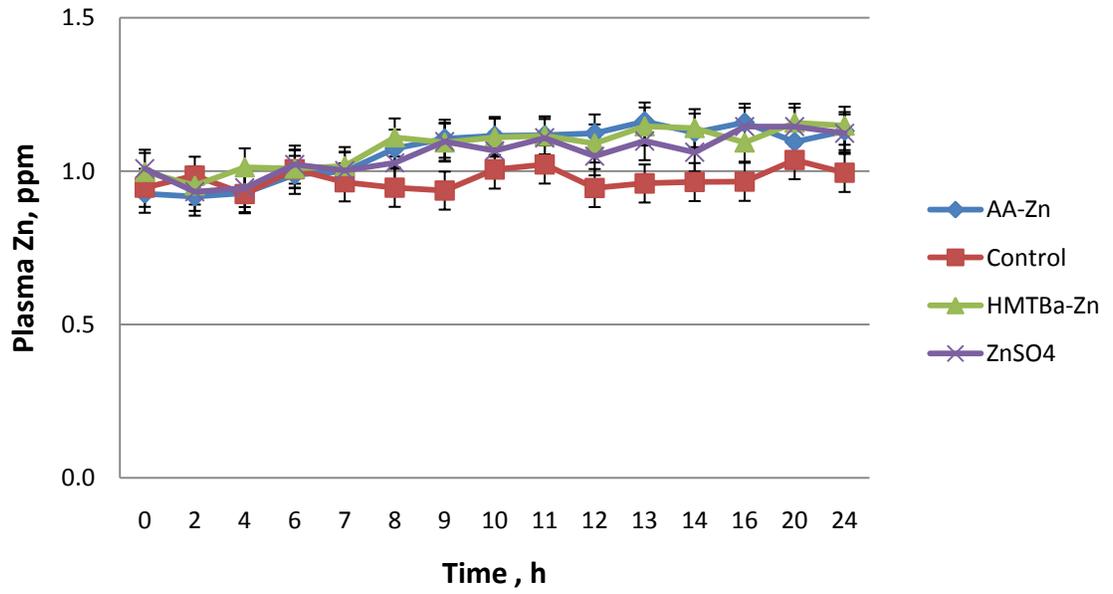


**Figure 8.** Average dry matter intake on day of infusion during Experiment 3. SEM = 0.974.



<sup>a,b</sup> Bars with unlike superscripts differ,  $P < 0.10$

**Figure 9.** Effect of ruminal infusions of 0 or 4 g of HMTBa Zn, AA-Zn, or ZnSO<sub>4</sub> on plasma zinc concentrations in Experiment 3. SEM = 0.489.



## Chapter 6

### APPENDIX

#### The Antioxidant System

Cells need energy to function and they generate this energy primarily through the aerobic metabolism of fuels such as carbohydrates, proteins and fats. Organisms metabolize  $O_2$  during this process and a molecule of  $O_2$  is completely reduced after sequentially accepting 4 electrons. Aerobic metabolism of fuels includes glycolysis, the citric acid cycle and oxidative phosphorylation. Glycolysis is important in carbohydrate metabolism and converts glucose into pyruvate. Pyruvate and products of fat and protein metabolism then enter the citric acid cycle where they are further metabolized to energy (adenosine triphosphate or ATP), reduced cofactors (NADH and  $FADH_2$ ) and  $CO_2$ . Oxidative phosphorylation is the final part of the metabolic pathway and the net result is that  $O_2$  accepts electrons from the reduced cofactors, generating  $H_2O$ , oxidized cofactors (NAD<sup>+</sup> and FAD) and energy. During oxidative phosphorylation, electrons are transferred from the reduced cofactors to  $O_2$  through a series of donors and acceptors in the electron transport chain. If an electron generated from metabolism escapes the oxidative phosphorylation process, it can be directly accepted by  $O_2$  and form superoxide ( $O_2^-$ ).

Superoxide is one type of reactive oxygen species (ROS) or free radical. Superoxide and other ROS, such as hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical

( $\cdot\text{OH}$ ), contain an unpaired electron and are hence unstable. When a ROS contacts another molecule, the free radical can either donate an electron to or accept an electron from that molecule to achieve a stable paired-electron state. However, during this process the electron balance of the previously stable molecule is permanently disrupted and sometimes becomes a free radical, initiating a chain reaction of destruction within a cell (Poli et al., 1999). Free radical propagation results in damage to cellular components that accept and donate electrons such as proteins, lipids or DNA.

Damage to DNA, proteins and lipids can cause irreversible loss of cell function. Oxidation, alkylation and hydrolysis of bases are some of the mechanisms by which DNA damage can occur (Poli et al., 1999). Protein damage can take place because of amino acid modification, fragmentation, or aggregation and can lead to a decrease or loss in biological function or an increase in proteolytic susceptibility (Salvi et al., 2001). Lipid damage occurs when a free radical interacts with the lipid molecule, typically at a carbon-carbon double bond, creating a lipid radical. When the lipid radical reacts with another unsaturated lipid molecule it breaks that double bond. This chain reaction finally terminates when two free radical lipids connect and form a covalent bond. Nonetheless, the lipids are permanently damaged by this process, similar to protein and DNA damage (Hanson, 2005).

In a healthy cell, antioxidant metabolites and enzymes are used to combat ROS-induced damage by one of two means, either by preventing the formation of free radicals, or by removing ROS before damage can occur. Antioxidants are classified into two broad fields based on solubility (Fridovich, 1978). Hydrophilic or water soluble antioxidants react with ROS in the cytosol and plasma. On the other hand, hydrophobic or lipid

soluble antioxidants act in the cell membrane. Superoxide dismutase is one hydrophilic antioxidant. It is an enzyme that contains metal ion cofactors used to convert  $O_2^-$  to  $H_2O_2$ . Copper/Zn SOD is present in the cytosol of cells, while Mn SOD is present in mitochondria (Fridovich, 1978). Copper/Zn SOD from bovine erythrocytes has been found to contain 2 subunits. Each subunit is a cylinder composed of a  $\beta$ -barrel with a pair of non-helical loops on the top and bottom which compose the active site (Richardson et al., 1975). The Cu in the Cu/Zn SOD participates in the catalytic cycle and oscillates from the cupric to the cuprous state, while the Zn appears primarily to play a structural role (Fridovich, 1978).

In an unhealthy cell there is an imbalance that occurs when ROS production exceeds the cell's ability to remove ROS. This imbalance is referred to as oxidative stress. This typically occurs during times of increased metabolism, such as heat stress or calving, when cellular metabolism increases and in turn ROS formation increases (Miller et al., 1993). When the cellular production of antioxidants cannot keep up with the rate of ROS formation from metabolism, damage to cellular components occurs, eventually leading to cell destruction (Hanson, 2005). As described in the following section, Zn supplementation has been found to sometimes enhance health and immune function. Because Zn has so many functions within the cell, most studies cannot pinpoint the precise mechanism of Zn action. However, reduced oxidative damage, particularly to white blood cells (WBC), appears to be one mechanism whereby Zn improves animal health. In fact, activity of Cu/Zn SOD in WBC themselves has been suggested to regulate WBC function (Marikovsky et al., 2003).

## Chapter 7

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