COPPER AND ZINC BALANCE IN EXERCISING HORSES

FED TWO FORMS OF MINERAL SUPPLEMENTS

A Dissertation

by

ELIZABETH LYNN WAGNER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Animal Science

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Approved by:

Co-Chair of Committee,	Gary D. Potter
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ABSTRACT

Copper and Zinc Balance in Exercising Horses

Fed Two Forms of Mineral Supplements. (December 2006)

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Co-Chairs of Advisory Committee: Dr. Gary D. Potter
Dr. Pete G. Gibbs

This study was undertaken to compare the absorption and retention of copper and zinc when supplemented to exercising horses in the form of sulfate or organic-chelate mineral supplements. Nine mature horses were used in a modified-switchback designed experiment consisting of seven 28-d periods. Horses were fed a diet consisting of 50% coastal Bermudagrass and 50% concentrate. All diets were balanced to meet the energy, protein, calcium and phosphorus requirements for horses performing moderate to intense exercise. Copper and zinc supplementation varied by period. During mineral depletion and repletion periods, horses respectively consumed diets with no supplemental mineral or Cu and Zn supplemented in the sulfate form to provide 100% of NRC (1989) values. In periods 4 and 7, horses were fed diets designed to provide 90% of NRC (1989) values for Cu and Zn supplied in the sulfate or organic-chelate forms. Horses were subjected to a standard exercise test on d 23 of periods 4 and 7 followed by a 4-d total fecal and urine collection. Blood samples were drawn every 28-d for determination of plasma Cu, Zn and ceruloplasmin concentration, and white blood cell counts and Cu,Zn-superoxide dismutase activity were evaluated in periods 4 and 7. Copper and zinc balance was

determined from feed, fecal, urine and water samples obtained during the total collections in periods 4 and 7.

Copper and Zn intake and fecal excretion were greater ($P<0.05$) for horses consuming the organic-chelate supplemented diet. Apparent Cu absorption as a percent of intake and retention as a percent of intake were also greater for this group. Plasma Cu, Zn and ceruloplasmin concentration was not different for horses consuming the two mineral supplement forms. White blood cell counts and superoxide dismutase activity were not affected by diet treatment. Formulation error and suspected sample contamination made it difficult to compare absorption and retention of Cu and Zn, but the use of a controlled repletion-depletion diet sequence appeared to be an effective experimental design component.

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First, I would like to begin by thanking my primary committee chair, Dr. Gary Potter, for his guidance over the past six years. I feel privileged to have had the opportunity to work with him in my graduate career, and especially appreciated his continued assistance over the past year. His wealth of knowledge and expertise is the result of a lifelong commitment to learning, and serves as an inspiration to those just starting in academe. His daily presence in the office has been greatly missed, but reminds all of us that the important things in life are not found in a research laboratory. I hope "retirement" continues to bring him exciting travels and many summer baseball games.

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Elena Eller has been much more than a lab technician and project manager over the past six years. I am thankful for her experience and oversight in carrying out a variety of research tasks, as well as her patience and sense of humor in dealing with my various "research moments" over the years.

I must also thank the efforts of a variety of student workers, 485s and graduate students that made my project possible. Somehow they managed to politely humor my "great attention to detail" and respected the measures necessary for a successful trace mineral project. I would be remiss if I did not specifically thank Trinette Ross for all of her help and mutual support in the animal, laboratory and writing phases of this research project.

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Finally, and most importantly, I extend my appreciation to my family and friends, both in Wisconsin and scattered across the country. School and work continue to keep us far apart, but I have appreciated their encouragement over the years in fulfilling my goals. To my parents for supporting the horse habit that lead to an unlikely career choice, your love and support have made this process a little easier. I am thankful for my personal guardian angels, watching out for me while I'm far from home. And, of course, to all of my teachers over the years, formal and informal, who fostered the desire to learn more and question why.

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CHAPTER I

INTRODUCTION

Copper and zinc play important roles in the growth and maintenance of tissues in horses, including bone and hoof wall. They have also been associated with mechanisms for managing oxidative stress, particularly the cytosolic enzyme superoxide dismutase (SOD). These trace minerals are manufactured for feed supplementation in several forms, but are most commonly added to diets in sulfate or organic-chelate forms. Research has examined the relative bioavailability of these minerals in many livestock species, as evidenced by growth, tissue mineral concentration and immune function. Several researchers have noted significant differences in the bioavailabilities of minerals provided in sulfate and organic-chelate forms, while others have noted comparable performance. Research in horses has not been as extensive as in other species, leaving much to be learned about trace mineral absorption and retention.

Previous research from this laboratory (Wagner et al., 2005) compared the absorption and retention of copper and zinc in mature, idle horses, concluding that there were no differences in absorption among sulfate, oxide and organic-chelate forms of supplementation for horses in this metabolic state. It was postulated that horses in a state of increased mineral demand, such as intense exercise, may be a more ideal model for comparing forms of trace mineral supplementation. Research in this area has the

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potential to answer several industry issues related to feed formulation. Large scale producers and individual horse owners seek a mineral supplement that will provide an economically efficient source of trace minerals to meet requirements. A better understanding of the amount of a given mineral absorbed by the horse will result in more accurate ration formulation, and a reduced potential for environmental pollution by over-supplementation of minerals.

The objectives of this dissertation research are two-fold: first, to compare absorption and retention of copper and zinc supplemented in either the sulfate or organic-chelate forms in a marginally-deficient diet; second, to document physiologic responses of plasma mineral concentrations, ceruloplasmin activity and erythrocyte SOD activity as horses are subjected to the experimental procedures described here-in.

CHAPTER II

REVIEW OF LITERATURE

Elemental copper (Cu) and zinc (Zn) are commonly added to formulated horse diets to correct or prevent deficiencies and a variety of related disorders. The NRC (1989) established minimum requirements for these minerals of 10 ppm Cu and 40 ppm Zn for all classes of horses. However, many processed feeds contain more than the NRC recommendations for Cu and Zn to compensate for mineral deficient forages, to ensure optimal absorption and utilization, or both. Additionally, it is frequently observed that individual owners oversupplement their horses with these trace minerals. Copper and Zn supplementation is often misunderstood, and sometimes unknowingly abused, in the feeding of horses. Not only is this economically wasteful, but environmentally challenging, as excess mineral is excreted back into the environment.

Copper received most of its attention in equine research due to its role in bone tissue formation and strength. Copper is required for lysyl oxidase function, a step in the formation of cross-links in collagen and elastin (Strause et al., 1986). As a result, lysyl oxidase is an enzyme of interest in bone development and formation. This explains why Cu research has been associated with developmental orthopedic diseases (DOD), particularly osteochondritis dissecans (Cymbaluk and Smart, 1993). Low Cu intake has been implicated in the incidence of DOD (Bridges and Harris, 1988; Knight et al., 1985), though it should be noted that copper deficiency is only part of a complex etiology (Cymbaluk and Smart, 1993). Beyond bone tissue integrity, Cu is involved in several

other prominent physiological processes. Cytochrome c oxidase and Cu,Zn-superoxide dismutase (SOD) both require Cu for proper metabolic function (Baker and Ammerman, 1995a). Copper deficiency has also been associated with impaired neutrophil activity, tissue mitochondrial degeneration and decreased heme synthesis (Mills, 1987).

Zinc is also associated with bone formation in horses, though some of the literature has addressed the antagonistic effect high Zn intake has on Cu absorption and utilization in this respect (Bridges and Moffitt, 1990). In addition to bone, epithelial integrity including hoof growth and quality is a focus of research in horses (Ott and Johnson, 2001). Zinc serves in a wide range of capacities in the body ranging from enzyme cofactor to protein structural component. Zinc works in concert with Cu in the activity of cytosolic SOD and also as part of the zinc-finger proteins associated with DNA translation and cell replication.

The ability of these minerals to perform their functions in the body is dependent upon their inclusion in the diet at appropriate concentrations and their subsequent absorption. Most equine diets need supplementation of Cu and Zn in order to meet NRC requirements. Mineral supplements can be divided into two groups: inorganic and organic-chelates. Inorganic minerals consist of the mineral of interest bound in an ionic salt molecule, most commonly paired with a sulfate or oxide group. Organic-chelates are minerals covalently bound to specific amino acid structures or non-descript proteinate complexes.

The proposed absorption mechanisms for these two groups reflect their structural differences (Ashmead et al., 1985). The inorganic salts ionize in the low pH of the

stomach and intestine, allowing the metal cations to covalently bind with integral membrane proteins of the intestinal mucosal cells. The mineral is absorbed against a concentration gradient, relying on changes in fluid pH and competition between various carrier proteins to assist the cation in its journey from the intestinal lumen to the exterior side of the basement membrane. Organic-chelate minerals differ in that the covalently bound structure formed by the mineral ion and the amino acid(s) is thought to be resistant to the acidic stomach environment, and is able to be absorbed intact in the small intestine. Once the molecule enters the cell via amino acid or small peptide absorption mechanisms, the mineral is believed to be released from its ring structure by a shift in pH at the basement membrane of the mucosal structure. Because the absorption of the organic-chelate mineral complex requires fewer steps and a less critical intestinal and cellular environment for its uptake than the naked mineral cations, it is believed that the organic-chelate is more available for absorption and incorporation into body tissues.

Other theories regarding absorption of metal chelates have also been proposed. Binding affinity between the metal and organic constituents determine the characteristics of the chelate, including its ability to remain bound in the gastrointestinal environment (Kratzer and Vohra, 1988). This can be extended to include the interaction of inorganic metal salts with various amino acids, proteins and digestive juices during the process of digestion, some of which may have such a strong binding affinity that the mineral becomes unavailable for absorption later. In a review of literature on organic mineral sources, Greene et al. (1995) noted the possibility that the metal chelate may exchange the metal with other dietary constituents prior to absorption, thereby influencing the

absorption of several nutrients. The authors also suggested metals already complexed in an organic-chelate form when entering the digestive tract may be more soluble and more likely to be absorbed. In this respect, minerals originating in feedstuffs and already bound to organic compounds may be more available than those supplemented in the inorganic salt forms. The specific mechanisms by which chelated minerals influence the absorption of their own metals and as well as other dietary constituents are still not understood, and would be an area for additional research.

Generally the normal absorptive mechanism of trace minerals depends on the metal's ability to chelate or bind with proteins, peptides and amino acids in the lumen of the intestine (Menard and Cousins, 1983). Copper is absorbed by several mechanisms in mammalian cells. Harris (2001) reviewed several identified copper uptake mechanisms, including the copper transport (Crt-1) protein as well as the natural resistance-associated microphage protein (Nramp2) system. Nramp2 is involved in the transport of a number of divalent cations including Cu^{2+} and Zn^{2+}, and has also been referred to as divalent metal transporter (DMT-1) or divalent cation transporter (DCT-1) for this reason. In the case of Zn, the ZIP family and DCT-1 transporters appear to be the best candidates for transporting the Zn^{2+} into the cell, though the exact metabolic pathway is still unknown (Harris, 2002).

In vivo experimentation in various species of animals has yielded mixed results depending on the amount of mineral supplemented, the species model, and the actual forms of mineral supplement. Baker et al. (1991) observed similar bioavailability comparisons with CuO, $CuSO_4$ and Cu-lysine when examining Cu concentrations in

chick livers. Rats supplemented Cu as Cu-proteinate or Cu-lysine had higher concentrations of Cu in the liver, spleen and heart compared to those supplemented with $CuSO_4$ (Du et al., 1996). Coffey et al. (1994) also noted mixed results from a series of experiments studying $CuSO_4$ and Cu-lysine fed to growing swine at different supplementation rates, but concluded that Cu as Cu-lysine was as effective as, and in some cases more than, $CuSO_4$ in enhancing feed and growth performance. Schell and Kornegay (1996) observed higher serum Zn concentrations with $ZnSO_4$ than ZnO, with intermediate values for Zn-methionine and Zn-lysine in weanling pig diets. Sheep supplemented with Zn-lysine had a greater accumulation of Zn in the liver, kidney and pancreas than Zn-methionine, $ZnSO_4$ and ZnO, while Zn-methionine was not significantly different from $ZnSO_4$ when comparing all four treatments (Rojas et al., 1995).

In general, the oxide supplement form is less available or as available as mineral supplemented as the sulfate (Baker and Ammerman, 1995a, b). Studies comparing organic-chelates and sulfates are less conclusive, due in part to the wide variability in organic-chelate forms. This can be attributed to differences in binding affinity with respect to the chelating compound. Metal amino acid complexes, such as Cu-lysine, consist of a specific mineral bound to a specific amino acid whereas mineral bound to a proteinate complex could be bound to a variety of constituent amino acids with various binding affinities (Greene et al., 1995).

Research illustrating differences in trace mineral supplementation in horses has not been as thorough as that seen in other species, but does reflect the inconsistent

results observed elsewhere. Siciliano et al. (2001a) supplemented one group of mares to meet NRC requirements with $CuSO_4$, MnO and $ZnSO_4$, and a second group received one half of the supplement in the same inorganic forms and the other half from Cu-lysine, Mn-methionine and Zn-methionine. No difference in liver mineral concentration or immune response was noted between the two groups. Ott and Asquith (1994) noted no differences in mare reproductive performance between broodmares supplemented with inorganic or a combination of inorganic and proteinated trace minerals, no differences in foal growth measurements and no differences in foal estimated bone mineral content. An increase in foal serum Zn and Cu was observed in the organic and inorganic supplementation group, though the response was thought to result from foals consuming the mares' concentrate. Ott and Johnson (2001) reported that yearlings supplemented with a proteinate trace mineral mix including Cu, Mn and Zn had greater hoof growth than yearlings supplemented with an inorganic supplement, but saw no differences in hoof strength or skeletal growth characteristics. Siciliano et al. (2001b) did not observe any significant differences in hoof wall growth rates, hardness, tensile strength or trace mineral content between mature horses fed an inorganic Cu, Mn and Zn supplement and those fed a supplement consisting of half inorganic and half organic sources. In exercised yearling geldings, there was no difference in radiographic bone aluminum equivalence measurements or carboxyterminal propeptide of type I procollagen concentrations in horses receiving an unsupplemented diet, an inorganic Cu and Zn supplemented diet or supplemented with an inorganic-organic blend (Baker et al., 2003).

Mineral balance studies in horses have also yielded mixed results. In work with yearling geldings, Miller et al. (2003) fed an unsupplemented concentrate or a concentrate containing approximately 50 ppm Cu and 200 ppm Zn as $CuSO_4$ and ZnO or with 45% of the supplemental mineral replaced by an organic source. Copper digestibility was greater in horses fed the organic supplement, as was mean apparent Cu balance and Cu balance as a percent of intake. Apparent Zn balance was also greater for yearlings consuming the organic supplement, though this concentrate contained 216.6 ppm Zn compared to 189.9 ppm in the inorganic supplemented concentrate. Baker et al. (2005) used mature horses in a study of similar diet treatment design, with concentrates containing 66.6 ppm Cu and 373.7 ppm Zn in the inorganic supplemented treatment and 53 ppm Cu and 305 ppm Zn in the inorganic-organic blend treatment. Apparent Cu digestibility and balance was greater for horses consuming the inorganic mineral source, as was apparent Zn digestibility and balance. Wagner et al. (2005) used adult Miniature Horses to compare absorption and retention of Cu and Zn when supplemented in the sulfate, oxide or organic-chelate forms. Horses consuming 13.5 ppm Cu and 56.2 ppm Zn had no difference in absorption or retention, both apparent and as a percent of intake. It should be noted that this project used a 10 d diet adaptation prior to total collection compared to longer adaptation periods in other studies.

Copper and zinc balance and status can be difficult to measure in horses. When fed in excess of body needs, both minerals are excreted through the bile in an unavailable form in the feces (Chesters, 1997; Harris, 1997). Work with bile-duct cannulated ponies given intravenous doses of ^{64}Cu also demonstrated the excretion of

excess Cu through the bile, where 70.7% of the radioactive dose was recovered from the bile compared to 3.6% from the feces after 5 d of collection (Cymbaluk et al., 1981). Hoyt et al. (1995) fed Miniature Horses 76.6, 168.1, 389.5 and 606.8 mg Zn/100 kg BW, and reported urinary Zn excretion of 0.8 to 1.2 mg/100 kg BW across all treatments.

Many researchers have attempted to define normal plasma and serum concentrations of Cu and Zn in an effort to establish a diagnostic reference for identifying deficiency. Researchers in the United Kingdom reported mean serum concentrations of 0.79 ± 16 μg/ml Cu and 1.70 ± 54 μg/ml Zn for stabled horses, and 1.01 ± 26 μg/ml Cu and 1.11 ± 45 μg/ml Zn for pastured horses (Stubley et al., 1983). In an Australian study with apparently normal horses, stabled Thoroughbreds had plasma concentrations of 0.76 ± 0.18 μg/ml Cu and 0.56 ± 0.14 μg/ml Zn (Auer et al., 1988). These values were lower than those reported by Hintz (1982), where the "normal" range for North American horses was defined as 0.80 μg/ml to 1.20 μg/ml for Cu and 0.55 μg/ml to 0.80 μg/ml for Zn. Cymbaluk et al. (1986) documented plasma Cu and Zn values of 22.5-28.3 μmol/L and 11.7-13.5 μmol/L, respectively, in yearlings on various feeding trials. Plasma Cu concentrations reflected liver Cu content, but were not directly related to dietary Cu intake. Liver Zn concentrations were not different across a range of dietary Zn intakes, but there was no correlation between liver Zn content and plasma Zn values. Ralston (1992) reported serum concentrations of 1.03-1.66 ppm Cu and 0.60-0.79 ppm Zn in horses with varying trace mineral intakes, but noted that Cu and Zn content of feed alone did not explain the variation observed.

Testing for the concentration or activity of Cu or Zn specific blood-borne proteins may be the least invasive technique for examining bioavailability. These indicators are examined in conjunction with plasma or serum mineral concentrations. Two such proteins are ceruloplasmin and Cu,Zn-superoxide dismutase. Both proteins have been implicated in antioxidant activity and inflammatory responses.

Seventy-three percent of plasma copper is found in the ceruloplasmin protein (Auer et al., 1988). In that respect, ceruloplasmin has been used as an evaluative measure in a variety of animals, including horses (Bingley and Dick, 1969; Milne et al., 1991). Ceruloplasmin is important not only as the main Cu carrier in the blood, but also for its role in a variety of oxidative reactions in the body. Ceruloplasmin is responsible for the oxidation of ferrous iron to the ferric state, making it readily available for binding the transferrin transport protein (Cranfield et al., 1979). The anti-inflammatory and anti-oxidant activities of ceruloplasmin have been reviewed (Auer, 1989). Ceruloplasmin has also been classified as an acute phase protein that increases in concentration in the intermediary or later phases of acute inflammation (Okumura et al., 1991).

Unlike plasma or serum Cu concentrations, ceruloplasmin concentrations in horses are not as well documented. In a sampling of 83 stabled Thoroughbreds, the mean plasma ceruloplasmin Cu concentration was 0.56 ± 0.14 μg/ml (Auer et al., 1988). Bingley and Dick (1969) reported 23.3 mg/100ml ceruloplasmin and 0.93 μg/ml total plasma Cu in a donkey. Ceruloplasmin oxidase activity in a group of Thoroughbreds was 23.7 U/ml (SD ± 6.0 U/ml), and had a linear relationship with the serum Cu concentration (Smith et al., 1983). It should be noted that assays, assay conditions and

expression of measurement are not consistent among research presented in this literature review, and therefore a clear comparison of ceruloplasmin values may be difficult to establish.

Like ceruloplasmin, superoxide dismutase (SOD) has been studied in conjunction with plasma trace mineral concentrations. There are two forms of SOD, a cytosolic isoform containing Cu and Zn (Cu,Zn-SOD), and a mitochondria-predominant isoform with manganese (Mn-SOD) (Minatel and Carfagnini, 2000). Erythrocyte Cu,Zn-SOD lends itself well to assessment of SOD activity as it pertains to Cu and Zn status, and is the isoform examined in this review of literature. Superoxide dismutase is considered a preventative anti-oxidant as it functions by catalyzing the conversion of the superoxide free radical ($O_2\cdot$) into hydrogen peroxide (H_2O_2), which is then further reduced by catalase or glutathione peroxidase (Auer, 1989).

It is thought that increased oxygen consumption during exercise in horses creates oxidative stress, including the formation of free radicals and subsequent peroxidation of membranes (Balogh et al., 2001). Balogh et al. (2001) observed no change in SOD activity in horses competing in a pentathlon contest even though there were elevated plasma lipid peroxide levels 24 h after exercise. Ji et al. (2001) reported no significant changes in SOD activity in response to acute exercise when measured at rest and at 2 and 30 minutes following 12 min exercise. The authors concluded there was sufficient antioxidant capacity to handle the exercise-induced oxidative stress. Greater SOD activity has been observed in horses fed Zn and Cu deficient diets, possibly as compensation for decreased levels of Cu, Zn and ceruloplasmin (Górecka et al., 1999).

Copper and zinc absorption and subsequent incorporation into SOD in exercising horses may have important implications for performance horses.

Also of concern in performance horses is the ability to summon an immune response while subjected to a stressful work and travel environment. Immune function can be classified as one of two forms: innate and acquired. Innate, or native, immunity is composed of mechanisms in place prior to an infection, ready to respond in a similar manner to repeated infections. This system relies on a number of components, including the phagocytic neutrophils and macrophages, to attack microbes as soon as they are presented. Acquired, or adaptive, immunity develops as a response to infection, adapting to individual circumstances. It may take several days for an adaptive response to be mounted, but the response is specific to the microbe presented. Responsive cells include the antibody producing B-lymphocytes of humoral immunity and the T-lymphocytes associated with cell-mediated immunity (Abbas and Lichtman, 2005).

Copper is involved in many aspects of immune function, and immunity can be impaired even when other indicators of Cu status appear normal (Percival, 1998). Copper deficiency has been implicated in changes in innate immune responses in cattle, including neutrophil function (Minatel and Carfagnini, 2000). However, Arthington et al. (1995) subjected heifers to copper depletion and subsequent repletion with $CuSO_4$ or Cu-proteinate and noted no change in neutrophil bactericidal function or lymphocyte blastogenesis using in vitro assays. Steers fed organic or inorganic sources of Cu at or above recommended concentrations had no difference in bovine rhinotracheitis antibody titers (Rhoads et al., 2003). Copper supplementation as either $CuSO_4$ or Cu-Lys had no

effect on antibody titers, cell-mediated immune response or in vitro lymphocyte blastogenesis in feedlot steers (Ward et al., 1993).

Like Cu, Zn also has strong ties to immune function. A review by Shankar and Prasad (1998) noted that Zn is crucial for both development and function of neutrophils as well as the development of acquired immunity. Livestock studies comparing supplementation forms have had difficulty in defining a superior supplement form using this study parameter. In vitro lymphocyte activity was not different in cattle supplemented Zn in the oxide or organic forms (Kincaid et al., 1997; Spears and Kegley, 2002). Kegley et al. (2001) compared immune function in calves fed an unsupplemented diet as well as those with supplemental Zn from sulfate or amino acid-complex sources. There was no difference in total white blood cell count among the three treatment groups, though animal fed the amino acid-complex supplement had higher antibody titers for bovine syncytial virus.

In studies where both Cu and Zn are supplemented, analyses of immune response also had mixed results. Organically supplemented heifers had greater total Ig concentrations than those receiving a sulfate-based mineral supplement 21 d following an immune challenge with porcine red blood cells, even though plasma Cu and Zn concentrations were not different (Ahola et al., 2005a). Calves from these heifers were used in a subsequent study, and were maintained on the same supplement form as their dams through finishing (Ahola et al., 2005b). There was no difference in cell-mediated immune response to intradermal injection of phytohemagglutinin among animal

receiving no supplementation or those fed inorganic or organic supplemented diets, nor was there a difference in IgG, IgM and total Ig among treatments.

There is a limited amount of work examining trace mineral balance and immune function in horses. In a companion study to work previously reviewed herein (Siciliano et al., 2001b), horses were inoculated with porcine red blood cells to elicit a primary humoral immune response (Siciliano et al., 2003). Mean total antibody titer was greater 7- and 14-d post-innoculation for horses consuming an organic trace mineral supplement than those on the inorganic supplement. Mean IgM titer was also greater for organically supplemented horses though there was no difference in IgG titer. Finding an appropriate test for immune function can be a challenge in equine studies due to limited animal numbers per treatment and length of time for feeding periods or recovery from immune challenges. Because T-cell function is Zn dependent, a hematological analysis including differential white cell count and lymphocyte count is the starting point for examining differences in immune function (Tizzard, 2005).

Copper and Zn balance is just one way to examine the availability of these minerals from various supplement forms when fed to horses. Equine studies rarely combine this information with other indicators of Cu and Zn status, such as plasma mineral profiles and function of Cu- and Zn-dependent enzymes or systems.

CHAPTER III

MATERIALS AND METHODS

Eight mature mares and one gelding of various breeds (two Arabians, two Paints and five Quarter Horses) were used in a modified switchback experimental design. Horses were maintained at the Texas A&M University Horse Center, following procedures in the approved Animal Use Protocol. Prior to and during the experiment, horses were dewormed and vaccinated in accordance with facility procedures. Horses were blocked by age and breed and randomly assigned to one of two groups for the switchback periods.

The experiment was conducted in seven 28-d periods as outlined in Table 1. Periods 1 and 2 served as exercise conditioning periods, where horses were acclimated to the mechanical walker and treadmill as well as adapted to the exercise demands of the subsequent periods. The concentration of dietary Cu and Zn changed each period, such that the horses were subject to a standardized repletion/depletion cycle prior to receiving the appropriate experimental diet.

Table 1. Exercise and Diet Treatments by Period

Period	Exercise	Diet
1	Conditioning	Basal diet (No Cu and Zn Supplementation)
2	Conditioning	Repletion Diet (Cu and Zn at 100% of NRC)
3	Standard Workload	Basal diet
4	Standard Workload	Experimental Diet (Cu and Zn at 90% of NRC)
5	Standard Workload	Repletion Diet
6	Standard Workload	Basal diet
7	Standard Workload	Experimental Diet

Diet Formulation

Horses were offered a 50:50 concentrate:hay diet at 1.5-2% of BW divided between two feedings daily. During the first three periods, the amount of feed offered was adjusted such that the horses maintained a body condition suitable for their workload, and this amount was held constant for the remainder of the project. The diet was formulated to provide DE at 175% of maintenance, with CP, Ca and P balanced to appropriate DE:nutrient ratios (NRC, 1989). Other nutrients were supplied to meet or exceed NRC (1989) requirements. Diet Cu and Zn concentrations varied by period, such that the horses received no supplemental Cu and Zn (basal/depletion diet), 100% of the NRC (repletion diet), or the experimental diet of Cu and Zn supplemented to meet 90%

Table 2. Background Ration Formulations

Basal (Depletion) Diet

		Provided on a Dry Matter Basis					
Ingredient	% of Diet As Fed	DE (Mcal)	% CP	Ca %	P %	Cu (ppm)	Zn (ppm)
Coastal Bermudagrass	50.0000%	1.05	5.90	0.22	0.12	3.00	14.00
Corn	27.1000%	1.04	2.82	0.01	0.08	1.14	5.96
Oats	8.0000%	0.26	1.06	0.01	0.03	0.54	3.12
Wheat Mids	5.0000%	0.17	0.93	0.01	0.05	0.90	5.45
Soybean Meal	5.0000%	0.18	2.50	0.02	0.04	1.12	0.65
Vegetable Fat	4.0000%	0.36	0.00	0.00	0.00	0.00	0.00
Calcium Carbonate	0.4000%			0.16			
Salt	0.5000%						
Total		3.05	13.20	0.42	0.32	6.69	29.18

Repletion Diet

		Provided on a Dry Matter Basis					
Ingredient	% of Diet As Fed	DE (Mcal)	% CP	Ca %	P %	Cu (ppm)	Zn (ppm)
Coastal Bermudagrass	50.0000%	1.05	5.90	0.22	0.12	3.00	14.00
Corn	27.0966%	1.04	2.82	0.01	0.08	1.14	5.96
Oats	8.0000%	0.26	1.06	0.01	0.03	0.54	3.12
Wheat Mids	5.0000%	0.17	0.93	0.01	0.05	0.90	5.45
Soybean Meal	5.0000%	0.18	2.50	0.02	0.04	1.12	0.65
Vegetable Fat	4.0000%	0.36	0.00	0.00	0.00	0.00	0.00
Calcium Carbonate	0.4000%			0.16			
Copper Sulfate	0.0012%					3.05	
Zinc Sulfate	0.0022%						8.00
Salt	0.5000%						
Total		3.05	13.20	0.42	0.32	9.74	37.18

of the NRC recommendations (Tables 2 and 3)**.** In this manner, horses were subject to controlled repletion and depletion of body mineral stores prior to feeding of a marginally deficient Cu and Zn diet, so that a systemic demand for Cu and Zn existed during the feeding of the experimental diet. Mineral supplementation in the repletion phases was provided by $CuSO_4$ and $ZnSO_4$, whereas supplementation in the experimental phases

Table 3. Experimental Ration Formulations

Sulfate Supplemented Diet

		Provided on a Dry Matter Basis					
Ingredient	% of Diet As Fed	DE (Mcal)	% CP	Ca %	P %	Cu (ppm)	Zn (ppm)
Coastal Bermudagrass	50.0000%	1.05	5.90	0.22	0.12	3.00	14.00
Corn	27.0966%	1.04	2.82	0.01	0.08	1.14	5.96
Oats	8.0000%	0.26	1.06	0.01	0.03	0.54	3.12
Wheat Mids	5.0000%	0.17	0.93	0.01	0.05	0.90	5.45
Soybean Meal	5.0000%	0.18	2.50	0.02	0.04	1.12	0.65
Vegetable Fat	4.0000%	0.36	0.00	0.00	0.00	0.00	0.00
Calcium Carbonate	0.4000%			0.16			
Copper Sulfate	0.0008%					2.04	
Zinc Sulfate	0.0012%						4.36
Salt	0.5000%						
Total		3.05	13.20	0.42	0.32	8.73	33.54

Organic-Chelate Supplemented Diet

		Provided on a Dry Matter Basis					
Ingredient	% of Diet As Fed	DE (Mcal)	% CP	Ca %	P %	Cu (ppm)	Zn (ppm)
Coastal Bermudagrass	50.0000%	1.05	5.90	0.22	0.12	3.00	14.00
Corn	27.0850%	1.04	2.82	0.01	0.08	1.14	5.96
Oats	8.0000%	0.26	1.06	0.01	0.03	0.54	3.12
Wheat Mids	5.0000%	0.17	0.93	0.01	0.05	0.90	5.45
Soybean Meal	5.0000%	0.18	2.50	0.02	0.04	1.12	0.65
Vegetable Fat	4.0000%	0.36	0.00	0.00	0.00	0.00	0.00
Calcium Carbonate	0.4000%			0.16			
Copper-Lysine	0.0040%					2.00	
Zinc-Methionine	0.0110%						4.40
Salt	0.5000%						
Total		3.05	13.20	0.42	0.32	8.69	33.58

was provided either by the sulfate forms or organic-chelate sources in the form of Cu-lysine and Zn-methionine (Zinpro Corp., Eden Prairie, MN). All concentrates were manufactured by Martindale Feed Mill, Valley View, TX.

Daily hay and concentrate amounts were divided into two separate feedings and offered at 600 and 1800 h. Horses were fed individually in concrete-floored stalls from

non-metal containers. Refusals, if any, were weighed and recorded. When not being fed, horses were group-housed in dry-lot pens with ad libitum access to tap water. Pens were cleaned twice daily to prevent coprophagy.

Exercise

During the standard workload phases, horses were exercised on a mechanical walker to create a DE demand of 175% of maintenance. Experimental workload was determined using the formula DE (Mcal/kg) = $5.97 + 0.021W + 5.03X – 0.48X^2$, where W is the body weight (kg) and X = (wt of horse, rider and tack) x km x 10^{-3} (Anderson et al., 1983). The horses were conditioned to perform an average daily workload of 3.93 kg*km*10^{-3} with a DE demand of 28.8 Mcal. Horses were exercised on the mechanical walker five days a week, with a workout consisting of 3 min warm-up at the walk, 10 min at 200 m/min, three 7 min bouts of cantering at 333 m/min, 10 min trotting and a 3 min cool down at the walk, with 3 min walk breaks between trotting and cantering bouts, for a total of 10.99 km of non-walking exercise.

Standard Exercise Test

On d 24 of periods 4 and 7, horses completed a standardized exercise test (SET). Prior to the SET, horses were fitted with jugular catheters and heart rate monitors (EquiPulse, San Jose, CA) to facilitate data and sample collection. The SET was performed on the treadmill at a 1.9% slope or on the mechanical walker when mechanical failure prevented use of the treadmill. The SET was designed to mimic the

typical exercise session. It consisted of 3 min warm-up at the walk, 10 min trot at 200 m/min, 3 min walking, 7 min cantering at 360 m/min, 3 min walking, 7 min cantering at 333 m/min, 3 min walking, 7 min cantering at 333 m/min, 3 min walking, 10 min trotting at 200 m/min and a 5 min hand-walking cool down. Horses traveled 11.18 km during non-walking exercise, for an approximate workload of 5.59 kg*km*10^{-3}. Heart rates were monitored to ensure the workload was within normal parameters. Rates were recorded prior to the start of the test, following the warm-up walk, and upon completion of the trotting and cantering bouts. Heart rates were also recorded at 1, 2 and 5 min post-exercise, at which point all horses had returned to pre-exercise, resting heart rates.

Mineral Intake and Balance

Random samples of hay and concentrate were obtained throughout the experiment for determination of Cu and Zn intake during the repletion and depletion diet periods.

A 4-d total collection for determination of Cu and Zn balance was conducted d 25 through d 28 of periods 4 and 7. Horses were confined to individual tie stalls, and stood on rubber mats so as to lessen physical stress to the legs from the concrete flooring. Horses were fitted with a urine collection harness to allow for separate and total collection of feces and urine. Feed and water was offered from non-metal containers. Frequent inspections of horses during collection reduced the risk of sample contamination, and included thorough sweeping of floor surfaces to remove dirt and

hair. In order to alleviate the effects of confinement, horses were walked and lightly trotted for 30-45 min on a mechanical walker each day of the total collection.

Concentrate and hay samples were obtained daily at feeding time during the total collection and stored in plastic bags for later analyses. Water intake was measured and recorded. One water sample per collection period was collected from the same source as the horses' drinking water. The samples were stored in Nagalene tubes and frozen at -20ºC until further analyses. Daily concentrate and hay samples were collected and stored in plastic bags.

Immediately upon defecation, fecal samples were placed in plastic buckets. Every 3 h, feces for each horse were weighed and recorded, with a 10% aliquot added to a daily collection bag. Contaminated fecal matter was weighed separately and weight recorded, but not added to the daily sample. Workers changed latex gloves between horses, and cleaned plastic weighing equipment between samples to prevent cross-contamination. Daily fecal samples were stored in plastic freezer-quality bags and frozen at -20ºC for later analyses.

Urine was collected after every void to prevent contamination. Urine volume was measured in a plastic graduated cylinder and recorded, with a 10% aliquot added to a daily collection bottle. Any contaminated urine was measured and volume recorded, but not added to the daily composite. Urine was initially refrigerated in mineral-free collection bottles. At the end of each collection period, daily urine samples were strained through three layers of cheesecloth and individually frozen at -20ºC in Nagalene tubes until further analyses. Collection and measuring equipment was rinsed with

distilled water between uses, and thoroughly cleaned and rinsed between collection periods.

In the laboratory, feed and fecal samples were dried at 65ºC for a minimum of 72 h and ground in a Wiley mill with a 2mm screen. Dried samples were stored in sealed, mineral-free plastic containers. Composites of hay, concentrate and fecal samples were generated from individual daily samples to provide one sample per horse per treatment.

Copper and zinc concentration of feed, fecal, urine and water samples was determined by the Soil, Water and Forage Testing Laboratory at Texas A&M University, College Station, TX. Prior to shipment to the lab, submitted samples were placed in mineral-free Nagalene tubes, and assigned a sample ID number so as to blind laboratory technicians to sample identity. Corn stalk standard samples were similarly processed, labeled, and inserted randomly among the experiment samples to serve as control samples.

Blood Sampling and Analyses

Blood samples were obtained prior to the morning feeding at various points during the research project. Blood samples were drawn by venipuncture using heparinized tubes.

Plasma for determination of Cu and Zn concentration was harvested from samples obtained on the first day of each period and at the completion of the experiment.

Plasma was frozen at -20ºC and sent to the Texas Veterinary Medical Diagnostic Laboratory (TVMDL) in College Station, TX for Cu and Zn analyses.

Ceruloplasmin concentration was determined from plasma obtained on the first day of each period and at the completion of the experiment. Because ceruloplasmin is an acute-phase protein responsive to inflammation, an additional sample was drawn the morning of d 24 of periods 4 and 7 prior to the stresses of the exercise test and total collection. Plasma was harvested and stored at -20ºC until analyzed. Ceruloplasmin concentration as indicated by oxidase activity was measured by the colorimetric method described by Bingley and Dick (1969), at pH 6.4 as reported by Milne et al. (1991). In this procedure, 1 ml of 0.1% p-phenylenediamine (PPD) solution, dissolved in 0.1 M sodium acetate buffer and pH adjusted, was added to 100 μL plasma. After incubation at 37ºC for 15 min, the oxidation reaction was stopped by the addition of 5 ml of a 0.1% sodium azide solution. Absorbance was read at a wavelength of 525 nm on an ultraviolet spectrophotometer (Pharmacia LKB Ultraspec III). A control was included in each run to correct for inherent colorimetric change of the PPD solution. Ceruloplasmin concentration was determined by the formula -1.7 + 150(corrected absorbance) = ceruloplasmin mg/100mL.

Samples for SOD activity analysis and hemoglobin concentration were drawn on the first day of periods 4 and 7 as well as at several points during the SET. For the SET, blood was drawn prior to exercise, upon completion of the final canter and trot phases of the test and at 30 and 60 min after the last trot phase. Erythrocytes were harvested, processed and frozen at -20ºC for later analyses. Erythrocyte SOD activity was analyzed

using RANSOD kits (RANDOX Laboratories Ltd., Antrium, UK). Hemoglobin concentration, used in determining SOD activity, was performed by TVMDL as part of the complete blood chemistry analysis.

Blood samples for white blood cell analyses were obtained on d 1, d 24 and upon completion of periods 4 and 7. Complete blood chemistry was performed by TVMDL, including total white blood cell count (WBC), differential white cell count and lymphocyte count.

Statistical Analyses

Resulting data were using STATA statistical software. Differences were considered significant at $P<0.05$. Mineral balance was evaluated using analysis of variance (ANOVA) procedures appropriate for the switchback design. Plasma mineral and ceruloplasmin concentrations were compared during the background and washout periods using paired t-tests when horses were subject to repeated diet treatments. Analysis of variance was used to evaluate plasma mineral and ceruloplasmin concentrations during the initial background periods as well as to compare response to the experimental diets in periods 4 and 7. Heart rate and response of SOD to exercise were analyzed by linear regression. Superoxide dismutase activity at rest was compared by ANOVA. White blood cell count data were analyzed by ANOVA, and paired t-tests were utilized in comparing means on the first day of periods 4 and 7.

CHAPTER IV

RESULTS AND DISCUSSION

One horse developed hind limb lameness during period 5 requiring several months of rest, and was removed from the project. All other horses remained sound and healthy through the course of the project.

One horse refused to consume concentrate during the final total collection. Consequently, all intake and mineral balance data for this horse during period 7 were omitted from statistical analyses. Data from this horse regarding the SET and blood sampling from this period were included in all other analyses.

Repletion and Depletion Diet Periods

Mean mineral concentrations of the diets on a dry matter basis are illustrated in Table 4. During the depletion phases, the diet contained 50-56% of NRC (1989) values for Cu and 78-83% of Zn. Zinc content of the repletion diet was slightly greater than the requirement of 40 ppm, analyzing at 102-113% of the target value. Copper content of the repletion diet varied during the course of the project. During the first repletion period, Cu concentration of the diet was similar to the requirement of 10 ppm. Copper concentration of the diet fed during the second repletion period was 7.05 ppm, or 71% of the requirement. At the onset of the project, sufficient quantities of feed were ordered such that all depletion and repletion period concentrates should have come from single,

Table 4. Mineral Concentrations of Depletion and Repletion Diets

		Dry Matter Basis	
Period	Diet	Cu (ppm)	Zn (ppm)
1	Depletion	5.05	33.35
2	Repletion	10.75	45.50
3	Depletion	5.05	33.35
5	Repletion	7.05	42.35
6	Depletion	5.60	31.00

uniform batches. However, rodent damage to feed bags necessitated re-ordering of the repletion and depletion phase concentrates during the study. Variations in mineral concentration can be attributed to inherent variation in trace mineral content in grains and forages.

Feed Intake and Mineral Consumption from Experimental Diets

Mean mineral concentration of the sulfate diet was 6.28 ppm Cu and 35.99 ppm Zn on a dry matter basis, while the chelate diet contained 23.62 ppm Cu and 90.67 ppm Zn on a dry matter basis. Mineral content of the diets was significantly different. A calculation error in the formulation of the organic-chelate diet resulted in the Cu-Lys and Zn-Met being included at ten times the rate they should have been added. Further complicating the differences between expected and observed dietary mineral concentrations was variation in the mineral content of the hay. All hay was purchased in one lot and sampled prior to the beginning of the project for the purpose of diet formulation. Pre- and post-project hay analysis was performed by the same laboratory.

The initial hay samples tested 6 ppm Cu and 28 ppm Zn on a dry matter basis, whereas grab samples taken throughout the project, including total collections, averaged 4.5 ppm Cu and 24.14 ppm Zn.

Daily dry matter consumption by horses averaged 1.52% of body weight during periods 4 and 7. Dry matter digestibility averaged 60.39% across treatment groups in these periods.

Water Intake and Mineral Consumption

Daily water intakes for each horse are noted in Appendices 1 and 2. Mineral composition of the tap water during periods 4 and 7 averaged 0.156 ppm Cu and 0.380 ppm Zn, representing 3.97% of daily Cu intake and 1.94% of daily Zn intake.

Absorption and Retention of Minerals

Balance data for each horse in periods 4 and 7 are in Appendices 1 and 2. Total daily mineral intake, including mineral consumed from both feed and water, was used to calculate balance data. The mean daily Cu and Zn absorption and retention for horses consuming the sulfate and organic-chelate experimental diets are illustrated in Tables 5 and 6.

Copper intake and fecal excretion was greater for horses consuming the organic chelate diet. While apparent absorption was not different between treatments, differences in absorption as a percent of intake reflected the disparity in Cu intake between diets. Urinary Cu excretion and apparent retention were not different.

Table 5. Apparent Daily Copper Absorption and Retention

	Treatment					
	Sulfate		Organic Chelate		Total	
	Mean	SEM	Mean	SEM	Mean	SEM
Intake, mg/100kg BW	10.53*	0.62	35.73*	1.27	24.71	3.31
Fecal Excretion, mg/100kg BW	13.15*	0.66	38.59*	1.89	27.46	3.43
Absorbed, mg/100kg BW	-2.62	0.63	-2.85	0.89	-2.75	0.55
% of intake	-26.14*	6.83	-7.71*	2.29	-15.77	3.91
Urinary Excretion, mg/100kg BW	3.43	1.02	3.46	1.50	3.45	0.92
Retained, mg/100kg BW	-6.05	1.19	-6.32	1.61	-6.20	1.01
% of intake	-57.94*	10.74	-17.21*	4.19	-35.03	7.26
% of absorbed	331.10	102.31	93.19	182.08	197.27	112.72

*Row means differ (P<0.05)

Table 6. Apparent Daily Zinc Absorption and Retention

	Treatment					
	Sulfate		Organic Chelate		Total	
	Mean	SEM	Mean	SEM	Mean	SEM
Intake, mg/100kg BW	57.72*	2.02	136.48*	5.17	102.02	10.51
Fecal Excretion, mg/100kg BW	72.14*	3.56	153.65*	5.38	117.99	10.95
Absorbed, mg/100kg BW	-14.43	4.54	-17.17	6.11	-15.97	3.86
% of intake	-26.34	9.04	-13.63	5.23	-19.19	5.02
Urinary Excretion, mg/100kg BW	3.53	0.77	3.69	0.52	3.62	0.43
Retained, mg/100kg BW	-17.96	4.81	-20.85	6.21	-19.58	3.97
% of intake	-32.48	9.67	-16.34	5.32	-23.40	5.40
% of absorbed	41.05	99.10	114.44	15.55	82.33	43.35

*Row means differ (P<0.05)

Copper retention between the diets was different when expressed as a percent of intake but not as a percent of the mineral absorbed. There was a trend (P=0.07) for a period difference and a significant effect of period by diet interaction for Cu absorbed as a

percent of intake, though no other significant differences or trends were noted for period or period by diet interactions regarding Cu balance.

Similarly, zinc intake and fecal excretion were greater for horses consuming the organic chelate supplemented diet. No other differences were found between the diets for absorption, absorption as a percent of intake, retention, retention as a percent of intake or retention as a percent of Zn absorbed. There was a significant difference by period for urinary Zn excretion, though no other period differences or trends were observed. Trends toward differences in period by diet interactions were observed for Zn absorption (P=0.06) and Zn retention (P=0.08) but not for other measures of Zn balance.

The low absorption values would normally indicate horses in a state of negative mineral balance, or deficient mineral intake. However, horses on the organic-chelate diet were consuming two to three times the NRC recommended intakes for Cu and Zn. Furthermore, the high urinary excretion of these minerals would normally indicate that horses were in a positive mineral balance.

The absorption and retention values in the present study are very different from those reported by other researchers. Ponies consuming 5.64-25.67 mg Cu/100 kg BW had fecal Cu excretion in the range of 2.88-14.18 mg Cu/100 kg BW while urinary excretion remained constant at 0.12 mg Cu/100 kg BW (Cymbaluk et al., 1981). Schryver et al. (1980) also reported low urinary excretion of Zn (0.7-1.0 mg Zn/100 kg BW) in ponies consuming 36.8-310.7 mg Zn/100 kg BW, with a subsequent fecal Zn excretion of 34.3-264.7 mg/100 kg BW. Ponies consuming an average of 11.65 mg Cu/100 kg BW while fed increasing concentrations of Zn had an average fecal excretion

of 5.9 mg/100 kg BW and urinary excretion of 0.05 mg/100 kg BW (Hoyt et al., 1995). Urinary excretion of Zn for these ponies averaged 1.0 mg/100 kg BW despite increasing intakes of Zn ranging 76.6-606.8 mg/100 kg BW.

Feed, fecal, urine and water analyses were performed by an outside laboratory, with blinded standard checks mixed in with the submitted samples. Because the check standards analyzed within accepted values, it can be assumed that the laboratory results are accurate. All samples were stored in mineral-free plastic containers and sealed to prevent outside contamination. Feed samples tested at values similar to those obtained from diet calculations. It is possible that fecal and urine samples may have been exposed to contaminants during the total collection process. However, great care was taken by all workers to minimize risk of sample contamination. As outlined in Chapter III, all collection equipment was checked and thoroughly cleaned throughout the collection phase. This included frequent sweeping to remove dust, dander and hair. If a fecal or urine sample became contaminated, it was not included the daily aliquot. Given these procedures, it seems unlikely that a single sample could be contaminated. A single contaminated sample would present itself as an outlier value during statistical analysis of the balance data. However, all fecal and urine samples, and subsequent calculations of daily mineral excretion, resulted in consistently higher-than-expected values. These results suggest either widespread sample contamination or some other event that caused the horses to excrete more mineral than was consumed.

Work by Schryver et al. (1980) and Cymbaluk et al. (1981) used balance studies in conjunction with isotope work to determine the sources of endogenous mineral losses

of Zn and Cu, respectively. Cymbaluk et al. (1981) constructed a regression analysis of Cu intake (x) and retention (y) generating the equation y = 0.66x – 3.47 (r=0.882). The predicted obligatory Cu loss in mature ponies was 3.47 mg Cu/100 kg BW/d. This prediction value was similar to the estimated obligatory Cu loss generated in work with bile duct-cannulated ponies in the same study. Schryver et al. (1980) reported recovery of 83-88% of an intravenous dose of ^{65}Zn in the feces of horses over a 14-d period. A review of the literature did not reveal documentation of obligatory Zn loss in horses. Hoyt et al. (1995) fed ponies varying concentrations of Zn while holding Cu intake constant. Using the approach of Cymbaluk et al. (1981) on the mean balance data reported by Hoyt et al. (1995), a basic regression of Zn retention (y) on intake (x) generated the equation y = 0.42x – 10.47 (r=.0.99), predicting an obligatory Zn loss of 10.47 mg Zn/100 kg BW/d. However, caution should be taken with this extrapolation as there is no validation of obligatory Zn loss through isotope work as was performed for Cu by Cymbaluk et al. (1981).

Using the estimated obligatory losses of 3.47 mg Cu/100 kg BW and 10.47 mg Zn/100 kg BW/d, true absorption and retention in the present study were calculated (Tables 7 and 8, Appendices 3 and 4). Adjusted fecal excretion of both Cu and Zn was different between the diets. There was no difference in true absorption of either Cu or Zn by diet. Copper absorbed as a percent of intake tended to be different by period (P=0.05) and had a diet by period interaction. There was a trend for Cu retention as a percent of intake to be different by diet (P=0.09). There were also trends for diet by

period interactions for true Zn absorption (P=0.06) and Zn retention (P=0.08). No other significant differences or trends were noted.

Table 7. True Daily Copper Absorption and Retention

	Treatment					
	Sulfate		Organic Chelate		Total	
	Mean	SEM	Mean	SEM	Mean	SEM
Intake, mg/100kg BW	10.53*	0.62	35.73*	1.27	24.71	3.31
Adjusted Fecal Excretion, mg/100kg BW	9.68*	0.66	35.12*	1.89	23.99	3.43
True Absorption, mg/100kg BW	0.85	0.63	0.62	0.89	0.72	0.55
% of intake	7.46	6.10	2.11	2.46	4.45	2.97
Urinary Excretion, mg/100kg BW	3.43	1.02	3.46	1.50	3.45	0.92
Retained, mg/100kg BW	-2.58	1.19	-2.85	1.61	-2.73	1.01
% of intake	-24.34	10.77	-7.39	4.36	-14.80	5.54
% of absorbed	-1960.38	1336.62	-1156.03	1156.38	-1507.93	851.13

*Row means differ (P<0.05)

Table 8. True Daily Zinc Absorption and Retention

	Treatment					
	Sulfate		Organic Chelate		Total	
	Mean	SEM	Mean	SEM	Mean	SEM
Intake, mg/100kg BW	57.72*	2.02	136.48*	5.17	102.02	10.51
AdjustedFecal Excretion, mg/100kg BW	61.67*	3.56	143.18*	5.38	107.52	10.95
True Absorption, mg/100kg BW	-3.96	4.54	-6.69	6.11	-5.50	3.86
% of intake	-8.06	8.57	-5.87	5.01	-6.83	4.52
Urinary Excretion, mg/100kg BW	3.53	0.77	3.69	0.52	3.62	0.43
Retained, mg/100kg BW	-7.49	4.81	-10.38	6.21	-9.11	3.97
% of intake	-14.20	9.22	-8.58	5.10	-11.04	4.82
% of absorbed	225.64	107.46	56.86	51.58	130.71	57.33

*Row means differ (P<0.05)

Plasma Mineral Concentrations

Changes in plasma Cu and Zn concentrations are illustrated in Figure 1. Mean Cu concentrations varied from 0.66 to 0.89 ppm and Zn values ranged 0.37 to 0.53 ppm (Appendices 5 and 6). These observed values, including those at the initiation of the study, are considerably lower than those reported by Ralston (1992) and the "normal" range for North American horses (Hintz, 1982). The present results are similar to the values obtained (0.81 ppm Cu and 0.52 ppm Zn) from horses in a known Cu and Zn deficient region (Górecka et al., 1999), as well as those observed in apparently normal horses in Australia (Auer et al., 1988). Cymbaluk et al. (1986) reported plasma mineral concentration can vary by age and breed of horse. Plasma Cu and Zn concentration may not be an adequate tool for comparing mineral status among separate research projects, but appears be a good tool for tracking mineral status within a given research project.

Diet did not affect plasma Cu and Zn concentrations when horses were fed the experimental diets, nor was there a difference in normalized values between diets. There was a trend (P=0.05) for plasma Cu concentration to increase while horses were on the experimental diets while Zn concentrations did not change. There was no diet by day interaction observed in periods 4 and 7. Initial plasma Zn concentrations were different between periods 4 and 7, but there was no difference in d 28 values or normalized values for the periods.

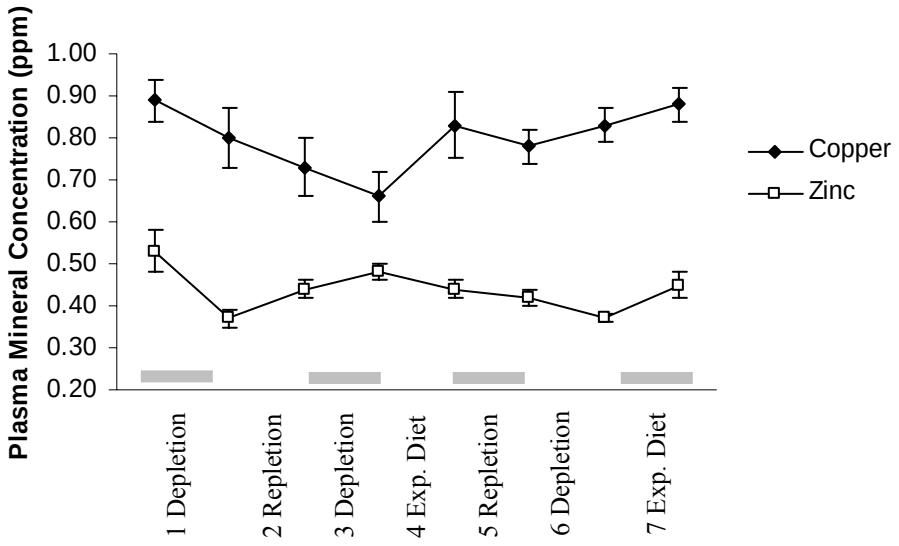


Figure 1. Plasma Mineral Concentration

The repletion-depletion diet cycle used in this study was designed in response to questions about pre-study mineral balance in work by Wagner et al. (2005). The purpose of the background and washout periods was to deplete body stores in an effort to standardize horses' responses to the experimental diets. This methodology proved to be effective for plasma Zn concentrations, which decreased from d 1 to d 29. Prior to the study, horses were maintained on pasture and fed a commercially available concentrate. The observed change in plasma Zn during period 1 demonstrated that feeding the mineral deficient diet led to depletion of Zn stores. Zinc concentrations remained unchanged from d 29 to d 85. Plasma Cu concentrations did not change significantly from d 1 to d 85.

In order to validate the use of the washout periods, t-tests were used to compare plasma mineral concentrations during repeated phases of the background and washout period sequences. Mean plasma Cu and Zn concentrations were not different between d 57 and d 141, the conclusion of the repletion periods. Plasma mineral concentrations were different at d 85 and d 169, the days representing the end of depletion periods and the start of feeding the experimental diets.

Differences were observed in plasma mineral concentrations following the depletion diets (Table 9). Plasma Cu concentration was greater on d 169 than d 85. Diet history did not influence this difference, as means were similar among horses coming off the background sequence and those previously subject to the sulfate and organic chelate diets in period 4. Plasma Zn concentration was greater on d 85. Diet history appeared to influence this observation (Table 9). Horses that just completed the background diet sequence in periods 1-3 had significantly greater plasma Zn concentrations than those fed the chelate diet during period 4. There was no difference between horses coming off the background sequence and sulfate experimental diet, nor was there a difference between horses previously fed the sulfate or organic chelate experimental diets. There was a numerical difference in the mineral concentration of the depletion diets fed in periods 3 and 6 (Table 1), though it is doubtful that such small variation could elicit the differences observed in plasma mineral concentrations between d 85 and 169.

Table 9. Plasma Mineral Concentrations with Respect to Diet History

	Day 85		Day 169	
Prior Treatment Sequence	**Background**		**Washout (Total)**	
	Mean	SEM	Mean	SEM
Copper (ppm)	0.66	0.05	0.83	0.04
Zinc (ppm)	0.48	0.02	0.37	0.01

	Day 169			
Prior Experimental Diet	**Sulfate**		**Organic Chelate**	
	Mean	SEM	Mean	SEM
Copper (ppm)	0.87	0.07	0.78	0.04
Zinc (ppm)	0.39	0.01	0.35	0.01

Ceruloplasmin Concentration

Mean ceruloplasmin concentrations by period are illustrated in Figure 2. There was no significant change in ceruloplasmin concentration as horses transitioned through the initial background diet sequence. In comparing the repeated phases of the study, there was no difference in ceruloplasmin concentrations between d 57 and d 141, when horses completed the repletion diet periods. This is similar to what was observed in plasma Cu concentrations during this time.

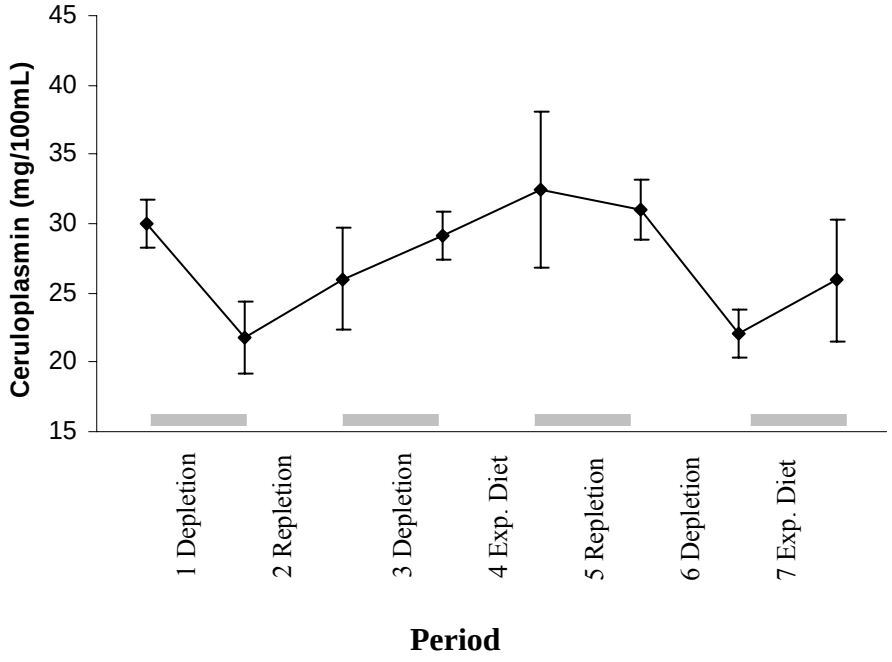


Figure 2. Mean Ceruloplasmin Concentration by Period

In contrast to observed plasma Cu values, ceruloplasmin concentration was greater on d 85 than d 169. Diet history appeared to influence this difference, as horses completing the background sequence had significantly greater ceruloplasmin concentrations than horses that were previously fed the sulfate supplemented experimental diet. There was no difference between horses from the background phase and those fed the chelate diet, nor was there a difference between horses recently fed the sulfate or organic chelate experimental diets.

Diet had no affect on ceruloplasmin concentrations when horses were fed the sulfate or organic chelate experimental diets, nor was there a period effect (Figure 3). There was a difference by day during periods 4 and 7, as ceruloplasmin increased from

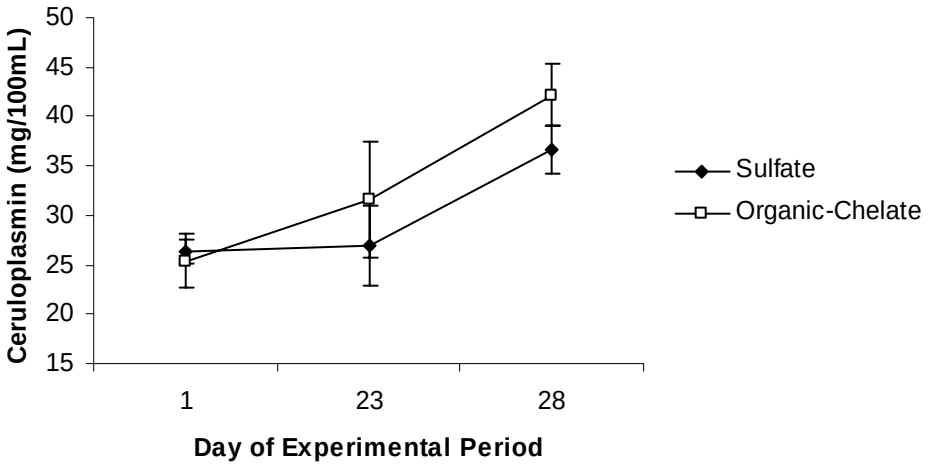


Figure 3. Changes in Ceruloplasmin Concentration During Experimental Diet Periods

25.84 mg/100mL to 39.57 mg/100mL d 1 to d 29 of the periods. Ceruloplasmin concentrations were not different between d 1 and d 23 of the experimental periods, but increased significantly during the total collection. This observation supports the role of ceruloplasmin as an acute-phase protein responsive to inflammation (Auer, 1989).

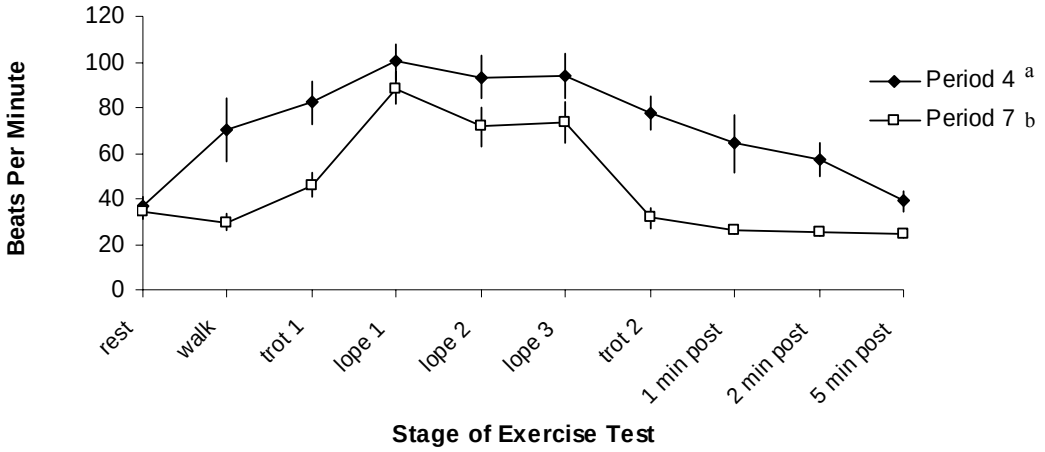
Ceruloplasmin assays used in equine research are not consistent in terms of conditions of the assay, oxidizing reagent used and unit of measurement, making it difficult to directly compare reported values. Values in the present study are similar to those reported in donkeys (Bingley and Dick, 1969). Ceruloplasmin activity was reduced in horses consuming 5.64 mg Cu/100 kg BW/d compared to those with a Cu intake of 16.60 mg/100 kg BW (Cymbaluk et al., 1981). In that study, the difference in ceruloplasmin activity mirrored the difference in plasma Cu concentration between the diets. That type of relationship was not as apparent in the present study. Ceruloplasmin

is a better indicator of Cu deficiency rather than adequacy (Harris, 2006), but little has been reported regarding ceruloplasmin response to exercise as seen in the present study. The novel interaction of exercise stress and marginally deficient diets may have resulted in ceruloplasmin responses unexplained by previous research.

Standard Exercise Test

One horse was not able to complete the second SET due to psychological and safety concerns, and the test was halted during the first cantering bout. Heart rate data collected prior to the cessation of the test was included in the statistical analyses. All other horses completed tests without incident. The observed heart rates were considered normal for this intensity of work.

Seven of the nine horses completed the first SET on the treadmill. Mechanical failure necessitated the use of the mechanical walker for the remaining SETs. Horses were subject to the same speeds and distances on both machines. There was no difference in heart rates between diets. Mean heart rates during the SET were greater during period 4 (Figure 4, Appendix 7). Statistical analysis could not determine if the difference was a period effect or a machine effect. Horses spent the first two periods of the research project in a conditioning program, and were performing the prescribed exercise regimen for the eight weeks leading up to the first SET. It is possible that the horses became more fit in the 12 weeks between SETs. The treadmill was set at an angle that should have created approximately the same workload as horses working over



a,b Periods lacking a common superscript differ (P<0.05)

Figure 4. Standard Exercise Test Heart Rates

the ground (Barrey et al., 1993; Courouce et al., 2000), though the angle could have caused horses to work harder than during over-ground work. Horses had several training sessions on the treadmill in the weeks leading up to the SET, so it is unlikely that the novelty of working on the treadmill caused the observed difference in heart rates.

Erythrocyte Superoxide Dismutase

There was no change in SOD activity from d 1 to d 23 of periods 4 and 7, nor was there a diet or period effect for SOD activity at rest on d 23 (Table 10). These values are similar to those reported by Górecka et al. (1999) who noted activity of 1752.03 ± 363.16 U/g Hb in horses assumed to be Cu and Zn deficient whereas horses presumed to be mineral adequate had a mean value of 1118.20 ± 430.15 U/g Hb. In that study, the group of horses assumed to be mineral deficient had plasma mineral

Table 10. Erythrocyte Superoxide Dismutase Activity

	Period 4		Period 7	
	Mean	SEM	Mean	SEM
Day 1	1614.53	192.58	1761.29	241.16
Day 23	1596.89	223.03	1504.64	300.95

concentrations of 0.81 ppm Cu and 0.52 ppm Zn which was greater than the d 85 Cu concentration and both d 85 and d 169 Zn values in the present study.

Polish researchers reported an increase in SOD activity in horses from a Cu and Zn deficient geographical region, suggesting a compensatory effect for decreased plasma mineral and ceruloplasmin concentrations (Górecka et al., 1999). Horses in the present study were on a Cu and Zn deficient diet prior to the first SOD sampling, and were either on a marginally deficient diet (sulfate supplementation) or a Cu and Zn sufficient diet (organic chelate supplementation) during periods 4 and 7. While this study did not reveal differences in erythrocyte SOD activity due to mineral intake status, it does not rule out possible effects from long term (i.e. more than 28 d) mineral deficiency.

Superoxide dismutase activity did not change over the course of the SET (Figure 5, Appendix 8). The Randox kit procedure uses hemoglobin concentrations to correct for changes in red blood cell count, an important consideration in horses where a splenic contraction increases circulating red blood cells in response to exercise. The observed lack of SOD response to this exercise test is in agreement with observations during short term, high intensity treadmill work (Ji et al., 2001) as well as observations before and after a pentathlon competition (Balogh et al., 2001).

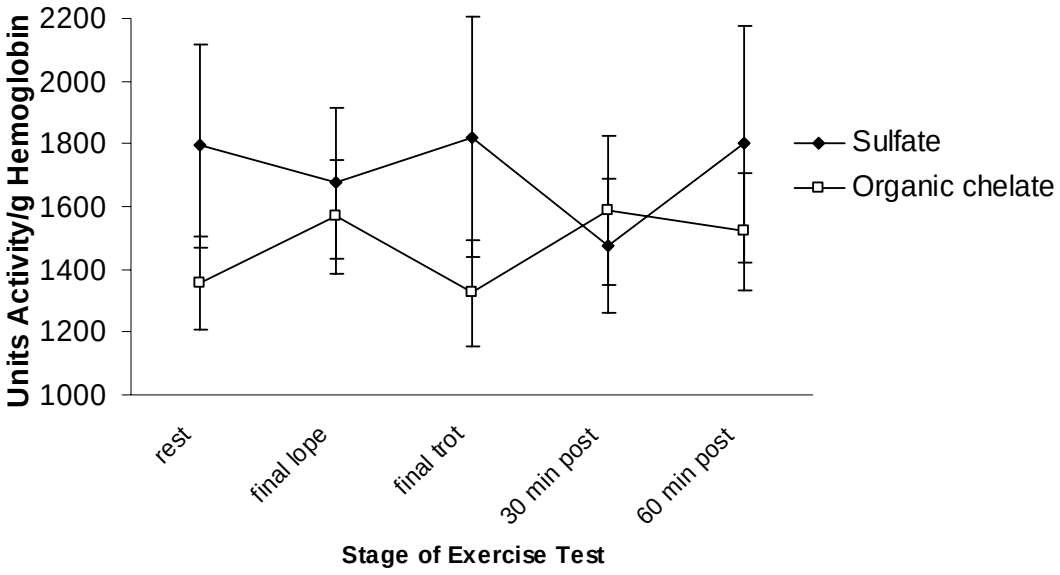
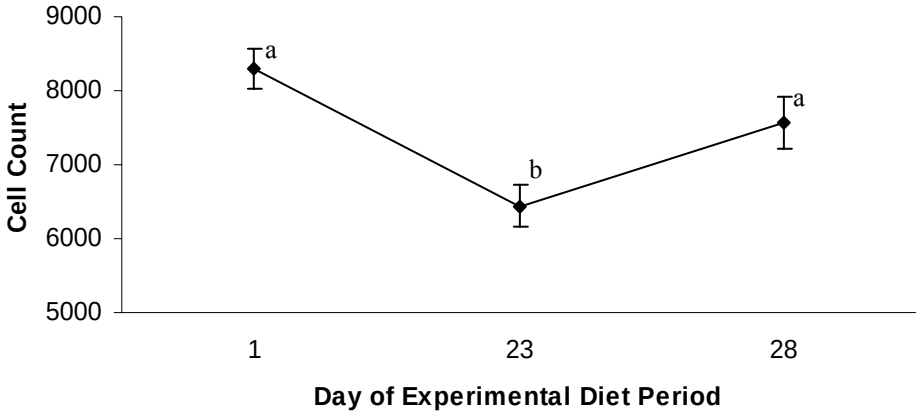


Figure 5. Erythrocyte Superoxide Dismutase Activity Response to Exercise

Immune Function

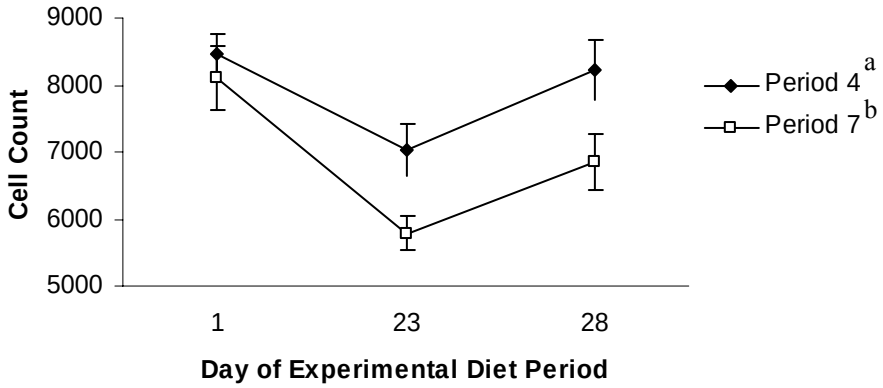
Mean total WBC was not different by diet, nor was there a diet by day interaction. There was a significant difference by day, where the mean d 1 and d 28 counts were greater than d 23, though d 1 and d 28 were not different from each other (Figure 6). White blood cell counts were higher for horses in period 4, though there was no difference in d 1 values between periods (Figure 7).

Diet did not influence absolute lymphocyte count (Figure 8), however there as a trend (P=0.07) for a difference by day as the lymphocyte count decreased from d1 to d 23. There was no diet by day interaction or period effect.



[a,b] Days lacking common superscripts are different (P<0.05)

Figure 6. Total White Blood Cell Count by Day



[a,b] Periods lacking common superscripts are different (P<0.05)

Figure 7. Total White Blood Cell Count by Period

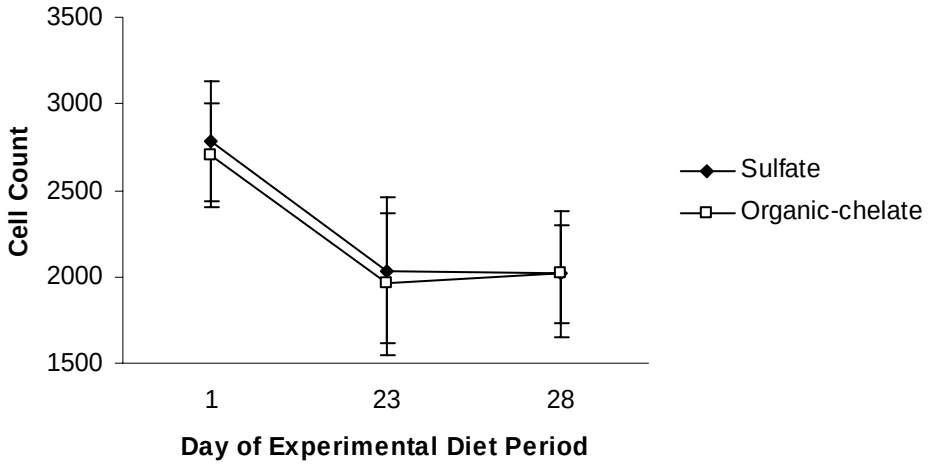


Figure 8. Lymphocyte Count

Differential cell counts indicated the percent of the total WBC constituted by neutrophils, lymphocytes, monocytes, eosinophils and basophils. Figures 9-14 illustrate the differential cell counts. There was no difference by diet, day, diet by day interaction or period for differential neutrophils, lymphocytes, eosinophils and basophils. Differential monocyte counts were different by day from d 1 to d 28 of the experimental periods, but there was no difference by diet, diet by day interaction or period.

The increase in WBC during the total collection period, d 23 to d 28, may indicate an immune challenge presented to the horses due to the stress of the collection. The lack of change in differential counts over the course of the total collection suggests that both acquired and innate immune functions were summoned in this response.

Lymphocytes are part of the adaptive immunity response whereas the other white cells are associated with some type of innate response (Abbas and Lichtman, 2005).

Means tables for all white blood cell data are found in Appendices 9 and 10.

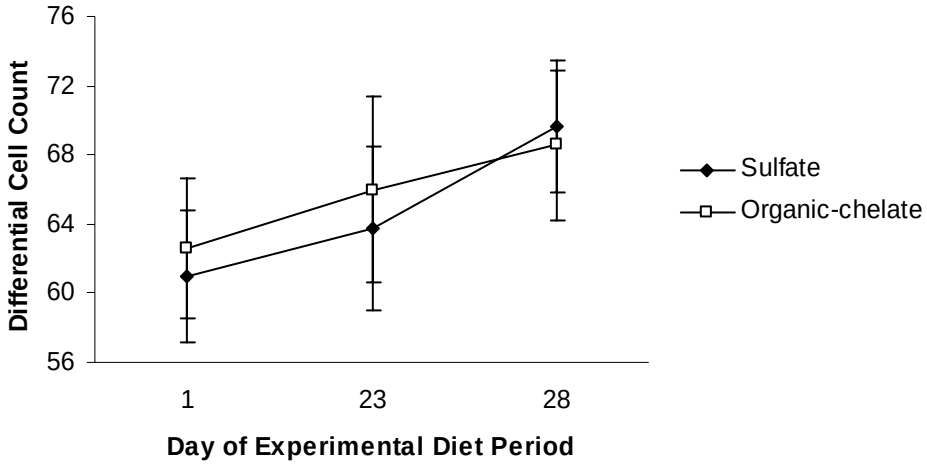


Figure 9. Differential Neutrophil Count

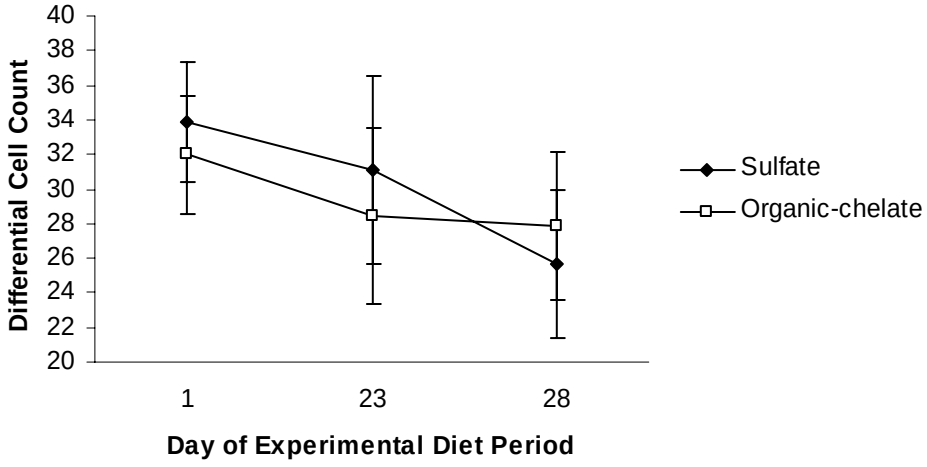


Figure 10. Differential Lymphocyte Count

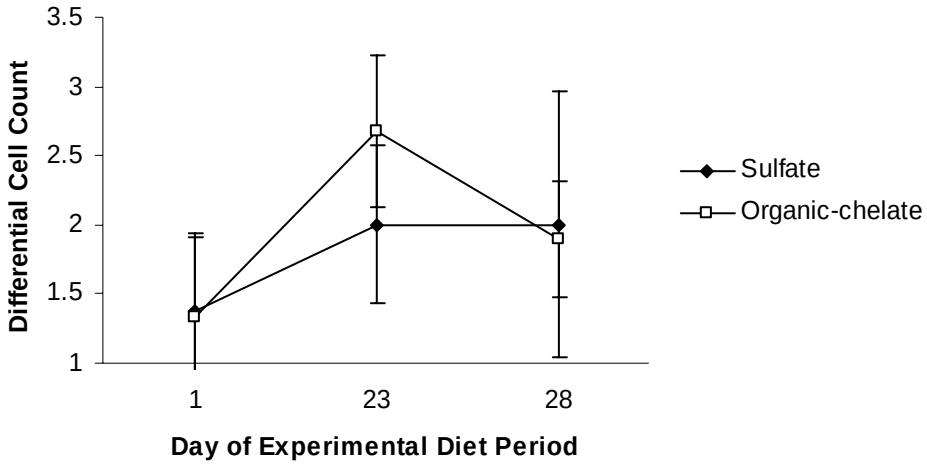


Figure 11. Differential Eosinophil Count

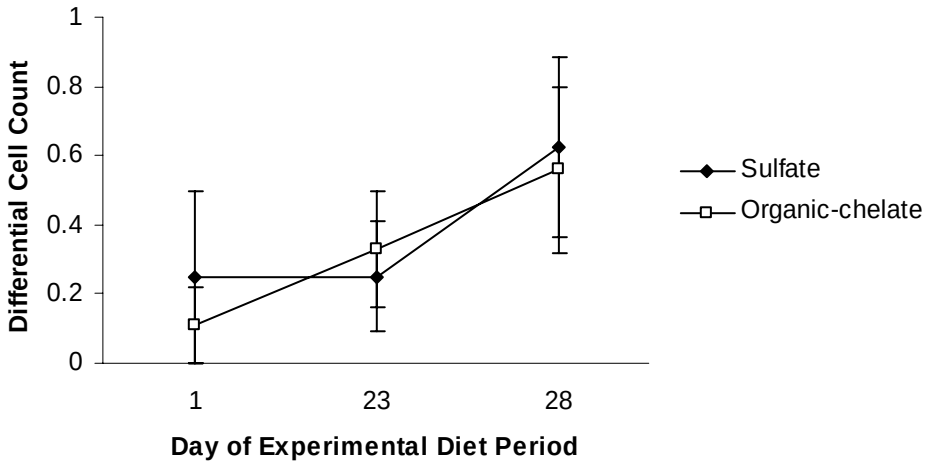


Figure 12. Differential Basophil Count

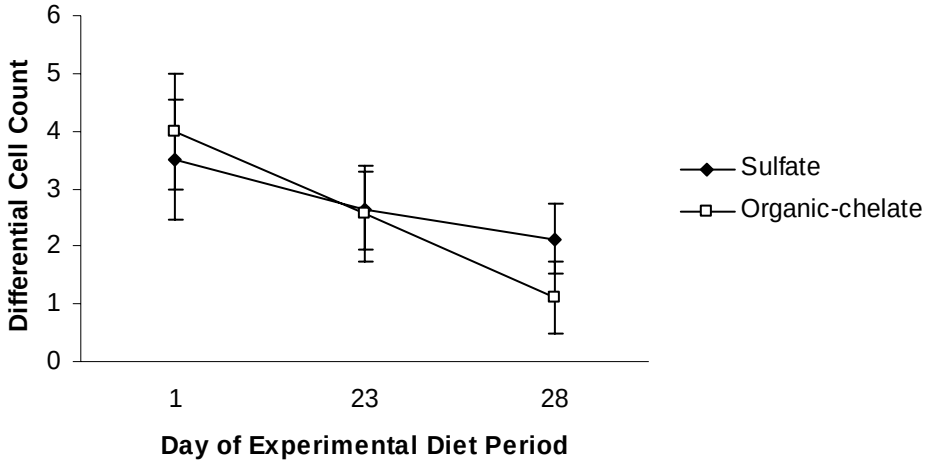
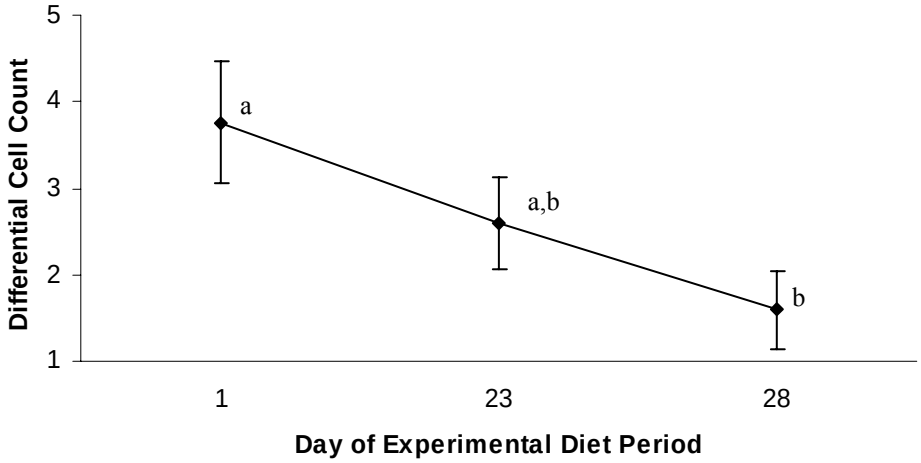


Figure 13. Differential Monocyte Count



[a,b] Days lacking common superscript are different (P<0.05)

Figure 14. Mean Differential Monocyte Count by Day

The lack of difference in total WBC by diet is in agreement with work using receiving steers (Kegley et al., 2001). While the present study did not use measures of acquired immunity such as antibody titers and immunoglobulin (Ig) counts, studies using those indices of immune function usually saw no difference by diet (Ahola et al., 2005a; Ahola et al., 2005b; Kegley et al., 2001). These indices were not possible in the present study as the switchback design would have created a carryover effect. A longer study, or one using completely separate groups of animals for the treatments, would be a better opportunity to examine antibody titers to vaccines for respiratory illness or even challenge horses with some type of antigen. While in vitro analyses, such as pokeweed mitogen and phytohemagglutinin challenge assays, have been used in supplementation studies in cattle (Kincaid et al., 1997; Spears and Kegley, 2002), they may not be the most effective tools for examining immune function in this context (Tizzard, 2005).

CHAPTER V

GENERAL DISCUSSION

Research in various species of livestock has examined differences in performance or body function when animals were fed mineral supplements of different chemical compositions. Results have been mixed, finding organic-chelates to be comparable to or better than sulfate or oxide supplement formulations. In horses, concerns regarding copper and zinc function in skeletal development and other physiological processes drive the need to define a superior supplementation form. As the horse industry becomes a target for environmental regulations already seen in other production livestock industries, trace mineral nutrition will take on another challenge in improving the efficiency of supplementation.

Previous research from this laboratory (Wagner et al., 2005) compared the absorption and retention of copper and zinc in mature, idle horses, concluding that there were no differences in absorption among sulfate, oxide and organic-chelate forms of supplementation for horses in this metabolic state. It was postulated that horses in a state of increased mineral demand, such as intense exercise, may be a more ideal model for comparing forms of trace mineral supplementation.

From this work, the present study was designed, keeping in mind the objectives of comparing absorption and retention of Cu and Zn in exercising horses as well as to document physiologic changes in select Cu and Zn dependent systems. To this end, plasma mineral concentrations and ceruloplasmin concentration were tracked throughout

the study, and SOD activity and immune function were examined during periods where horses consumed Cu and Zn from sulfate or organic-chelate sources.

A formulation error resulted in horses on the organic-chelate diet consuming about three times the amount of Cu and Zn of that provided in the sulfate supplemented diet. Fecal mineral excretion for the groups reflected this disparity in mineral intake. Horses also had negative absorption of Cu and Zn when consuming the experimental diets, including those consuming Cu and Zn concentrations well above NRC (1989) requirements. Mineral deficiency, as characterized by negative absorption, was also unlikely as horses were also excreting Cu and Zn in the urine. There was a significant difference in apparent absorption of Cu as a percent of intake as well as retention of Cu as a percent of intake between the sulfate and organic-chelate supplemented diets. There was no difference in apparent retention or retention as a percent of absorption for Cu. No significant differences were noted for absorption as a percent of intake, retention, retention as a percent of intake and retention as a percent of mineral absorbed for Zn.

When Cu and Zn absorption was corrected for endogenous fecal mineral losses, true absorption values remained lower than those reported in other balance studies. Great care was taken in the feeding and management of horses to prevent mineral intake from undocumented sources, and sample collection and processing was carried out using practices designed to minimize sample contamination. The most plausible explanation for the values reported would be fecal and urine sample contamination, though the source of contamination cannot be determined at this time. It is also possible that the

depletion diet periods were not sufficient in reducing body mineral stores to a deficient status.

The research project was designed to subject horses to periods of controlled depletion and repletion of mineral stores. There was a significant decrease in plasma Zn concentration as horses were removed from their commercially available concentrate and fed a basal diet with no supplemental Cu and Zn. Plasma Cu and Zn decreased numerically, but not significantly, through the first 85-d of the project which served as a background period. In the 56-d washout period between periods 4 and 7, horses were again fed a repletion-depletion diet sequence. While there was no difference in plasma mineral concentrations between d 56 and d 141, there was a difference in d 85 and d 169 values, the days preceding the experimental diet periods. Despite a significant difference in mineral intake during periods 4 and 7, plasma mineral concentrations were not different between treatment groups.

As a Cu-dependent protein, ceruloplasmin changes reflected the mineral intake of horses during the research project. As was observed with plasma mineral concentrations, there was no difference by diet on ceruloplasmin concentration in periods 4 and 7. In contrast to the difference in plasma Cu concentrations between d 85 and d 169, ceruloplasmin concentrations were actually lower on d 169. During period 4 and 7, ceruloplasmin concentrations increased significantly during the total collection period. This change was most likely a response to the physical stress of standing in tie stalls for the collection period, as ceruloplasmin has been identified as acute-phase protein for inflammation.

There was a difference in heart rate response to the exercise tests performed in periods 4 and 7, though it could not be determined if it was a period or machine effect. Source of mineral supplementation did not have an effect on heart rate. Superoxide dismutase activity did not change over the course of the exercise test. There was no difference in resting SOD activity between d 1 and d 23 of the experimental diet periods, nor was SOD activity influence by mineral source.

Immune function as measured by total white blood cell count, lymphocyte count and differential white blood cell count was not different by diet treatment in periods 4 and 7. While total WBC was similar on d 85 and d 169, there was a period effect. Studies in cattle reported similar findings regarding WBC in response to supplement form. Antibody titers may be a more effective test for comparing immune response, but the present project design did not allow for this type of analysis.

There continues to be a need to define the advantage, if any, of preferably feeding one mineral supplement form over another. The present study was designed to examine this issue, but formulation and sample contamination issues complicated a comparison of mineral balance data. Copper provided in the organic-chelate supplement was more available when considering apparent absorption as a percent of intake and retention as a percent of intake. However there was no difference in apparent Zn absorption as a percent of intake, nor was there no significant difference between the sulfate and organic chelate forms of Cu and Zn when comparing true absorption. Based on changes in plasma mineral concentrations, the use of a repletion-depletion diet sequence appears to be effective in depleting and standardizing body mineral stores.

While liver biopsies are considered a risky procedure in horses, it may be interesting to see how liver mineral stores compare to these observed changes in plasma mineral concentrations in animals subject to a similar diet treatment sequence. Ceruloplasmin concentrations can also serve as an indicator of a Cu deficient state, but its function as an acute-phase protein has the potential to confound results if horses are subject to inflammation-inducing events. Based on results from the present study, Cu,Zn-SOD and WBC are not responsive to changes in diet Cu and Zn concentration over a 23 d period.

The confounding circumstances of the experimental diet periods in the present study made it difficult to assess the use of the mature, exercising horse as a model for mineral balance studies. Moderate to intense exercise does place stress on body systems, though more intense exercise may be more effective in evaluating mineral balance. Mineral balance in the mature equine athlete should be re-evaluated before looking at the more complicated model of the juvenile athlete, where growth and exercise stress can influence Cu and Zn demand.

Regardless of age or use of animal, future balance studies must also consider mineral absorption efficiency in terms of environmental impact. Mineral consumption in excess of body needs will result in more mineral excreted from the horse. As small and large scale equine facilities struggle with waste management and removal, nutrient disposal concerns will become a bigger issue in the equine industry.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The present study was designed to compare absorption and retention of Cu and Zn in exercising horses when supplemented in either the sulfate or organic-chelate form. To accomplish this, nine horses were subject to a modified switchback designed experiment consisting of seven 28-d periods. A controlled repletion-depletion diet sequence was fed prior to consumption of an experimental diet in periods 4 and 7, where Cu and Zn were supplemented at 90% of NRC (1989) values using sulfate or specific amino acid chelate supplements. Horses were conditioned to perform an average daily workload of 3.93 kg*km*10^{-3}, creating a DE demand of 28.8 Mcal. Blood samples were drawn at regular intervals for analysis of plasma Cu, Zn and ceruloplasmin concentrations. White blood cell counts and SOD activity were assayed during periods 4 and 7. Horses were subjected to a standard exercise test on d 23 of periods 4 and 7 for the purpose of measuring SOD activity during exercise. A 4-d total collection beginning d 24 of periods 4 and 7 was used to assess Cu and Zn balance.

Dietary copper and zinc intakes were different between supplement forms in periods 4 and 7 due to a formulation error. This resulted in horses on the organic-chelate supplemented diet consuming nearly three times the amount of mineral as those on the sulfate supplemented diet. Apparent and true absorption of Cu and Zn were different by diet. Absorption as a percent of intake and retention as a percent of intake were negative, suggesting the possibility of sample contamination. Copper-lysine was more

available than $CuSO_4$ when measured as apparent absorption as a percent of intake, but there were no differences in apparent Zn or true Cu and Zn absorption as a percent of intake. Plasma Cu, Zn and ceruloplasmin concentrations, as well as immune function and SOD activity were not influenced by diet in periods 4 and 7. Plasma Cu, Zn and ceruloplasmin concentrations fluctuated throughout the experiment, and there was a significant difference in all three when comparing d 85 and d 169. Ceruloplasmin concentration increased significantly during the total collection period. Erythrocyte Cu,Zn-SOD activity remained consistent throughout the exercise test.

Conclusions regarding availability of Cu and Zn provided in the sulfate or organic-chelate forms are mixed. The use of a repletion-depletion diet sequence appeared to be somewhat effective in standardizing individual mineral status in adult horses, however 28-d periods may not be long enough to deplete body stores. Superoxide dismutase and WBC did not respond to changes in diet in the present study, and may not be suitable measures for similar project designs. The mature, exercising horse still appears to be an appropriate model for mineral balance studies. Further investigation is needed to generate data suitable for evaluating differences in Cu and Zn absorption, retention and availability in the horse.

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APPENDICES

APPENDIX 1. COPPER BALANCE

Horse	Period	Diet	Body Weight kg	Conc. Intake DM kg/d	Conc. Cu mg/kg	Conc. Cu mg/d	Hay Intake DM kg/d	Hay Cu mg/kg	Hay Cu Intake mg/d	Total Feed Cu mg/d	Water Intake L/d
1A	4	sulfate	429.11	3.18	7.00	22.27	3.22	4.60	14.80	37.07	14.07
2A	4	sulfate	514.39	4.30	7.00	30.13	4.34	4.60	19.97	50.09	23.29
3A	4	sulfate	530.26	3.58	7.00	25.07	3.62	4.60	16.65	41.72	36.76
5A	4	sulfate	494.42	4.16	10.00	41.56	4.15	5.30	21.99	63.55	22.16
1B	4	chelate	429.56	2.53	41.00	103.76	2.91	4.60	13.41	117.17	14.98
2B	4	chelate	533.44	4.61	41.00	189.15	4.68	4.60	21.51	210.66	23.68
3B	4	chelate	546.13	4.28	41.00	175.59	4.20	4.60	19.33	194.93	20.49
4B	4	chelate	514.39	4.10	41.00	168.17	4.14	4.60	19.03	187.20	22.27
5B	4	chelate	516.65	3.63	42.00	152.46	3.68	5.30	19.51	171.98	15.46
1A	7	chelate	458.13	3.15	45.00	141.86	3.22	3.50	11.27	153.13	15.21
2A	7	chelate	527.54	4.29	45.00	192.99	4.35	3.50	15.24	208.23	20.63
3A	7	chelate	519.37	3.57	45.00	160.58	3.63	3.50	12.69	173.27	29.33
5A	7	chelate	530.71	4.00	46.00	183.87	3.92	3.90	15.27	199.15	32.71
1B	7	sulfate	400.98	2.05	9.00	18.44	2.61	3.50	9.13	27.57	8.96
2B	7	sulfate	544.32	4.75	9.00	42.79	4.70	3.50	16.46	59.26	19.06
4B	7	sulfate	522.55	4.23	9.00	38.05	4.08	3.50	14.27	52.32	21.19
5B	7	sulfate	534.34	3.66	9.00	32.96	3.62	3.90	14.10	47.06	20.65

APPENDIX 1. CONTINUED

Horse	Period	Diet	Water Cu mg/L	Water Cu Intake mg/d	Total Cu Intake mg/d	Cu Intake mg/100kg BW	Urine L/d	Conc. Cu mg/L	Urine Cu mg/d	Urine Cu mg/100kg BW	Fecal DM kg/d
1A	4	sulfate	0.20	2.87	39.94	9.31	4.42	0.83	3.66	0.85	2.26
2A	4	sulfate	0.20	4.75	54.84	10.66	8.08	2.55	20.58	4.00	3.78
3A	4	sulfate	0.20	7.50	49.22	9.28	17.50	1.34	23.39	4.41	2.98
5A	4	sulfate	0.17	3.85	67.41	13.63	9.62	1.70	16.38	3.31	3.18
1B	4	chelate	0.20	3.06	120.23	27.99	3.80	1.16	4.40	1.02	2.10
2B	4	chelate	0.20	4.83	215.49	40.40	7.97	1.15	9.14	1.71	3.98
3B	4	chelate	0.20	4.18	199.11	36.46	5.36	4.18	22.37	4.10	3.22
4B	4	chelate	0.20	4.54	191.75	37.28	8.63	1.17	10.11	1.97	2.92
5B	4	chelate	0.17	2.69	174.67	33.81	7.45	1.70	12.69	2.46	3.36
1A	7	chelate	0.14	2.19	155.32	33.90	4.65	1.20	5.57	1.22	2.24
2A	7	chelate	0.14	2.97	211.20	40.03	5.71	1.09	6.21	1.18	3.74
3A	7	chelate	0.14	4.22	177.49	34.17	16.32	0.75	12.25	2.36	2.81
5A	7	chelate	0.01	0.16	199.31	37.56	14.77	5.45	80.48	15.17	3.03
1B	7	sulfate	0.14	1.29	28.86	7.20	5.10	6.73	34.32	8.56	1.61
2B	7	sulfate	0.14	2.74	62.00	11.39	6.91	6.79	46.90	8.62	3.65
4B	7	sulfate	0.14	3.05	55.37	10.60	7.86	1.24	9.71	1.86	3.13
5B	7	sulfate	0.01	0.10	47.16	8.83	7.06	0.73	5.11	0.96	3.07

APPENDIX 1. CONTINUED

Horse	Period	Diet	Fecal Cu mg/kg	Fecal Cu mg/d	Fecal Cu mg/100kg BW	Cu Absorbed mg/100kg BW	Cu Abs. % Intake	Cu Retained mg/100kg BW	Cu Ret. % Intake	Cu Ret. % Abs.	% Digestible DM
1A	4	sulfate	19.97	45.13	10.52	-1.21	-13.00	-2.06	-22.17	170.58	64.68
2A	4	sulfate	19.08	72.13	14.02	-3.36	-31.51	-7.36	-69.03	219.09	56.27
3A	4	sulfate	19.13	56.99	10.75	-1.47	-15.79	-5.88	-63.31	401.04	58.63
5A	4	sulfate	21.85	69.46	14.05	-0.42	-3.05	-3.73	-27.36	896.98	61.72
1B	4	chelate	59.10	124.35	28.95	-0.96	-3.43	-1.98	-7.09	206.76	61.36
2B	4	chelate	63.70	253.71	47.56	-7.16	-17.73	-8.88	-21.98	123.92	57.13
3B	4	chelate	67.70	217.84	39.89	-3.43	-9.41	-7.53	-20.64	219.38	62.08
4B	4	chelate	65.20	190.33	37.00	0.28	0.74	-1.69	-4.54	-614.07	64.57
5B	4	chelate	60.00	201.61	39.02	-5.22	-15.43	-7.67	-22.69	147.09	54.04
1A	7	chelate	69.00	154.65	33.76	0.15	0.43	-1.07	-3.16	-736.15	64.83
2A	7	chelate	64.40	240.96	45.68	-5.64	-14.09	-6.82	-17.03	120.86	56.70
3A	7	chelate	67.40	189.30	36.45	-2.27	-6.65	-4.63	-13.55	203.81	60.96
5A	7	chelate	68.20	206.85	38.98	-1.42	-3.78	-16.59	-44.16	1167.09	61.67
1B	7	sulfate	18.51	29.80	7.43	-0.23	-3.26	-8.79	-122.19	3748.05	65.44
2B	7	sulfate	22.00	80.40	14.77	-3.38	-29.67	-12.00	-105.32	354.97	61.36
4B	7	sulfate	23.16	72.50	13.87	-3.28	-30.94	-5.14	-48.47	156.68	62.31
5B	7	sulfate	24.46	75.01	14.04	-5.21	-59.05	-6.17	-69.89	118.37	57.86

APPENDIX 2. ZINC BALANCE

Horse	Period	Diet	Body Weight kg	Conc. Intake DM kg/d	Conc. Zn mg/kg	Conc. Zn mg/d	Hay Intake DM kg/d	Hay Zn mg/kg	Hay Zn Intake mg/d	Total Feed Zn mg/d	Water Intake L/d
1A	4	sulfate	429.11	3.18	47.00	149.56	3.22	26.20	84.28	233.84	14.07
2A	4	sulfate	514.39	4.30	47.00	202.27	4.34	26.20	113.73	316.00	23.29
3A	4	sulfate	530.26	3.58	47.00	168.30	3.62	26.20	94.84	263.14	36.76
5A	4	sulfate	494.42	4.16	48.00	199.50	4.15	20.20	83.81	283.31	22.16
1B	4	chelate	429.56	2.53	148.00	374.56	2.91	26.20	76.36	450.92	14.98
2B	4	chelate	533.44	4.61	148.00	682.79	4.68	26.20	122.53	805.32	23.68
3B	4	chelate	546.13	4.28	148.00	633.85	4.20	26.20	110.11	743.96	20.49
4B	4	chelate	514.39	4.10	148.00	607.06	4.14	26.20	108.40	715.46	22.27
5B	4	chelate	516.65	3.63	153.00	555.40	3.68	20.20	74.37	629.77	15.46
1A	7	chelate	458.13	3.15	164.00	516.98	3.22	24.10	77.61	594.60	15.21
2A	7	chelate	527.54	4.29	164.00	703.34	4.35	24.10	104.91	808.26	20.63
3A	7	chelate	519.37	3.57	164.00	585.21	3.63	24.10	87.40	672.61	29.33
5A	7	chelate	530.71	4.00	174.00	695.52	3.92	23.80	93.21	788.73	32.71
1B	7	sulfate	400.98	2.05	48.00	98.33	2.61	24.10	62.89	161.22	8.96
2B	7	sulfate	544.32	4.75	48.00	228.23	4.70	24.10	113.35	341.58	19.06
4B	7	sulfate	522.55	4.23	48.00	202.92	4.08	24.10	98.27	301.18	21.19
5B	7	sulfate	534.34	3.66	48.00	175.77	3.62	23.80	86.06	261.83	20.65

APPENDIX 2. CONTINUED

Horse	Period	Diet	Water Zn mg/L	Water Zn Intake mg/d	Total Zn Intake mg/d	Zn Intake mg/100kg BW	Urine L/d	Conc. Zn mg/L	Urine Zn mg/d	Urine Zn mg/100kg BW	Fecal DM kg/d
1A	4	sulfate	0.44	6.22	240.05	55.94	4.42	1.71	7.55	1.76	2.26
2A	4	sulfate	0.44	10.29	326.30	63.43	8.08	3.77	30.46	5.92	3.78
3A	4	sulfate	0.44	16.25	279.39	52.69	17.50	1.93	33.77	6.37	2.98
5A	4	sulfate	0.41	9.02	292.32	59.12	9.62	2.39	22.98	4.65	3.18
1B	4	chelate	0.44	6.62	457.54	106.51	3.80	2.53	9.60	2.24	2.10
2B	4	chelate	0.44	10.47	815.78	152.93	7.97	2.64	21.04	3.94	3.98
3B	4	chelate	0.44	9.05	753.02	137.88	5.36	7.43	39.79	7.29	3.22
4B	4	chelate	0.44	9.84	725.30	141.00	8.63	2.44	21.06	4.09	2.92
5B	4	chelate	0.41	6.29	636.06	123.11	7.45	2.51	18.70	3.62	3.36
1A	7	chelate	0.37	5.66	600.25	131.02	4.65	2.48	11.52	2.51	2.24
2A	7	chelate	0.37	7.68	815.93	154.67	5.71	2.14	12.21	2.31	3.74
3A	7	chelate	0.37	10.91	683.52	131.61	16.32	1.35	22.03	4.24	2.81
5A	7	chelate	0.16	5.33	794.06	149.62	14.77	1.05	15.51	2.92	3.03
1B	7	sulfate	0.37	3.33	164.55	41.04	5.10	1.56	7.96	1.98	1.61
2B	7	sulfate	0.37	7.09	348.68	64.06	6.91	1.54	10.64	1.95	3.65
4B	7	sulfate	0.37	7.88	309.07	59.15	7.86	1.36	10.68	2.04	3.13
5B	7	sulfate	0.16	3.37	265.20	49.63	7.06	1.52	10.72	2.01	3.07

APPENDIX 2. CONTINUED

Horse	Period	Diet	Fecal Zn mg/kg	Fecal Zn mg/d	Fecal Zn mg/100kg BW	Zn Absorbed	Zn Abs. % Intake	Zn Retained	Zn Ret. % Intake	Zn Ret. % Abs.	% Digestible DM
1A	4	sulfate	105.70	238.88	55.67	0.27	0.49	-1.49	-2.66	-545.42	64.68
2A	4	sulfate	92.40	349.29	67.90	-4.47	-7.05	-10.39	-16.38	232.49	56.27
3A	4	sulfate	153.80	458.16	86.40	-33.71	-63.99	-40.08	-76.07	118.89	58.63
5A	4	sulfate	109.20	347.15	70.21	-11.09	-18.76	-15.74	-26.62	141.91	61.72
1B	4	chelate	310.70	653.73	152.19	-45.67	-42.88	-47.91	-44.98	104.89	61.36
2B	4	chelate	244.00	971.83	182.18	-29.25	-19.13	-33.20	-21.71	113.48	57.13
3B	4	chelate	275.10	885.21	162.09	-24.21	-17.55	-31.49	-22.84	130.10	62.08
4B	4	chelate	254.80	743.80	144.60	-3.60	-2.55	-7.69	-5.45	213.79	64.57
5B	4	chelate	244.50	821.56	159.02	-35.90	-29.16	-39.52	-32.10	110.08	54.04
1A	7	chelate	254.20	569.74	124.36	6.66	5.08	4.15	3.16	62.25	64.83
2A	7	chelate	232.10	868.42	164.62	-9.95	-6.43	-12.26	-7.93	123.26	56.70
3A	7	chelate	277.20	778.53	149.90	-18.29	-13.90	-22.53	-17.12	123.18	60.96
5A	7	chelate	251.80	763.72	143.90	5.72	3.82	2.80	1.87	48.90	61.67
1B	7	sulfate	99.70	160.52	40.03	1.01	2.45	-0.98	-2.38	-97.12	65.44
2B	7	sulfate	113.50	414.77	76.20	-12.14	-18.96	-14.10	-22.01	116.10	61.36
4B	7	sulfate	120.10	375.96	71.95	-12.80	-21.64	-14.85	-25.10	115.97	62.31
5B	7	sulfate	133.60	409.71	76.68	-27.05	-54.49	-29.05	-58.54	107.42	57.86

APPENDIX 3. TRUE COPPER ABSORPTION

Horse	Period	Diet	Cu Intake mg/100 kg BW	Urine Cu mg/100 kg BW	Fecal Cu mg/100 kg BW	Endog. Cu mg/100 kg BW	Adj. Fecal Cu	True Cu Abs.	True Cu Abs % Int	True Cu Ret	True Cu Ret % Int	True Cu Ret % Abs
1A	4	sulfate	9.31	0.85	10.52	3.47	7.05	2.26	24.28	1.41	15.11	62.22
2A	4	sulfate	10.66	4.00	14.02	3.47	10.55	0.11	1.04	-3.89	-36.49	-3523.07
3A	4	sulfate	9.28	4.41	10.75	3.47	7.28	2.00	21.60	-2.41	-25.93	-120.04
5A	4	sulfate	13.63	3.31	14.05	3.47	10.58	3.05	22.40	-0.26	-1.90	-8.50
1B	4	chelate	27.99	1.02	28.95	3.47	25.48	2.51	8.97	1.49	5.31	59.17
2B	4	chelate	40.40	1.71	47.56	3.47	44.09	-3.69	-9.14	-5.41	-13.39	146.39
3B	4	chelate	36.46	4.10	39.89	3.47	36.42	0.04	0.11	-4.06	-11.13	-10381.42
4B	4	chelate	37.28	1.97	37.00	3.47	33.53	3.75	10.05	1.78	4.77	47.50
5B	4	chelate	33.81	2.46	39.02	3.47	35.55	-1.75	-5.16	-4.20	-12.43	240.70
1A	7	chelate	33.90	1.22	33.76	3.47	30.29	3.62	10.66	2.40	7.08	66.35
2A	7	chelate	40.03	1.18	45.68	3.47	42.21	-2.17	-5.42	-3.35	-8.36	154.19
3A	7	chelate	34.17	2.36	36.45	3.47	32.98	1.20	3.50	-1.16	-3.40	-97.00
5A	7	chelate	37.56	15.17	38.98	3.47	35.51	2.05	5.46	-13.12	-34.93	-640.18
1B	7	sulfate	7.20	8.56	7.43	3.47	3.96	3.24	44.95	-5.32	-73.98	-164.57
2B	7	sulfate	11.39	8.62	14.77	3.47	11.30	0.09	0.79	-8.53	-74.85	-9419.56
4B	7	sulfate	10.60	1.86	13.87	3.47	10.40	0.19	1.81	-1.67	-15.72	-868.67
5B	7	sulfate	8.83	0.96	14.04	3.47	10.57	-1.74	-19.73	-2.70	-30.58	154.96

APPENDIX 4. TRUE ZINC ABSORPTION

Horse	Period	Diet	Zn Intake mg/100 kg BW	Urine Zn mg/100 kg BW	Fecal Zn mg/100 kg BW	Endog. Zn mg/100 kg BW	Adj. Fecal Zn	True Zn Abs.	True Zn Abs % Int	True Zn Ret	True Zn Ret % Int	True Zn Ret % Abs
1A	4	sulfate	55.94	1.76	55.67	10.47	45.20	10.74	19.20	8.98	16.06	83.62
2A	4	sulfate	63.43	5.92	67.90	10.47	57.43	6.00	9.46	0.08	0.12	1.31
3A	4	sulfate	52.69	6.37	86.40	10.47	75.93	-23.24	-44.11	-29.61	-56.20	127.40
5A	4	sulfate	59.12	4.65	70.21	10.47	59.74	-0.62	-1.05	-5.27	-8.91	850.49
1B	4	chelate	106.51	2.24	152.19	10.47	141.72	-35.20	-33.05	-37.44	-35.15	106.35
2B	4	chelate	152.93	3.94	182.18	10.47	171.71	-18.78	-12.28	-22.73	-14.86	121.00
3B	4	chelate	137.88	7.29	162.09	10.47	151.62	-13.74	-9.96	-21.02	-15.25	153.04
4B	4	chelate	141.00	4.09	144.60	10.47	134.13	6.87	4.87	2.78	1.97	40.43
5B	4	chelate	123.11	3.62	159.02	10.47	148.55	-25.43	-20.66	-29.05	-23.60	114.23
1A	7	chelate	131.02	2.51	124.36	10.47	113.89	17.13	13.07	14.62	11.16	85.32
2A	7	chelate	154.67	2.31	164.62	10.47	154.15	0.52	0.34	-1.79	-1.16	-344.76
3A	7	chelate	131.61	4.24	149.90	10.47	139.43	-7.82	-5.94	-12.06	-9.17	154.21
5A	7	chelate	149.62	2.92	143.90	10.47	133.43	16.19	10.82	13.27	8.87	81.95
1B	7	sulfate	41.04	1.98	40.03	10.47	29.56	11.48	27.97	9.49	23.13	82.71
2B	7	sulfate	64.06	1.95	76.20	10.47	65.73	-1.67	-2.61	-3.63	-5.66	216.88
4B	7	sulfate	59.15	2.04	71.95	10.47	61.48	-2.33	-3.94	-4.38	-7.40	187.70
5B	7	sulfate	49.63	2.01	76.68	10.47	66.21	-16.58	-33.40	-18.58	-37.44	112.11

APPENDIX 5. MEAN PLASMA COPPER, ZINC AND CERULOPLASMIN CONCENTRATION BY DAY

	Copper		Zinc		Ceruloplasmin	
Day	Mean	SEM	Mean	SEM	Mean	SEM
1	0.89	0.05	0.53	0.05	30.03	1.71
29	0.80	0.07	0.37	0.02	21.80	2.61
57	0.73	0.07	0.44	0.02	26.02	3.62
85	0.66	0.06	0.48	0.02	29.14	1.76
108	-	-	-	-	32.46	5.58
113	0.83	0.08	0.44	0.02	39.62	3.59
141	0.78	0.04	0.42	0.02	31.05	2.17
169	0.83	0.04	0.37	0.01	22.12	1.74
192	-	-	-	-	25.89	4.41
197	0.88	0.04	0.45	0.03	39.51	2.22

APPENDIX 6. PLASMA COPPER AND ZINC CONCENTRATION BY TREATMENT DURING PERIODS 4 AND 7

	Sulfate		Organic-Chelate	
	Mean	SEM	Mean	SEM
Plasma Cu (ppm)				
d 29	0.91	0.06	0.81	0.06
Normalized	-0.21	0.07	0.03	0.07
Plasma Zn (ppm)				
d 29	0.46	0.03	0.43	0.02
Normalized	-0.25	0.07	-0.40	0.07
Ceruloplasmin (mg/100 ml)				
d 23	26.92	4.09	31.54	5.87
Normalized	0.56	3.58	6.17	5.23
d 29	36.62	2.48	42.20	3.18
Normalized	10.26	3.00	16.82	2.50

APPENDIX 7. STANDARD EXERCISE TEST HEART RATE

	Period 4		Period 7		Treadmill		Trojan	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
rest	36.78	3.71	33.88	2.68	37.43	4.82	34.00	2.12
walk	70.00	14.07	29.50	3.69	79.67	17.16	32.38	3.30
trot 1	82.22	9.61	45.88	5.23	87.00	11.74	49.80	5.05
lope 1	100.22	7.89	88.50	7.12	110.00	5.85	84.00	6.40
lope 2	93.22	9.28	71.57	8.82	104.29	7.34	67.78	7.29
lope 3	93.89	10.16	73.71	9.00	103.71	10.22	70.56	7.19
trot 2	77.44	7.14	31.43	4.15	82.86	8.09	37.44	5.09
1 min post	64.29	12.56	25.86	1.44	70.60	17.00	30.89	3.78
2 min post	57.11	7.32	25.29	1.23	59.57	9.16	30.44	3.81
5 min post	38.78	4.44	24.14	1.53	37.71	5.35	28.22	3.34

APPENDIX 8. SUPEROXIDE DISMUTASE ACTIVITY

	Sulfate		Organic-Chelate		Period 4		Period 7	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
rest	1793.47	323.00	1355.70	148.32	1596.89	223.03	1504.64	300.95
final lope	1675.51	240.64	1567.48	180.03	1742.10	217.76	1451.00	159.17
final trot	1822.60	381.05	1324.92	171.00	1275.05	163.61	1562.15	205.44
30 min post	1476.16	213.40	1589.14	239.35	1378.22	153.00	1747.35	303.38
60 min post	1799.59	376.13	1520.79	187.04	1363.32	134.33	1665.15	265.39

APPENDIX 9. MEAN WHITE BLOOD CELL COUNTS BY DAY OF PERIODS 4 AND 7

	Sulfate		Organic-Chelate		Period 4		Period 7		Total	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
d 1										
Total White Blood Cell Count	8162.50	415.30	8411.11	377.29	8466.67	299.54	8100.00	484.40	8294.12	272.31
Absolute Lymphocyte Count	2785.75	348.35	2705.22	299.36	2615.00	230.96	2887.25	403.57	2743.12	220.92
Differential Neutrophil	61.00	3.81	62.56	4.02	65.00	2.60	58.25	4.82	61.82	2.70
Differential Lymphocyte	33.88	3.47	32.00	3.42	30.89	2.39	35.13	4.31	32.88	2.37
Differential Monocyte	3.50	1.04	4.00	1.00	2.00	0.55	5.75	0.96	3.76	0.70
Differential Eosinophil	1.38	0.56	1.33	0.58	1.89	0.68	0.75	0.25	1.35	0.39
Differential Basophil	0.25	0.25	0.11	0.11	0.22	0.22	0.13	0.13	0.18	0.13
d 23										
Total White Blood Cell Count	6275.00	421.20	6588.89	382.04	7022.22	382.52	5787.50	262.84	6441.18	276.94
Absolute Lymphocyte Count	2034.38	423.20	1959.89	409.68	2695.56	302.81	1206.75	331.34	1994.94	285.29
Differential Neutrophil	63.75	4.72	66.00	5.40	56.11	3.58	74.88	4.12	64.94	3.52
Differential Lymphocyte	31.13	5.44	28.44	5.12	38.22	3.57	20.13	4.77	29.71	3.63
Differential Monocyte	2.63	0.68	2.56	0.83	3.33	0.69	1.75	0.75	2.59	0.53
Differential Eosinophil	2.00	0.57	2.67	0.55	1.89	0.56	2.88	0.52	2.35	0.39
Differential Basophil	0.25	0.16	0.33	0.17	0.44	0.18	0.13	0.13	0.29	0.11

APPENDIX 9. CONTINUED

	Sulfate		Organic-Chelate		Period 4		Period 7		Total	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
d 29										
Total White Blood Cell Count	7787.50	445.39	7377.78	542.82	8222.22	457.78	6837.50	419.58	7570.59	348.50
Absolute Lymphocyte Count	2015.25	365.08	2015.33	282.63	2015.33	220.71	2015.25	416.36	2015.29	220.42
Differential Neutrophil	69.63	3.79	68.56	4.36	70.89	3.87	67.00	4.29	69.06	2.83
Differential Lymphocyte	25.63	4.30	27.89	4.29	25.56	3.46	28.25	5.15	26.82	2.96
Differential Monocyte	2.13	0.61	1.11	0.61	1.78	0.70	1.38	0.53	1.59	0.44
Differential Eosinophil	2.00	0.96	1.89	0.42	1.11	0.42	2.88	0.83	1.94	0.49
Differential Basophil	0.63	0.26	0.56	0.24	0.67	0.24	0.50	0.27	0.59	0.17

APPENDIX 10. NORMALIZED WHITE BLOOD CELL COUNTS BY DAY OF PERIODS 4 AND 7

	Sulfate		Organic-Chelate		Period 4		Period 7		Total	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
d 23										
Total White Blood Cell Count	-1887.50	495.13	-1822.22	491.81	-1444.44	547.24	-2312.50	338.29	-1852.94	338.48
Absolute Lymphocyte Count	-751.38	527.30	-745.33	500.39	80.56	351.41	-1680.50	454.58	-748.18	351.46
Differential Neutrophil	2.75	6.39	3.44	7.16	-8.89	3.99	16.63	6.10	3.12	4.69
Differential Lymphocyte	-2.75	7.11	-3.56	6.16	7.33	3.57	-15.00	6.73	-3.18	4.53
Differential Monocyte	-0.88	1.02	-1.44	1.69	1.33	1.04	-4.00	1.10	-1.18	0.99
Differential Eosinophil	0.63	0.82	1.33	0.67	0.00	0.71	2.13	0.55	1.00	0.51
Differential Basophil	0.22	0.22	0.00	0.33	0.22	0.32	0.00	0.19	0.12	0.19
d 29										
Total White Blood Cell Count	-375.00	351.91	-1033.33	418.66	-244.44	388.41	-1262.50	331.09	-723.53	280.43
Absolute Lymphocyte Count	-770.50	265.48	-689.89	224.66	-599.67	233.04	-872.00	245.82	-727.82	167.25
Differential Neutrophil	8.63	3.63	6.00	3.84	5.89	4.16	8.75	3.11	7.24	2.59
Differential Lymphocyte	-8.25	3.43	-4.11	2.69	-5.33	2.96	-6.88	3.30	-6.06	2.14
Differential Monocyte	-1.38	1.46	-2.89	1.41	-0.22	1.09	-4.38	1.43	-2.18	1.00
Differential Eosinophil	0.63	1.03	0.56	0.93	-0.78	0.91	2.13	0.69	0.59	0.67
Differential Basophil	0.38	0.18	0.44	0.18	0.44	0.18	0.38	0.18	0.41	0.12

APPENDIX 11. ANOVA TABLE FOR COPPER AND ZINC BALANCE

Source	Partial SS	df	MS	F-value	P-value
Cu Intake					
Model	2505.0526	3	835.0175	77.93	0.0000
Period	0.5902	1	0.5902	0.06	0.8184
Diet	2478.7353	1	2478.7353	231.32	0.0000
Period*Diet	2.7370	1	2.7370	0.26	0.6224
Residual	128.5851	12	10.7154		
Total	2633.6377	15	175.5758		
Cu Urine					
Model	17.3029	3	5.7676	0.37	0.7763
Period	11.1460	1	11.1460	0.71	0.4145
Diet	0.0732	1	0.0732	0.00	0.9465
Period*Diet	4.1165	1	4.1165	0.26	0.6168
Residual	187.2169	12	15.6014		
Total	204.5198	15	13.6347		
Cu Fecal					
Model	2554.8064	3	851.6021	38.12	0.0000
Period	4.3626	1	4.3626	0.20	0.6664
Diet	2481.4117	1	2481.4117	111.07	0.0000
Period*Diet	2.6790	1	2.6790	0.12	0.7351
Residual	268.0996	12	22.3416		
Total	2822.9059	15	188.1937		
Cu Absorption					
Model	11.8680	3	3.9560	0.77	0.5338
Period	1.7435	1	1.7435	0.34	0.5715
Diet	0.0007	1	0.0007	0.00	0.9908
Period*Diet	10.8315	1	10.8315	2.10	0.1727
Residual	61.8317	12	5.1526		
Total	73.6997	15	4.9133		
Cu Absorption as a Percent of Intake					
Model	2350.2422	3	783.4141	7.10	0.0053
Period	427.6086	1	427.6086	3.87	0.0726
Diet	1598.7300	1	1598.7305	14.48	0.0025
Period*Diet	709.5684	1	709.5684	6.43	0.0262
Residual	1324.5855	12	110.3821		
Total	3674.8277	15	244.9885		

APPENDIX 11. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Cu Retention					
Model	22.4377	3	7.4792	0.40	0.7541
Period	21.7057	1	21.7057	1.17	0.3012
Diet	0.0885	1	0.0885	0.00	0.9461
Period*Diet	1.9532	1	1.5932	0.09	0.7748
Residual	223.1690	12	18.5974		
Total	245.6066	15	16.3738		
Cu Retention as a Percent of Intake					
Model	8020.7121	3	2673.5707	6.94	0.0058
Period	1065.4861	1	1065.4861	2.77	0.1221
Diet	7019.1655	1	7019.1655	18.23	0.0011
Period*Diet	605.0330	1	605.0330	1.57	0.2339
Residual	4620.6120	12	385.0510		
Total	12641.3241	15	842.7549		
Cu Retention as a Percent of Absorbed					
Model	365823.9840	3	121941.3280	0.55	0.6606
Period	1519.9720	1	1519.9720	0.01	0.9357
Diet	175960.2370	1	175960.2370	0.79	0.3925
Period*Diet	142852.7560	1	142852.7560	0.64	0.4397
Residual	2683683.3600	12	223640.2800		
Total	3049507.3500	15	203300.4900		
Zn Intake					
Model	24627.1389	3	8209.0463	51.87	0.0000
Period	82.8944	1	82.8944	0.52	0.4831
Diet	24345.3093	1	24345.3093	153.82	0.0000
Period*Diet	89.7097	1	89.7097	0.57	0.4660
Residual	1899.2044	12	158.2670		
Total	26526.3433	15	1768.4229		
Zn Urine					
Model	15.7440	3	5.2480	2.17	0.1441
Period	14.7963	1	14.7963	6.13	0.0292
Diet	0.3007	1	0.3007	0.12	0.7303
Period*Diet	1.9915	1	1.9915	0.83	0.3816
Residual	28.9645	12	2.4137		
Total	44.7085	15	2.9806		

APPENDIX 11. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Zn Fecal					
Model	26654.3076	3	8884.7692	50.24	0.0000
Period	85.9817	1	85.9817	0.49	0.4989
Diet	24998.2237	1	24998.2237	141.37	0.0000
Period*Diet	357.1894	1	357.1894	2.02	0.1807
Residual	2121.9984	12	176.8332		
Total	28775.3059	15	1918.4204		
Zn Absorption					
Model	1328.3539	3	442.7846	2.36	0.1232
Period	337.7250	1	337.7250	1.80	0.2049
Diet	4.3199	1	4.3199	0.02	0.8820
Period*Diet	804.9098	1	804.9098	4.28	0.0607
Residual	2254.7963	12	187.8997		
Total	3583.1502	15	238.8767		
Zn Absorption as a Percent of Intake					
Model	1622.6796	3	540.8932	1.47	0.2726
Period	97.2901	1	97.2901	0.26	0.6167
Diet	808.8244	1	808.8244	2.20	0.1642
Period*Diet	801.0528	1	801.0528	2.17	0.1661
Residual	4420.8206	12	368.4017		
Total	6043.5002	15	402.9000		
Zn Retention					
Model	1431.6524	3	477.2175	2.43	0.1153
Period	493.9012	1	493.9012	2.52	0.1384
Diet	6.9000	1	6.9000	0.04	0.8543
Period*Diet	726.8265	1	726.8265	3.71	0.0782
Residual	2352.1035	12	196.0086		
Total	3783.7559	15	252.2504		
Zn Retention as a Percent of Intake					
Model	1990.4274	3	663.4758	1.59	0.2430
Period	236.4074	1	236.4074	0.57	0.4659
Diet	1200.7829	1	1200.7829	2.88	0.1154
Period*Diet	614.2778	1	614.2778	1.47	0.2481
Residual	5002.5537	12	416.8795		
Total	6992.9811	15	466.1987		

APPENDIX 11. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Zn Retention as a Percent of Absorbed					
Model	53020.1238	3	17673.3746	0.53	0.6684
Period	6368.6072	1	6368.6072	0.19	0.6691
Diet	14817.1554	1	14817.1554	0.45	0.5166
Period*Diet	28385.7260	1	28385.7260	0.86	0.3732
Residual	398066.0510	12	33172.1709		
Total	451086.1740	15	30072.4116		

APPENDIX 12. ANOVA TABLE FOR COPPER AND ZINC BALANCE ADJUSTED FOR TRUE ABSORPTION

Source	Partial SS	df	MS	F-value	P-value
Cu Fecal - Adjusted					
Model	2554.7991	3	851.5997	38.12	0.0000
Period	4.3625	1	4.3625	0.20	0.6664
Diet	2481.4055	1	2481.4055	111.07	0.0000
Period*Diet	2.6789	1	2.6789	0.12	0.7351
Residual	268.1002	12	22.3417		
Total	2822.8993	15	188.1933		
True Cu Absorption					
Model	11.8680	3	3.9560	0.77	0.5338
Period	1.7433	1	1.7433	0.34	0.5716
Diet	0.0007	1	0.0007	0.00	0.9908
Period*Diet	10.8316	1	10.8316	2.10	0.1727
Residual	61.8315	12	5.1526		
Total	73.6995	15	4.9133		
True Cu Absorption as a Percent of Intake					
Model	1037.2356	3	345.7452	3.86	0.0382
Period	404.8029	1	404.8029	4.52	0.0550
Diet	48.8752	1	48.8752	0.55	0.4744
Period*Diet	635.5339	1	635.5339	7.09	0.0207
Residual	1075.1327	12	89.5943		
Total	2112.3682	15	140.8246		
Cu Retention					
Model	22.4376	3	7.4792	0.40	0.7541
Period	21.7057	1	21.7057	1.17	0.3012
Diet	0.0885	1	0.0885	0.00	0.9461
Period*Diet	1.5932	1	1.5932	0.09	0.7748
Residual	223.1696	12	18.5975		
Total	245.6072	15	16.3738		
Cu Retention as a Percent of Intake					
Model	2529.1718	3	843.0573	2.09	0.1553
Period	1029.3086	1	1029.3086	2.55	0.1363
Diet	1354.6282	1	1354.6282	3.35	0.0919
Period*Diet	536.8233	1	536.8233	1.33	0.2714
Residual	4845.5241	12	403.7937		
Total	7374.6959	15	491.6464		

APPENDIX 12. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Cu Retention as a Percent of Absorbed					
Model	20686669.1000	3	6895556.3700	0.54	0.6638
Period	386588.2650	1	386588.2650	0.03	0.8647
Diet	4550359.3100	1	4550359.3100	0.36	0.5616
Period*Diet	18133876.3000	1	18133876.3000	1.42	0.2563
Residual	153175021.0000	12	12764585.1000		
Total	173861690.0000	15	11590779.3000		
Zn Fecal - Adjusted					
Model	26654.3085	3	8884.7695	50.24	0.0000
Period	85.9817	1	85.9817	0.49	0.4989
Diet	24998.2242	1	24998.2242	141.37	0.0000
Period*Diet	357.1896	1	357.1896		
Residual	2121.9981	12	176.8332		
Total	28776.3066	15	1918.4204		
True Zn Absorption					
Model	1328.3539	3	442.7846	2.36	0.1232
Period	337.7251	1	337.7251	1.80	0.2049
Diet	4.3199	1	4.3199	0.02	0.8820
Period*Diet	804.9097	1	804.9097	4.28	0.0607
Residual	2254.7964	12	187.8997		
Total	3583.1502	15	238.8767		
True Zn Absorption as a Percent of Intake					
Model	948.2040	3	316.0680	0.96	0.4446
Period	89.1110	1	89.1110	0.27	0.6130
Diet	58.8333	1	58.8333	0.18	0.6805
Period*Diet	757.5451	1	757.5451	2.29	0.1559
Residual	3965.4192	12	330.4516		
Total	4913.6231	15	327.5749		
Zn Retention					
Model	1431.6525	3	477.2175	2.43	0.1153
Period	493.9012	1	493.9012	2.52	0.1384
Diet	6.8999	1	6.8999	0.04	0.8543
Period*Diet	726.8266	1	726.8266	3.71	0.0782
Residual	2352.1033	12	196.0086		
Total	3783.7558	15	252.2504		

APPENDIX 12. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Zn Retention as a Percent of Intake					
Model	1032.2384	3	344.0795	0.91	0.4653
Period	223.5573	1	223.5573	0.59	0.4570
Diet	192.7312	1	192.7312	0.51	0.4891
Period*Diet	576.2539	1	576.2539	1.52	0.2408
Residual	4540.3528	12	378.3627		
Total	5572.5912	15	371.5061		
Zn Retention as a Percent of Absorbed					
Model	155432.0710	3	51810.6902	0.98	0.4338
Period	41187.6062	1	41187.6062	0.78	0.3944
Diet	109736.0480	1	109736.0480	2.08	0.1749
Period*Diet	362.6196	1	362.6196	0.01	0.9353
Residual	633307.0730	12	52775.5894		
Total	788739.1440	15	52582.6096		

APPENDIX 13. ANOVA TABLE FOR PLASMA COPPER, ZINC AND CERULOPLASMIN

Source	Partial SS	df	MS	F-value	P-value
Cu d0-d85					
Model	0.2591	3	0.0864	2.61	0.0687
Day	0.2591	3	0.0864	2.61	0.0687
Residual	1.0600	32	0.3300		
Total	1.3190	35	0.0377		
Cu d57 & d141					
Model	0.0103	2	0.0052	0.18	0.8362
Diet history	0.0103	2	0.0052	0.18	0.8362
Residual	0.4275	15	0.0285		
Total	0.4378	17	0.0258		
Cu d85 & d169					
Model	0.1353	2	0.0676	3.27	0.0682
Diet history	0.1353	2	0.0676	3.27	0.0682
Residual	0.2892	14	0.0207		
Total	0.4245	16	0.0265		
Cu periods 4 & 7					
Model	0.1767	3	0.0589	2.04	0.1287
Diet	0.0005	1	0.0005	0.02	0.9004
Day	0.1194	1	0.1194	4.15	0.0506
Diet*day	0.0667	1	0.0667	2.32	0.1385
Residual	0.8643	30	0.0288		
Total	1.0411	33	0.0315		
Cu periods 4 & 7					
Model	0.0940	1	0.0940	3.18	0.0842
Period	0.0940	1	0.0940	3.18	0.0842
Residual	0.9471	32	0.0296		
Total	1.0411	33	0.0315		
Cu periods 4 & 7 normalized					
Model	0.0667	1	0.0667	2.62	0.1153
Diet	0.0667	1	0.0667	2.62	0.1153
Residual	0.8146	32	0.0255		
Total	0.8813	33	0.0267		

APPENDIX 13. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Zn d0-d85					
Model	0.1286	3	0.0429	4.62	0.0085
Day	0.1286	3	0.0429	4.62	0.0085
Residual	0.2968	32	0.0093		
Total	0.4255	32	0.0122		
Zn d57 & d141					
Model	0.0103	2	0.0052	0.18	0.8362
Diet history	0.0103	2	0.0052	0.18	0.8362
Residual	0.4275	15	0.0285		
Total	0.4378	17	0.0258		
Zn d85 & d169					
Model	0.0493	2	0.0246	7.02	0.0077
Diet history	0.0493	2	0.0246	7.02	0.0077
Residual	0.0492	14	0.0035		
Total	0.0984	16	0.0062		
Zn periods 4 & 7					
Model	0.0169	3	0.0056	1.03	0.3927
Diet	0.0130	1	0.0130	2.39	0.1326
Day	0.0030	1	0.0030	0.54	0.4667
Diet*day	0.0007	1	0.0007	0.13	0.723
Residual	0.1635	30	0.0055		
Total	0.1804	33	0.0055		
Zn periods 4 & 7					
Model	0.0180	1	0.0180	3.55	0.0685
Period	0.0180	1	0.0180	3.55	0.0685
Residual	0.1624	32	0.0051		
Total	0.1804	33	0.0055		
Zn periods 4 & 7 normalized					
Model	0.0460	1	0.0460	0.089	0.3536
Diet	0.0460	1	0.0460	0.089	0.3536
Residual	1.6600	32	0.0519		
Total	1.7060	33	0.0517		

APPENDIX 13. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Cp d0-d85					
Model	373.8655	3	124.6218	2.14	0.1149
Day	373.8655	3	124.6218	2.14	0.1149
Residual	1865.4899	32	58.2965		
Total	2239.3555	33	63.9816		
Cp d57 & d141					
Model	120.6751	2	60.3376	0.71	0.5075
Diet history	120.6751	2	60.3376	0.71	0.5075
Residual	1274.9985	15	84.9999		
Total	1395.0000	17	82.0985		
Cp d85 & d169					
Model	263.4366	2	131.7183	5.44	0.0179
Diet history	263.4366	2	131.7183	5.44	0.0179
Residual	338.9361	14	24.2097		
Total	602.3727	16	37.6483		
Cp periods 4 & 7					
Model	1956.0676	5	391.2135	3.46	0.0099
Diet	120.0065	1	120.0065	1.06	0.3084
Day	1684.0703	2	842.0351	7.45	0.0016
Diet*day	106.5679	45	113.0622		
Residual	5087.7969	50	140.8773		
Total					
Cp periods 4 & 7					
Model	264.9742	1	264.9742	1.92	0.1726
Period	264.9742	1	264.9742	1.92	0.1726
Residual	6778.8903	49	138.3447		
Total	7043.8645	50	140.8773		
Cp periods 4 & 7 normalized					
Model	2045.25616	5	409.0512	5.06	0.0009
Diet	209.1952	1	209.1952	2.59	0.1147
Day	1684.07	2	842.0351	10.41	0.0002
Diet*day	106.56788	2	53.2894		
Residual	3638.1772	45	80.8484		
Total	5683.4334	50	113.6687		

APPENDIX 13. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Cp, d 113 & d 197 dropped					
Model	980.4457	7	140.0637	1.52	0.176
day of trial	980.4457	7	140.0637	1.52	0.176
residual	5699.1581	62	91.9219		
total	6679.6038	69	96.8059		

APPENDIX 14. REGRESSION TABLE FOR HEART RATE

Source	SS	df	MS
Model	93987.9358	6	15497.9893
Residual	76875.8126	152	505.7619
Total	169863.7480	158	1075.0870

r-square	0.5474
Adj. R-square	0.5296

	Coefficient	Std. Error	t	P>|t|
time	20.4791	3.3598	6.10	0.0000
time*diet	-0.3446	4.5585	-0.08	0.9400
$time^2$	-2.4403	0.3613	-6.75	0.0000
$time^2$*diet	-0.0288	0.4877	-0.06	0.9530
diet	-1.9784	8.8059	-0.22	0.8230
machine	-30.6210	3.6144	-8.47	0.0000
intercept	57.2831	6.7583	8.48	0.0000

APPENDIX 15. REGRESSION TABLE FOR SUPEROXIDE DISMUTASE DURING SET

Source	SS	df	MS
Model	1039101.8300	3	346367.2780
Residual	30880965.4000	71	434943.1750
Total	31920067.3000	74	431352.2600

r-square	0.0326
Adj. R-square	-0.0083

	Coefficient	Std. Error	t	P>|t|
time	9.6545	54.2356	0.18	0.8590
diet	234.2033	152.9969	1.53	0.1300
period	3.3170	51.5555	0.06	0.9490
intercept	1195.5210	389.8371	3.07	0.0030

APPENDIX 16. ANOVA TABLE FOR SUPEROXIDE DISMUTASE

Source	Partial SS	df	MS	F-value	P-value
Resting SOD					
Model	924795.7550	3	308265.2520	0.75	0.5329
Day	87132.7485	1	87132.7485	0.21	0.6493
Diet	171485.419	1	171483.419	0.42	0.5242
Day*Diet	671131.0760	1	671131.0760	1.63	0.2125
Residual	11544705.7000	28	412310.9190		
Total	12469501.5000	31	402241.9840		
Resting SOD					
Model	16366.3223	1	16366.3223	0.04	0.8439
Period	16366.3223	1	16366.3223	0.04	0.8439
Residual	12453135.2000	30	415104.5060		
Total	12469501.5000	31	402241.9840		

APPENDIX 17. ANOVA TABLE FOR WHITE BLOOD CELL COUNTS

Source	Partial SS	df	MS	F-value	P-value
Total WBC					
Model	31040637.3000	5	6208127.4500	3.84	0.0055
Diet	32952.0697	1	32952.0697	0.02	0.8871
Day	29706116.6000	1	14853058.3000	9.20	0.0004
Diet*Day	1357096.9500	2	678548.4750	0.42	0.6595
Residual	72675833.3000	45	1615018.5200		
Total	103716471.0000	50	2074329.4100		
Total WBC					
Model	12588507.6000	1	12588507.6000	6.77	0.0122
Period	12588507.6000	1	12588507.6000	6.77	0.0122
Residual	91127963.0000	49	1859754.3500		
Total	103716471.0000	50	2074329.4100		
Change WBC					
Model	31504223.9000	5	6300844.7700	5.59	4.0000
Diet	496538.6710	1	49538.6710	0.44	0.5102
Day	29706116.6000	2	14853058.3000	13.18	0.0000
Diet*Day	1357096.9500	2	678548.4750	0.60	0.5519
Residual	50699305.6000	45	1126651.2300		
Total	82203529.4000	50	1644070.5900		
Lymphocyte Count					
Model	6227115.9700	5	1245423.1900	1.15	0.3466
Diet	3887.2617	1	33887.2617	0.03	0.8602
Day	6175327.9700	2	3087663.9800	2.86	0.0677
Diet*Day	17075.6541	2	8537.8271	0.01	0.9921
Residual	48576977.3000	45	1079488.3800		
Total	54804093.3000	50	1096081.8700		
Lymphocyte Count					
Model	2089708.5000	1	2089708.5000	1.94	0.1697
Period	2089708.5000	1	2089708.5000	1.94	0.1697
Residual	52714384.8000	49	1075803.7700		
Total	54804093.3000	50	1096081.8700		

APPENDIX 17. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Lymphocyte Change					
Model	6203829.2400	5	1240765.8500	1.36	0.2588
Diet	10600.5231	1	10600.5231	0.01	0.9148
Day	6175327.9700	2	3087663.9800	3.37	0.0431
Diet*Day	17075.6541	2	8537.8271	0.01	0.9907
Residual	41179034.8000	45	915089.6610		
Total	47382864.0000	50	947657.2800		
Diff. Neutrophil					
Model	484.3374	5	96.8675	0.58	0.7135
Diet	10.5689	1	10.5689	0.06	0.8022
Day	456.4744	2	228.2372	1.37	0.2642
Diet*Day	25.9646	2	12.9823	0.08	92.5100
Residual	7489.8194	45	166.4404		
Total	7974.1569	50	159.4831		
Diff. Neutrophil					
Model	93.1985	1	93.1985	0.58	0.4502
Period	93.1985	1	93.1985	0.58	0.4502
Residual	7880.9583	49	160.8359		
Total	7974.1569	50	159.4831		
Change Diff. Neutrophil					
Model	479.0302	5	95.8060	0.55	0.7343
Diet	5.2617	1	5.2617	0.03	0.8623
Day	456.4744	2	228.2372	1.32	0.2772
Diet*Day	25.9646	2	12.9823	0.08	0.9278
Residual	7779.5972	45	172.8799		
Total	8258.6275	50	165.1725		
Diff. Lymphocyte					
Model	379.3031	5	75.8606	0.46	0.8039
Diet	7.4142	1	7.4142	0.04	0.8331
Day	323.6144	2	161.8072	0.98	0.3828
Diet*Day	59.6144	2	29.8072	0.18	0.8353
Residual	7422.7361	45	164.9497		
Total	7802.0392	50	156.0408		

APPENDIX 17. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Diff. Lymphocyte					
Model	176.0392	1	176.0392	1.13	0.2927
Period	176.0392	1	176.0392	1.13	0.2927
Residual	7626.0000	49	155.6327		
Total	7802.0392	50	156.0409		
Change Diff. Lymphocyte					
Model	387.5752	5	77.5150	0.52	0.7621
Diet	15.6863	1	15.6863	0.10	0.7479
Day	323.6144	2	161.8072	1.08	0.3486
Diet*Day	59.6144	2	29.8072	0.20	0.8205
Residual	6748.1111	45	149.9558		
Total	7135.6862	50	142.7137		
Diff. Monocyte					
Model	45.7859	5	9.1572	1.61	0.1767
Diet	0.4804	1	0.4804	0.08	0.7726
Day	38.5997	2	19.2998	3.39	0.0424
Diet*Day	4.9526	2	2.4763	0.44	0.6496
Residual	255.8611	45	5.6858		
Total	301.6471	50	6.0329		
Diff. Monocyte					
Model	4.3924	1	4.3924	0.72	0.3990
Period	4.3924	1	4.3924	0.72	0.3990
Residual	297.2546	49	6.0664		
Total	301.6471	50	6.0329		
Change Diff. Monocyte					
Model	51.4330	5	10.2866	0.88	0.5042
Diet	6.1275	1	3.1275	0.52	0.4736
Day	38.5997	2	19.2998	1.65	0.2043
Diet*Day	4.9526	2	2.4763	0.21	0.8105
Residual	527.8611	45	11.7302		
Total	579.2941	50	11.5859		
Diff. Eosinophil					
Model	10.5302	5	2.1060	0.65	0.6661
Diet	0.3728	1	0.3728	0.11	0.7369
Day	8.2358	2	4.1179	1.26	0.2627
Diet*Day	1.5691	2	0.7846	0.24	0.7872
Residual	146.7639	45	3.2614		
Total	157.2941	50	3.1459		

APPENDIX 17. CONTINUED

Source	Partial SS	df	MS	F-value	P-value
Diff. Eosinophil					
Model	3.6645	1	3.6645	1.17	0.2849
Period	3.6645	1	3.6645	1.17	0.2849
Residual	153.6296	49	3.1353		
Total	157.2941	50	3.1459		
Change Diff. Eosinophil					
Model	10.7337	5	2.1467	0.50	0.7722
Diet	0.5763	1	0.5763	0.14	0.7149
Day	8.2358	2	4.1179	0.97	0.3886
Diet*Day	1.5692	2	0.7846	0.18	0.8326
Residual	191.9722	45	4.2660		
Total	202.7059	50	4.0541		
Diff. Basophil					
Model	1.6609	5	0.3322	0.94	0.4675
Diet	0.0221	1	0.0221	0.06	0.8043
Day	1.5212	2	0.7606	2.14	0.1293
Diet*Day	0.1095	2	0.0547	0.15	0.8576
Residual	15.9861	45	0.3552		
Total	17.6471	50	0.3529		
Diff. Basophil					
Model	0.4804	1	0.4804	1.37	0.2473
Period	0.4804	1	0.4804	1.37	0.2473
Residual	17.1667	49	0.3503		
Total	17.6471	50	0.3529		
Change Diff. Basophil					
Model	1.7590	5	0.3518	1.16	0.3438
Diet	0.1201	1	0.1209	0.40	0.5324
Day	1.5212	2	0.7606	2.51	0.0928
Diet*Day	0.1095	2	0.0547	0.18	0.8355
Residual	13.6528	45	0.3034		
Total	15.4118	50	0.3082		

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Refereed Publication

Wagner, E.L., G.D. Potter, E.M. Eller, P.G. Gibbs, and D.M. Hood. 2005. Absorption and retention of trace minerals in adult horses. *Professional Animal Scientist.* 21:207

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