THE EFFECT OF DIETARY COBALT CONCENTRATIONS ON FIBER DIGESTION IN HORSES

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ABSTRACT

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Preliminary research suggests that increasing dietary Cobalt (Co) concentrations above NRC (2007) requirements appears to increase fiber digestion in horses, but the effects of its supplementation have not yet been evaluated. This study aimed to determine if the addition of varying concentrations of Co held implications for increasing fiber digestion. The study utilized four standardbred geldings and followed a 4x4 Latin square design; four 5-wk periods, each containing a 2-wk washout phase followed by a 3-wk treatment phase. Two feedings a day were accompanied by 5 treatment tablets, which were formulated to deliver 0.0 mg, 2.8 mg, 8.4 mg, or 14.0 mg of Co to the horses. Feces and urine were to evaluate fiber digestion and mineral digestibility. Although there was a trend showing a linear increase in dry matter and neutral detergent fiber digestibility ($P=0.06$), there was no effect of Co supplementation on the digestibility of neutral detergent fiber, acid detergent fiber, or lignin. There was an inverse linear relationship between supplemental Co concentration and serum cobalamin levels ($P=0.003$), while folate levels were unaffected. Treatment differences wee seen in Co digestibility with a trends in Co apparent absorption and retention. Additionally, serum Co showed differences between treatments ($P <0.0001$), as the high treatment (4.7 μg/mL) was greater than the control treatment (0.8 μg/mL).

(Key Words: cobalt, digestion, fiber, horse, mineral, nutrition)

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INTRODUCTION

The compilation of non-starch polysaccharides and lignins, more commonly known as dietary fiber (DF), is a critical component of animal feedstuffs. The complex micro-organization of these fibers renders them resistant to enzymatic digestion within the mammalian digestive system (Wenk, 2001). Due to this inability of herbivorous animals to digest DF, they have developed a symbiotic relationship with the microflora of the GI tract in order to efficiently degrade the indigestible matter. In foregut fermenters, the breakdown of DF takes place *before* it enters the small intestine (primary site of absorption) and thus the rate of absorption is maximized; coupled with a slow rate of passage, these factors work together to increase utilization of available energy.

In the hindgut fermenter, however, DF remains mostly intact throughout the small intestine, thus resulting in an inevitable decrease in the absorption of available nutrients (Graham and Aman, 1991). In principal, an upsurge in the microbial colonies within the hindgut will cause an elevation in fiber digestion and thus lead to increased energy efficiency. In addition to bacterial colonies in the GI tract, other plausible factors including feed additives, non-forage fiber sources, forage composition, and forage/concentrate ratios have been evaluated for their potential assistance in hindgut fermentation. Of particular interest to this study, however, is the addition of varying concentrations of supplemental Co and its potential influence on non-ruminant fiber digestion. Additionally, it is of interest to

explore the role of Co supplementation in serum cobalamin (vitamin B_{12}) synthesis; as Co acts as a precursor for the endogenous synthesis of this vitamin.

CHAPTER 1. Review of the Literature

Fiber

Fiber is defined as the indigestible components of roughage, and is considered an important factor in a balanced diet (Mayo Clinic, 2012). Also known as non-starch polysaccharides (NSP), this dietary component is found in the cell walls of long stem forage. These cell walls are comprised of elements such as cellulose, hemicellulose, and lignin, (Lattimer and Haub, 2010) which play a major role as the "building blocks" of the polysaccharides (Theander et al., 1989). These dietary fibers are indigestible in the small intestine and require fermentation in the large intestine for digestion and maximal absorption. Non-starch polysaccharides can be categorized as either soluble or insoluble polysaccharides. Soluble NSPs, such as beta-glucans, pectins, and fructans, dissolve in liquid to form a gel-like substance (Kumar et al., 2012). This gel can then undergo digestion via bacterial fermentation by the microflora population of the large intestine. Insoluble NSPs, such as lignin, cellulose, and hemicellulose, however, cannot dissolve in solution; thus reducing the rate and extent to which they may be digested by microbes (Wong and Jenkins, 2007).

Fiber's Role in the Diet

Equine. In both ruminants and nonruminants, fiber plays an important role in a well-balanced diet. A healthy system requires fiber for gastrointestinal health, efficient digestive function, and many other bodily functions. Fiber plays an

especially important role in digestive system of grazing animals, such as horses. The digestive system of the horse was designed to support a grazing eating pattern, one in which food intake is relatively constant throughout the day. The high fiber content of the consumed forage allows for efficient movement of the bolus through the intestines, and promotes proper water and electrolyte balance. Feeding a high carbohydrate diet deficient in fiber will increase the chance of impaction in the GI tract, and can ultimately lead to colic. Additionally, a massive influx of carbohydrates without adequate fiber tends to overwhelm the microbial population in the gut ultimately resulting in laminitis (Canning, 2014).

Digestibility of Dietary Fiber

Since forage plays such a significant role in the diet of large grazing animals, it is of great importance to quantify the capacity of its available energy. Due to the fact that nearly two-thirds of DF remains indigestible to "simple stomached" animals (non-ruminants), a large portion of these forages is not utilized as an energy substrate (Buxton and Redfearn, 1997).

The cell walls of long stem forage, such as hay, are primarily comprised of thousands of linearly-linked glucose monomers called cellulose (Theander et al., 1989), which encapsulate the nutrient dense components of some feeds. As an indigestible fiber, cellulose consequently limits the usable energy of hay and other sources of fodder in the equine diet. Cellulose can, however, be digested via microbial fermentation in the cecum and colon these animals. Therefore, the

process of hindgut fermentation plays a critical role in the digestion and absorption of the DF found in many types of forage (Fonty et al., 1989).

Equine fiber digestibility has been documented at 24-45% for acid detergent fiber (ADF), 38-55% for neutral detergent fiber (NDF), and 40-61% for dry matter (DM) (Crozier et al., 1997; Ordakowski-Burk et al., 2006; Cymbaluk, 1990; Pagan and Jackson, 1991). The wide range in these values is due to the maturity of the grasses as time of harvest.

Factors Affecting the Digestibility of Fiber

Starch Additives. The question is then raised as to what factors can possible affect the digestibility of fiber. Few studies have examined these possible variables in the equine model; however, a ruminant study at the University of Georgia set out to determine if the addition of starches to long stem forage had any impact on the rate of ruminal digestion, lag time of fiber, and the overall digestibility of the fiber itself. Purified corn and wheat starches were added to alfalfa, coastal Bermudagrass, fescue, and orchardgrass hays. The differing hays were then fed to Holstein cows and the rate/extent of digestion and lag time of fiber were measured in vitro. The results of the study concluded that despite decrease in the rate of passage, the extent of the fiber digestion itself was not affected (Mertens and Loften, 1978). Therefore, adding starches to forage does not appear to greatly alter fiber digestion in the ruminant.

Composition of Diet. Additionally, it was thought that the composition of the dietary fiber itself might play a role in its digestibility. In ruminant animals, the

available ruminal energy limits the growth of bacteria used in the process of digestion. Varga and Kolver (1997) concluded that changing the forage to concentrate ratio of the ruminant's diet leads to an increase in usable energy. It has been suggested that a diet higher in concentrate (nonstructural carbohydrates), and lower in forage (structural carbohydrates), may lead to a decrease in fiber digestion. This digestive reduction is due to the rapid breakdown of these nonstructural carbohydrates (Varga and Kolver, 1997) leading to a possible decrease in retention time in the gut, and ultimately resulting in an overall reduction in the digestion of fiber.

Physical Form of Roughage. Alterations in the physical form of forage have also been evaluated for consequent effects on digestibility. Many studies (Weston and Hogan, 1967; Alwash and Thomas, 1971; Beever et al., 1972) found that the grinding and pelleting of alfalfa and coastal Bermudagrass leads to a decrease in digestibility, most likely due to the increase in passage rate through the GI tract. However, due to the fact that horses digest forage at about 66% efficiently as cows, it is important to consider possible differences in these values when using an equine model (Hintz et al., 1969). Non-ruminants are often claimed to be at a disadvantage when it comes to fiber digestion, as their fermentation occurs past the principal site of digestion and absorption, giving fiber little time to fully digest (Hintz et al., 1978).

A Michigan State University study was conducted to evaluate the effects of chopping hay prior to feeding, using an equine model. Researchers hypothesized that the increase in surface area resulting from chopping the hay would lead to

increased levels of microbial fermentation, ultimately allowing for a more complete digestion of feedstuffs. However, the results concluded that chopping forage did not lead to changes in fecal particle size, and therefore had limited physiological implications on digestion (LeCompte et al., 2013).

Measuring the Digestible Energy of Fiber

In 1963, Van Soest coined the most commonly used and accepted method of fiber analysis. As his work on fiber analysis developed, he discovered a separation of plant protein from lignin; both of which are alkali-soluble. In an effort to circumvent this confounding factor, experiments were conducted using various detergents under varying temperatures and pH conditions. The final chemical analyses derived from his series of experimental fiber extractions were neutral detergent fiber (NDF) analysis, acid detergent fiber (ADF) analysis, and lignin analysis; these methods are widely used and accepted today (Van Soest, 1978).

Neutral Detergent Fiber. Neutral detergent fiber is an insoluble structural carbohydrate comprised of three main elements: cellulose, hemicellulose, and lignin. In addition to being indigestible itself, lignin also hinders the digestion of the associated cellulose and hemicellulose (Robinson, 1999). The original NDF method has been altered over the years to accommodate for interferences. The addition of amylase was incorporated in order to remove starches from the sample materials being analyzed. Later, sodium sulfite became a common supplement to the NDF procedure. The purposes of this additive are to lower protein levels and remove

keratinaceous animal residues. The sulfite acts to cleave the disulfide bonds and dissolve cross-linkages between proteins (Van Soest et al., 1991).

Acid Detergent Fiber. Acid detergent fiber is designed to determine the cellulose, lignin, and acid-insoluble ash. Sodium sulfite is not used in measuring ADF because the sulfite attacks lignin; hence making it an impractical additive to the process, as ADF and lignin are sequential processes. Although ADF and lignin are acceptable sequential processes, NDF is not. Due to the fact that biogenic silica and pectin are soluble in the neutral detergent reagents, and insoluble in the acid detergent reagents, hemicellulose estimated by way of subtracting ADF from NDF would be inaccurate (Van Soest et al., 1991).

Lignin. In his 1978 article on fiber analysis, Van Soest explained that the ADF procedure leaves behind a residue known as lignocellulose. Lignin, as it is more commonly known, is a network of phenylpropane units, which are partially linked to the hemicellulose in the cell wall (Theander et al., 1989). This residual substance contains other fractions such as silica, and unavailable proteins.

Cobalt

Discovered by Swedish chemist George Brandt in 1739, Co is a chemical element most commonly known for its magnetism and deep blue pigment. Cobalt can be found in two ore forms; cobaltite (CoAsS) and erythrite ($Co_3(AsO_4)_2$). However, it is primarily produced as a byproduct of copper and nickel mining. With

an atomic number of 27, Co^{59} is not only the most stable cobalt isotope, but is also the only cobalt isotope known to exist naturally on Earth. Co^{60}, however, is produced commercially for use in the production of gamma rays and as an agent for radioactive labeling (Jefferson Lab, 2014).

As a ferromagnetic metal, cobalt is a fundamental component of permanent magnets, and is also coined for its role in pigmentation. Furthermore, Co is involved in much of modern day engineering. The temperature stability of these Co alloys deem them useful in the construction of aircraft engines and gas turbines, while their corrosion and wear-resistant qualities make them highly suitable for orthopedic implants such as prosthetic hip and knee replacements (Royal Society of Chemistry, 2015).

Cobalt's Role in the Digestive System

In addition to its role in engineering and modern day technology, Co is also an essential element of a well-balanced diet of many animals. Metal ions are essential in proteins as they are responsible for electron transfer, nucleophilic catalysis, and maintaining the structure of the protein itself (Jelkmann, 2012). It is theorized that lignin may interfere with the microbial breakdown of fiber via physical barriers due to cross-linked polysaccharides (Buxton and Redfearn, 1997). It is believed, however, that supplementation of cobalt may play a role in increasing the digestibility of these fibers (Hussein et al., 1994). A preliminary study suggested that supplementing dietary Co beyond the NRC (2007) recommendations could

increase fiber digestion in horses (Larson, 2013). It is thought that the properties of Co as a divalent cation serve a bridge between both negatively charged gut microflora and plant cell walls. In doing this, the cobalt may aid in enhancing bacterial activity and thus ultimately improving fiber digestion (Hussein et al., 1994).

Cobalt Deficiency

Ailments as a direct result of depleted Co levels include "pining," "nakuruitis," "vinquish," and "wasting disease," all of which have been successfully treated with small doses of Co (Underwood, 1956). A pilot study conducted by Lines (1935) illustrated a dramatic increase in appetite, growth rate, and hemoglobin levels in sheep treated with 1 mg of Co per day, administered orally. However, due to the nonspecific symptoms of Co-deficiency, it is nearly indistinguishable from emaciation (Underwood, 1956).

A Co-deficiency has not been reported in horses; however, a vitamin B_{12} deficiency could be a likely symptom indicative of an underlying lack of Co (Vervuert and Kienzel, 2013). Most feed included in the equine diet ranges from 0.05 to 0.6 mg Co/kg DM, making a deficiency in Co rather unlikely. The variation in the Co concentration of feed is dependent upon where it is grown, as the soil in which it grows may be deficient in Co. Therefore, feed harvested from areas in Australia, East Africa, New Zealand, Norway, and even some parts of the United States, are at risk of containing levels of Co inadequate for optimal equine health (Ammerman, 1970).

Cobalt Requirements and Toxicity

Cobalt has been proven an essential mineral in sheep (Underwood and Filmer, 1937), cows (Neal and Ahmann, 1937), and in dogs (Frost et al., 1940), however, the research behind the role of Co in the equine diet is quite limited. The 2005 NRC set the maximum tolerable concentration of Co at 25 mg/kg of DM intake for most species. Although Verveurt and Kienzle (2013) suggest that horses have a tolerance for high concentrations of Co, the European Food Safety Authority has suggested limiting the concentration of Co to 0.3 mg Co/kg of complete feed (EFSA, 2009). The 2007 NRC set the minimum requirement for horses at 0.05 mg Co/kg DM, lowering it from 0.1 mg Co/kg DM as suggested by the 1989 NRC recommendations.

Cobalt is moderately toxic in instances of single-dose over exposure; however, chronic abuse can lead to severe long-term side effects. Consuming high doses of Co can interfere with proper organ function and lead to thyroid impairments, cardiotoxicity, and even heart failure (Mørkeberg, 2013). Some studies have gone as far as to illustrate a potential for tumor propagation as a result of overexposure (Semenza, 2003).

Cobalt's Role in Erythropoesis

Cobalt is known for its hypoxia-inducing qualities, and has been used pharmaceutically in the past to treat anemia. However, it is no longer utilized for its hematopoietic effects due to undesirable adverse complications (Simonsen, 2012). It functions altering the hypoxia inducible factor (HIF) pathway, which allows for

the cells and tissues to acclimate to decreases in oxygen availability (Ho et al., 2015). Due to its ability to concertedly inhibit HIF regulatory enzymes and stabilize the involved transcription factors, Co^{2+} supplementation increases the expression of erythropoietin (EPO) and thus increases red blood cell (RBC) populations in hypoxic conditions (Dery et al., 2005).

Cobalt as a Performance-Enhancing Drug.

Over the past several decades, the usage of Co as a performance enhancement tool has shown an increased prevalence in human athletes. Cobalt has proven capable of stimulating the aforementioned cellular hypoxia in an effort to induce elevated expression of HIF, ultimately resulting in an increase in EPO and, subsequently, RBC accumulation (Mørkeberg, 2013). Much research has been conducted on the common practices behind human blood doping and performance enhancement via tissue manipulation. Results show that Co produces effects similar to recombinant human EPO (Mørkeberg, 2013).

In addition to its short four-hour window of detection, most drug test panels commonly used do not include testing for Co levels (Indiana Horse Racing Commission, 2014). Being that this mineral is easily obtained and difficult to test for, it is marketed as an opportune means of modern human blood doping. Accompanying these "favorable" characteristics, however, is the fact that Co can also be fatal if administered chronically in exceedingly large doses (Kynch et al., 2014).

As of late, the practice of Co supplementation for performance enhancement has been causing health problems in the horse industry. When exploring the topic of equine performance-enhancing drugs, Co has not been a usual suspect until recently. Before December of 2014, Co was not even measured in post-mortem necropsy reports due to its nearly non-existent prevalence as an equine performance-enhancing drug. Although Co is not among the 15 pages of substances listed in the Association of Racing Commissioners International (ARCI) *Uniform Classification Guidelines for Foreign Substances*, its recent abuse on the racetrack has been considered grounds for the banishment of two New Jersey trainers. A string of race-related deaths at Hollywood Park racetrack in California has officials speculating that Co is to blame for the sudden death of 7 equine athletes over a 16-month period of time; all horses belonged to the same trainer and were under the medical care of the same veterinarian (Paulick, 2014).

Cobalt as an Essential Precursor to Cobalamin Synthesis

The microflora in the colon and cecum of the large intestine require adequate levels of Co for the synthesis of vitamin B_{12}, also known as cobalamin (Davies et al., 1971; Mitchell et al., 2007). Therefore, clinical signs of cobalt deficiency are most likely expressions of a reduction in vitamin B_{12} concentrations in the tissues.

Vitamin B_{12}

As a water-soluble molecule, B_{12} is the only metal-containing vitamin (Stillman, 2010), which makes it more complex than any other essential dietary vitamin (Banerjee, 1997). Vitamin B_{12} is formed from cobalt-containing compounds called corrinoids; when these corrinoids are assembled with fifth and sixth position ligands, they are considered cobalamins.

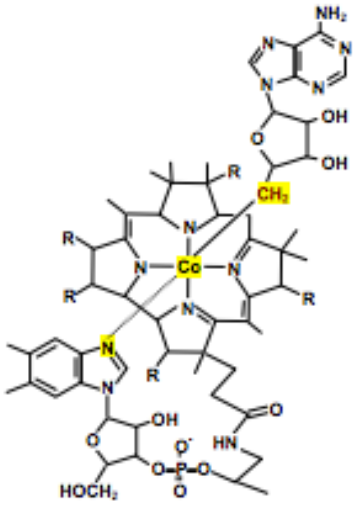

Figure 1. The structural composition of cobalamin: a nucleotide attached to a corrin ring with Co at its center.

Function. Cobalamin exists as either cyanocobalamin or hydroxycobalamin. Cyanocobalamin is the synthetic form found in common over-the-counter B_{12} supplements (National Institutes of Health, 2011). As an enzyme cofactor, it exists as one of two activated forms: methylcobalamin or adenosylcobalamin. There are

several enzymatic reactions in mammalian cells that require these enzymatic forms of cobalamin. One reaction requires methylcobalamin to act as a methyl group donor in the conversion of homocysteine to methoinine; this methyl transferase is crucial in the production of RBC (Ammerman, 1970). The other reaction requires adenosylcobalamin, which acts as a cofactor in the isomeration of L-methylmalonyl-CoA to succinyl CoA in the metabolism of propionate and several amino acids (Takahashi-Iniguezet al., 2012). Additionally, methyltethydropholate (the methyl group of methylcobalamin) provides adequate levels of both folate and cobalamin for normal erythropoiesis, while succinyl CoA (cofactor adenosylcobalamin) is required for heme porphorin and therefore is a vital contributor to hemoglobin synthesis (Diwan, 2008).

Transport and Digestion. The breakdown of cobalamin is inhibited by a glycoprotein called haptocorrin (HC), which is secreted by the salivary glands during mastication (Jelkmann, 2012). After ingestion, pepsin and gastric acids (such as HCl) work together to release the cobalamin from the ingested proteins, thus allowing for HC binding. In the stomach, cobalamin binds to a glycoprotein known as intrinsic factor (IF). This cobalamin-IF complex ensures resistance to enzymatic breakdown as it travels through the GI tract. Once the pair reach the distal portion of the small intestine, IF then recognizes receptors on the surface of iliac mucosal cells allowing for absorption. Due to their symbiotic relationship, a deficiency in IF can cause a subsequent vitamin B_{12} deficiency. The consequent decrease in B_{12} levels (considering the role vitamin B_{12} plays in erythropoesis) may result in

pernicious anemia and a secondary deficiency in folic acid, as vitamin B_{12} is required for the conversion of N5-mtheyl-tetrahydroflate to tetrahydropholate.

Cobalamin Deficiency

A deficiency in Co can lead to many health disorders and developmental problems. Studies have concluded that cobalt deficiency in many animals can result in mild to severe anemia and even death. This anemia is a direct result of cobalt's relationship with copper and their synergistic involvement in hematopoesis (Ammerman, 1970).

Deficiencies in cobalamin are widespread across the globe and affect at least 15% of the elderly population. The most common cause of cobalamin deficiency is gastric atrophy, which leads to a decrease in the secretion of IF and HC (Jelkmann, 2012). In addition to gastric atrophy, GI-related diseases such as pancreatic disorders and intestinal parasites can interfere with absorption of cobalamin and result in deficiency. "Manifest cobalamin deficiency" is expressed by general weakness of the body and megoblastic anemia due to an inability of erythropoiesis to maintain proper function. However, it takes many years to experience symptoms of cobalamin deficiency due to the fact that endogenous cobalamin stores can supply daily needs for 2 to 3 years. Cobalamin deficiency can also cause a neurological disease due to demyelination of the spinal cord. This disease results in symptoms such as paresthesia of the extremities, slowed reflexes in the deep tendons, and eventually loss of cognitive function (Lindenbaum et al., 1988)

Supplementation

A 1987 study conducted by Quirk and Norton concluded that supplementing pregnant ewes with Co increased the birth weight of the lambs. In a study out of Nairobi, Kenya, researchers observed the effects of cobalt supplementation on short-horned East African goats. After being fed a Co-deficient diet for 23 weeks, Mburu et al. (1993) observed higher serum B_{12} levels after 3 weeks of administering an oral Co drench to goats; these levels remained elevated for the remaining 20 weeks of the project.

A 1947 study determined that oral supplementation was more productive than IV supplementation, as inadequate amounts of Co reached the rumen and thus was unable to aid in the synthesis of B_{12} (Comar and Davis). However, Co supplementation has not been evaluated in horses.

CHAPTER II: Does supplemental cobalt effect fiber digestion?

Materials and Methods

Housing. The project utilized 4 mature Standardbred geldings (n=4) with an average body weight of 503 kg $\pm$ 37. The horses were housed at the Michigan State University Horse Teaching and Research Center in 3.7 x 3.7 m box stalls bedded with recycled and shredded newspaper. The Michigan State University Institutional Animal Care and Use Committee approved all methods. Weather permitting, the horses were turned out daily in a fenced paddock. To prevent potential confounding factors caused by consumption of feedstuffs other than the provided diet, horses were not permitted turn out once the snow cover melted. During times when turnout was unavailable, horses were exercised on a mechanical walker for 30 minutes each morning, with exception to total collection periods.

Feeding. All horses were provided with ad libitum access to water, and were fed grass hay at the rate of 1.6% BW, divided into two equal feedings per day. After each feeding, hay rations were assessed for any orts. Prior to the onset of the study, adequate quantities of a single hay source were sampled and analyzed to determine mineral concentration (Table 1). Hay samples were analyzed periodically throughout the duration of the study to ensure that the mineral intake remained consistent with the initial hay source analyzed.

Table 1. Analysis of forage composition measured prior to the start of the study, analyzed by Equi-Analytical Laboratories, Ithaca, New York.

Unit Measured	As Fed	Dry matter
% Moisture	13.1	
% Dry Matter	86.9	
% Ca	.56	.65
% P	.29	.34
% Mg	.24	.27
% K	2.24	2.58
% Na	0.015	0.017
% S	0.20	0.23
ppm Cu	6.5	7.4
ppm Zn	17.4	20.0
ppm Mn	59.6	68.6
ppm Mo	4.1	4.7
ppm Co	0.07	0.08

Body Weight and Condition. Each week, the horses were assessed for body condition score (BCS) (Henneke et al., 1983) by two researchers and weighed on a digital scale to detect changes in body mass. Over the course of the study, the forage intake was adjusted to meet the daily caloric needs of each horse in an effort to maintain starting weight and BCS.

*Cobalt Supplementation***.** The 20-week study followed a 4x4 Latin square design divided into four 5-week periods. The first 2 weeks of each period were "wash out" phases, during which the horses consumed only hay and were not provided a Co supplement. The washout period was then followed by a 3-week treatment phase. Each of the 4 horses was randomly assigned to one of the 4 cobalt-supplementation treatments: 0.0 mg (control), 5.6 mg (low), 16.8 mg (medium) or 28.0 mg (high). The treatments were administered in tablet form via apple-flavored sucrose reward tablets produced by Purina Test diet. The tablets were formulated to contain 2.0% COPRO®25 (Zinpro Corporation, Eden Prairie, MN) and deliver Co in the form of Co Glucoheptonate (0.1 g COPRO/5 g tablet). Plain sucrose tablets were administered as a placebo in order to create a control group. The tablets were color-coded to denote mineral content; green tablets contained only sucrose, while orange tablets were fortified with 2.8 mg of Co. The tablets were split between 2 equal daily feedings; 5 tablets were fed to each horse per feeding according to treatment group, as illustrated in Table 2. Thus, for the 5.6 mg (Low) dosage, the horse was provided one 2.8 mg Co tablet along with 4 sucrose tablets at each feeding. For the 16.8 mg or (Medium) dosage, the horse was provided three 2.8 mg tablets and 2 sucrose tablets per feeding. For the 28 mg (High) dosage, five 2.8 mg tablets were provided at each feeding. The 0.0 mg dosage (Control) received 5 sucrose tablets at each feeding. Table 3 shows the concentration of Co supplemented for each treatment. The mineral contents of both the sucrose and treatment are listed in Table 4.

Table 2. Treatment level administered to each horse during treatment periods.

Horse	Period 1	Period 2	Period 3	Period 4
A	High	Low	Control	Medium
C	Control	High	Medium	Low
D	Medium	Control	Low	High
H	Low	Medium	High	Control

Table 3. Amount and concentration of Co supplement provided to each treatment group

Treatment	Supplemental dietary Co provided (mg Co/day)	Total dietary Co (including from hay) on a mg/kg DM basis
Control	0.0	0.06
Low	5.6	0.7
Medium	16.8	2.0
High	28.0	3.0

Table 4. Mineral concentrations of supplemental tablets as analyzed by Michigan State University Diagnostic Center for Population and Animal Health.

	Treatment Tablet	**Sucrose Tablet**
Ca (mg/g)	2.4	0.02
P (mg/g)	-0.006	0.0
Mg (mg/g)	0.4	0.5
Cu (mg/g)	0.0002	0.0001
Zn (mg/g)	0.04	0.002
Se (mg/g)	0.000008	0.000008
Mn (mg/g)	0.003	0.0001
Co (mg/g)	.56	0.008

*Total Collections***.** Total collections of urine and feces were performed on days 11 to 14 and days 32 to 35 of each period. These collections served as a means to compare fiber and mineral apparent digestibility within treatment groups, as well as measure mineral retention. Feces and urine were collected via a total collection harness (Equisan Marketing Pty Ltd, Melbourne). Sampling took place for three days

and excrement was collected every 8 hours, resulting in 9 samples collected per horse during each collection. Each horse was fitted with a padded harness worn only for the duration of each collection. The harnesses were thoroughly washed with hot water and bleach after each usage, and were properly stored between collections. The harness remained with the respective horse for the remainder of the collections. When samples were gathered, the harnesses were emptied of all excrement; urine was transferred into clean vessels and feces were placed into a plastic sanitary bag. Both the urine and the feces were well mixed and homogenized to ensure the sub-sample collected was an accurate representation of the entire collection. Fecal samples were broken down and mixed by hand. Urine samples were thoroughly blended using a clean stirring rod. All collected feces were weighed and recorded. Ten percent of each collection was retained in sanitary bags and frozen for further analysis. Similarly, each of the urine samples was measured and recorded. Again, 10% of each urine collection was retained and frozen for further analysis. Total fecal weights and urine volumes can be found in Appendix A (Tables 1 and 2).

Blood samples were drawn via jugular venipuncure with a 20 G needle and vacuum tube (BD Vacutainer®, Becton Dickenson, Franklin Lakes) on days 14 and 35 of each period, and preserved for serum analysis.

Sample Analysis

*Serum Evaluation***.** Blood samples from days 14 and 35 of each period were spun in a centrifuge at 3,500 x g for 20 minutes. Three aliquots of blood serum from

each sample were collected and frozen at -20° C. The frozen serum samples were analyzed by Michigan State's Diagnostic Center for Population and Animal Health via ICP mass spectroscopy for concentration of Co, Cu, Mn, and Se. Duplicate serum samples were sent to be analyzed for cobalamin and folate levels at the Gastrointestinal Laboratory in the Department of Small Animal Clinical Sciences at Texas A&M University. The samples were analyzed using the Immulite 2000 system (Siemens AG, Munich, Germany). Each serum sample was placed in a reaction tube and treated with dithlothreitol (DTT) and a sodium hydroxide/potassium cyanide solution (NaOH/KCN). After a 30-minute incubation period, the sample was transferred to a second reaction tube containing porcine intrinsic factor (HIF) and a vitamin B_{12} coated polystyrene bead. Again, the tube was incubated for 30 minutes. Then, during the third and final incubation period, alkaline phosphatase-labeled anti-HIF was introduced to bind any HIF immobilized on the B12 coated bead. Any residual unbound enzyme conjugate was removed via centrifugal wash.

Fecal Samples. At the end of each 72-hour total collection, all urine and fecal samples were brought into the laboratory for further preparation. An individual composite of the 9 fecal samples from each horse was mixed, and two 0.5 kg sub-samples from each horse were then frozen at -20° C and retained for further analysis. Later, one fecal sub-sample per horse from each collection was freeze-dried, while the other remained unaltered in deep freeze. A portion of the freeze-dried feces was then broken up, well-mixed, and ground in a Cyclone mill (1093 Cyclotec Sample Mill, Foss Tecator, Eden Prairie, MN). The ground feces, as well as

the remaining freeze-dried feces, were retained in sterile storage bags for further evaluation. The hay samples collected on days 11-14 and 32-35 of each period were ground in a Wiley Mill (Parr Instrument Company Inc., Moline, IL) using a 1-mm screen, and stored in sterile bags for later analysis. Although researchers observed each horse for feed refusals, there were no orts from any of the subjects.

Urine Evaluation. Similarly, upon the completion of each 72-hour collection, the 9 urine samples from each horse were combined to create one composite sample per horse; two 100-mL sub-samples from each were retained. One sub-sample remained unaltered while the second was treated with 2 µl of 12 M HCl acid to dissolve urine sediment. Each of these samples were then further acidified with varying amounts of additional 12 M HCl to achieve a pH of 4 (Vitros Chemistry, Version 5). The dilution factor of each sample was calculated and accounted for accordingly. The acidified urine samples were then sent to Michigan State's Diagnostic Center for Population and Animal Health to be analyzed via ICP mass spectroscopy for mineral concentration.

*Dry Matter***.** Each sub-sample of feces (freeze-dried and unaltered) and feed was then evaluated for dry matter content. The unaltered feces were allowed to thaw at room temperature. Portions from each fecal and feed sample were weighed out at 0.5 g, placed in pre-weighed aluminum crucibles, and oven dried (Thelco®, Precision Scientific, Winchester, VA) at 105° C for 24 hours. The samples were then weighed back to determine dry matter content. The freeze-dried fecal samples were evaluated for dry matter using a hot weight method. A 0.5 g portion from each

sample was placed in a pre-weighed ceramic crucible, dried at 105° C for 24 hours, and weighed back for dry matter content. All dry matters were performed in triplicate with accepted CV's under 3%.

*Microwave Digestion***.** Each of the ground, freeze-dried fecal samples was then prepared for microwave digestion. Bovine liver powder and peach leaf powder (1577b; NIST, Gaithersburg, MD) were digested along with each sample to serve as a control. Each vessel was loaded with 0.4 g of ground feces and 10 mL of concentrated HNO_3. The vessels were left slightly covered at room temperature overnight to allow for initial digestion. After the allotted time period, all vessels were placed into protective sleeves and assembled into support modules. One module acted as a control vessel, which housed a temperature port and a pressure probe. All vessels were then loaded into the turntable and placed in the microwave digester at 180° C, 1200 W, 200 PSI, for 30 minutes. After the digestion was complete, the turntable was removed from the microwave and allowed to cool for 10 minutes beneath the fume hood. Each vessel was vented to alleviate pressure build up, and the modules were then disassembled and cooled. After the vessels reached room temperature, 2.0 mL of 30% H_2O_2 was added to each sample. After 20 minutes, the samples were then poured into individual 25 mL volumetric flasks. In order to ensure nothing was left behind, deionized water was used to rinse the remaining sample from each vessel into the respective flask. The samples were then brought up to volume (25 mL) using deionized water, and carefully poured into

labeled 50 mL conical bottom tubes. The final samples were then analyzed for mineral content via inductively coupled plasma mass spectrometry (ICP-MS) by Michigan State's Diagnostic Center for Population and Animal Health. Percent apparent digestibility of minerals was calculated as per the suggestions of the NRC (2007). The NRC uses the following equation to determine apparent digestibility:

$$\frac{\text{(Mass of mineral ingested – mass of mineral in the feces)}}{\text{Mass of mineral ingested}} \times 100$$

Acid Detergent Fiber. To determine the acid-detergent fiber (ADF) of the samples, the following procedure was used. The samples previously ground through the cyclone mill were measured into 0.5 g sub-samples and placed into 600 mL Berzelius beakers. The weight of each sample was recorded along with the corresponding beaker number. Then, 100 mL of cold acid-detergent was added to the samples, and the beakers were then placed on a refluxing apparatus and heated to a boil. Once the sample began to boil, the heat was reduced and adjusted to maintain constant temperature, prevent foaming, and to ensure all particles were suspended in the liquid. The reflux was timed for 60 minutes from the onset of boiling. After the 60-minute reflux was completed, the content of the Berzelius beaker was poured into a Gooch crucible. Boiling water was used to rinse the sample into the crucible to ensure no particles were left behind in the beaker. The residue was soaked in hot water for 60 seconds to wash the detergent from the particles. The liquid was then filtered, and the wash procedure was repeated with acetone and placed under the exhaust hood to speed the drying process. Once the acetone was completely evaporated and reached room temperature, the crucibles

were placed in the oven at 100° C overnight and hot weighed. The crucibles were then ignited in a muffle furnace at 500° C for 5 hours, cooled to 100° C and hot weighed again for ash content. The acid detergent fiber was calculated as a fraction of dry matter using the equation:

ADF (g) = (R-A)/DM

where

R = the weight of the crucible and ADF residue

A = the weight of the crucible and ash residue

DM = weight of sample dry matter

ADF digested = ADF intake – ADF output

% ADF digested = (ADF intake – ADF output)/ ADF intake x 100

Neutral Detergent Fiber. To determine the neutral-detergent fiber (NDF) of the samples, a very similar procedure was used. Again, the samples previously ground through the cyclone mill were measured into 0.5 g sub-samples and placed into 600-mL Berzelius beakers. The weight of each sample was recorded along with the corresponding beaker number. Then, 100 mL of cold neutral-detergent, 0.5 g of sodium sulphate, and 4.0 mL of amylase were added to each sample. Each beaker was then placed on a refluxing apparatus to be heated. Once the sample began to boil, the heat was reduced and adjusted to maintain a constant temperature, prevent foaming, and ensure all particles were suspended in the liquid. The reflux was timed for 60 minutes from the onset of boiling. After the 60-minute reflux was completed, the content of the Berzelius beaker was poured into a Gooch crucible. Boiling water

was used to rinse the sample into the crucible to ensure no particles were left behind in the beaker. The residue was soaked in hot water for 60 seconds to wash the detergent from the particles. The liquid was then filtered, and the wash procedure was repeated with acetone and placed under the exhaust hood to speed the drying process. Once the acetone was completely evaporated and reached room temperature, the crucibles were allowed to dry at 100° C overnight before hot weighing. The crucibles were then ignited in a muffle furnace at 500° C for 5 hours, cooled to 100° C and hot weighed again for ash content. The NDF was calculated as a fraction of dry matter using the following equation (Goering, 1984):

NDF = (R-A)/DM

where

R = the weight of the crucible and NDF residue

A = the weight of the crucible and ash residue

DM = weight of sample dry matter

NDF digested = NDF intake – NDF output

% NDF digested = (NDF intake – NDF output) / NDF intake x 100

Acid Detergent Lignin. Once the ADFs were calculated, the remaining ash residue was then used to measure acid-detergent lignin content (ADL). A 72% sulfuric acid (H_2SO_4) solution was cooled to 15° C on ice, and the crucibles were arranged in a Pyrex dish to prevent leaking. The ADF ash in each crucible was then saturated in the H_2SO_4 solution, mixed with individual Teflon rods, and allowed to soak for three hours. The crucibles were monitored closely, and the H_2SO_4 solution

was replaced continuously to ensure that the fiber mat never became dry. After three hours, the acid was neutralized with boiling water. Each crucible was thoroughly rinsed with boiling water, vacuumed to remove as much moisture as possible, and transferred to a drying oven where they rested at 100° C for 8 hours. After the dried crucibles were hot weighed for ADL content, they were then ignited in a muffle furnace at 500° C for 5 hours, cooled to 100° C, and hot weighed again for ash content. The ADL was calculated as a fraction of dry matter using the following equation (Goering, 1984):

ADL = (L-A)/DM

where

L = the weight of the crucible and ADL residue

A = the weight of the crucible and ash residue

DM = weight of sample dry matter

ADL digested = ADL intake – ADL output

% ADL digested = (ADL intake – ADL output) / ADL intake x 100

Phosphorous Determination. Serum and urine phosphorous concentration was analyzed using a colorimetric assay (Gomori, 1942). This assay uses spectrophotometric measurements to compare each concentration of molybdenum-blue against a standard curve of known P concentrations. The 2 reagents used to create the standards were molybdate-sulfuric (MS) solution and P-methylaminophenol sulfate (Elon) solution. The MS solution was comprised of 10 g of $Na_2MoO_42H_2O$, and 28 mL of concentrated H_2SO_4 combined in a 2,000 mL

volumetric flask and brought to volume with deionized water. The Elon solution consisted of 6 g of $NaHSO_3$ and 2 g of Elon combined in a 200-mL flask and brought to volume with deionized water. Table 3 provides the dilutions of each reagent used to prepare the P standards. After the reagents and standards were prepared, 50 μl of each standard, digested feed sample, and digested fecal sample were placed onto a 96-well plate in duplicate. 250 μl of MS solution and 25 μl of Elon solution were added to each well. The samples were then set to incubate on a plate shaker at 600 rpm for 45 minutes, and read at 700 nm.

Statistical Analysis

Data from all measured variables were analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Variables BW, BCS, DMI, mineral intake, mineral excretion, mineral retention, and mineral digestibility, and serum mineral concentration were analyzed using repeated measures of the Proc Mixed Procedure with a Tukey-Kramer adjustment. Analysis of treatment groups had fixed parameters of horse, treatment, and period, while analysis of washout groups used only a fixed effect of period. Lignin, NDF, ADF, and DM digestibility were analyzed using repeated measures of the GLM Procedure. Blood serum levels of cobalamin and folate were analyzed using repeated measures of the GLM Procedure as well; results were interpreted using linear contrast. All results are reported as LSM $\pm$ SEM.

Results

Body Weight and Condition. There were no observed treatment differences in BCS or BW (P = 0.9 and 0.08 respectively). The mean BCS during the study was 5.0 $\pm$ 0.9 kg while the mean BW was 503 $\pm$ 36 kg (Table 5).

Dry Matter Intake. There were no treatment differences in DMI as seen in Figure 1 (P = 0.3). Figure 2, however, illustrates an increase in DMI during the second two washout periods (P = 0.001). Although DM digestibility did not differ by treatment (P = 0.2) there was a trend seen for a significant cubic contrast (P = 0.06) (Table 6), however, no differences were observed between DM digestibility and period, or horse.

Table 5. Combined means for both body condition score and average body weight for respective treatments

	Control	Low	Medium	High	SEM	P Value
BCS	4.8	5.1	5.0	5.0	0.9	0.08
BW (kg)	502	499	502	508	37	0.9

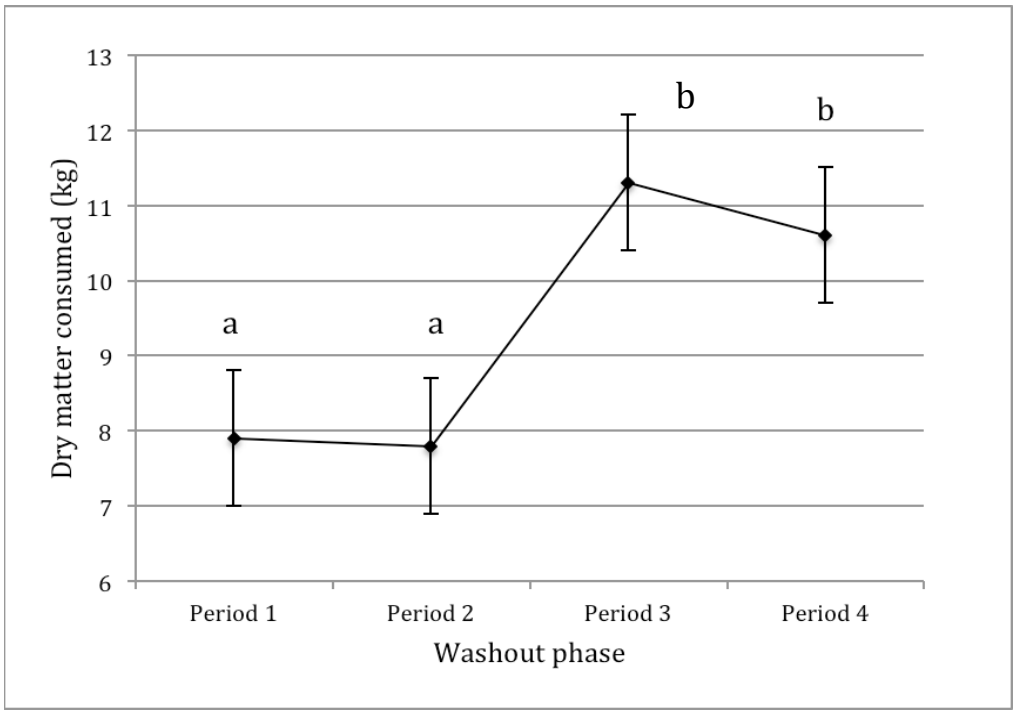

Figure 2. Combined means for DMI during washout phases as measured on d 14 of each phase. Means that do not share similar superscripts are different[ab] ($P \leq 0.05$).

Table 6. Average digestibility of DM, ADF, NDF, and lignin during treatment periods, reported by treatment group.

	Control	Low	Medium	High	SEM	P Value
DM %	52	55	52	53	0.9	0.2
ADF %	49	50	46	48	1.3	0.3
NDF %	51	54	51	52	1.4	0.5
Lignin %	4	0.7	-5	-2	3	0.3

Blood Serum. Table 7 illustrates a lack of treatment differences in blood serum levels of folate ($p = 0.09$). There was, however, an observed treatment difference in blood serum levels of cobalamin ($p = 0.003$). The highest serum cobalamin concentration was seen in the control group (2,684 $\pm$ 25 pg/mL), while the lowest was seen in the high treatment group (2,526 $\pm$ 25 pg/mL). While these differences are significant, it represents only about a 6% difference.

Table 7. Average concentrations of serum cobalamin and folate during treatment periods, reported by treatment group. Means not sharing similar superscripts are different.

	Control	Low	Medium	High	SEM	P Value
Cobalamin (pg/mL)	2,684[a]	2,612[b]	2,591[c]	2,526[d]	25	0.02
Folate (ng/mL)	8.9	8.3	8.8	8.2	0.3	0.4

Fiber Digestibility. There were no treatment differences for the digestibility of NDF, ADF, or lignin (P = 0.2, 0.2, and 0.3 respectively) as seen in Table 6, nor were there any significant correlations between the aforementioned variables and period (Table 8), or horse (Table 9). There was, however, a trend in cubic contrast seen between treatment and NDF digestibility (P = 0.1) The fiber content of forage fed can be found in Appendix B (Table 1).

Minerals. Similarly, there were no treatment differences in intake (Table 8), urinary output (Table 9), fecal output (Table 10), percent retention of daily intake (Table 11), or retention (Table 12) of Ca, Mg, P, Cu, Zn, Se, or Mn. There were, however, treatment differences seen in the intake and output of Co, which corresponds with the varying concentrations of administered Co supplement. Co intake was highest during the high treatment (29.0 $\pm$ 0.1 mg; $p < 0.0001$) and lowest during the control treatment (6.3 $\pm$ 0.1 mg; $p < 0.0001$). Co excretion (urinary and fecal) and digestibility shows the same pattern. However, there were no treatment differences in Co retention ($p = 0.3$). Blood serum concentrations for minerals Cu, Mn, and Se were not affected by treatment. However, there were significant differences seen between treatments in blood serum concentrations of Co ($P < 0.0001$). As illustrated in Table 14, the lowest concentration of serum Co was observed during the control treatment (0.8 $\pm$ 0.3 ng/mL) and the highest during the high treatment (4.7 $\pm$ 0.3 ng/mL).

Table 8. Average daily intake of minerals consumed by horses fed varying concentrations of dietary cobalt. Means not sharing similar superscripts are different.

	Control	Low	Medium	High	SEM	P Value
Consumed Ca (g)	54.1	55.1	52.2	57.7	6.6	0.3
Consumed P (g)	20.3	20.7	19.6	21.7	2.5	0.2
Consumed Mg (g)	24.8	25.8	23.9	26.4	2.3	0.3
Consumed Cu (mg)	75	76	72	80	8	0.3
Consumed Zn (mg)	175	177	168	186	18	0.3
Consumed Se (mg)	0.45	0.46	0.43	0.48	0.05	0.3
Consumed Mn (mg)	675	687	647	729	80	0.1
Consumed Co (mg)	0.06^{a}	6.3^{b}	17.6^{b}	28.9^{d}	1.4	<.0001

Table 9. Average daily urinary mineral excretion by horses fed varying concentrations of dietary cobalt. Means not sharing similar superscripts are different.

	Control	Low	Medium	High	SEM	P Value
Excreted Ca (g)	6.2	7.0	11.4	13.3	3.3	0.4
Excreted P (g)	0.02	0.02	0.02	0.02	0.002	0.2
Excreted Mg (g)	3.6	3.8	3.6	4.0	0.7	0.9
Excreted Cu (mg)	0.16	0.28	0.15	0.27	0.08	0.3
Excreted Zn (mg)	0.9	0.9	0.7	1.6	0.3	0.3
Excreted Se (mg)	0.17	0.21	0.15	0.27	0.06	0.6
Excreted Mn (mg)	0.2	0.2	0.2	0.2	0.06	0.7
Excreted Co (mg)	0.008^{a}	0.03^{ab}	0.08^{bc}	0.14^{c}	0.02	0.009

Table 10. Average daily fecal mineral excretion by horses fed varying concentrations of dietary cobalt. Means not sharing similar superscripts are different.

	Control	Low	Medium	High	SEM	P Value
Excreted Ca (g)	35.5	32.7	33.6	34.9	3.6	0.5
Excreted P (g)	22.1	19.6	20.6	20.7	1.9	0.7
Excreted Mg (g)	16.5	16.6	15.2	18.3	1.6	0.1
Excreted Cu (mg)	84	79	83	82	7.5	0.7
Excreted Zn (mg)	179	180	181	189	17	0.8
Excreted Se (mg)	0.75	0.69	0.56	0.62	0.12	0.2
Excreted Mn (mg)	653	621	565	750	77	0.03
Excreted Co (mg)	0.8^{a}	5.1^{a}	13.9^{b}	24.5^{c}	1.8	<0.0001

Table 11. Average daily % retention of intake ((daily dietary intake – [urinary and fecal excretion])/ daily dietary intake x 100%) of minerals fed to horses receiving varying concentrations of dietary cobalt. Means not sharing similar superscripts are different.

	Control	Low	Medium	High	SEM	P Value
% Ca	21	26	10	12	12	0.5
% P	-12	3	-8	4	8	0.1
% Mg	18	19	22	15	4	0.3
% Cu	-14	-7	-17	-3	11	0.3
% Zn	-5	-4	-9	-3	8	0.9
% Se	-104	-97	-64	-90	21	0.5
% Mn	0.4	8	11	-7	13	0.1
% Co	-34^{ac}	19^{b}	20^{b}	15^{bc}	13	0.04

Table 12. Average daily retention (daily dietary intake-[urinary and fecal excretion]) of minerals fed to horses receiving varying concentrations of dietary Co.

	Control	Low	Medium	High	SEM	P Value
Ca (g)	12.4	15.4	7.2	9.5	7.1	0.6
P (g)	-1.9	0.9	-1.0	0.9	1.4	0.4
Mg (g)	4.7	4.9	5.2	4.1	1.2	0.9
Cu (mg)	-9.3	-3.0	-10.5	-2.5	7.2	0.3
Zn (mg)	-5.1	-3.8	-13.5	-5.3	13.5	0.9
Se (mg)	-0.47	-0.45	-0.28	-0.41	0.09	0.3
Mn (mg)	22.3	65.3	81.6	-21.6	75.2	0.1
Co (mg)	-0.2	1.2	3.6	4.3	1.7	0.3

Table 13. Average % apparent digestibility ([daily dietary intake – fecal excretion]/ daily dietary intake x 100%) of minerals fed to horses receiving varying concentrations of dietary cobalt. Means not sharing similar superscripts are different.

	Control	Low	Medium	High	SEM	P Value
% Ca	33	39	33	39	8	0.9
% P	-12	3	-8	4	8	0.4
% Mg	32	34	36	31	5	0.9
% Cu	-14	-7	-17	-3	1	0.7
% Zn	-4	-3	-8	-2	8	0.9
% Se	-68	-50	-31	-27	20	0.4
% Mn	0.5	8	11	-7	13	0.8
% Co	-33^{ac}	19^{b}	21^{b}	16^{bc}	13	0.04

Table 14. Average blood serum concentrations of minerals measured in horses receiving varying concentrations of dietary Co. Means not sharing similar superscripts are different.

	Control	Low	Medium	High	SEM	P-value
Co ng/mL	0.8[a]	1.3[a]	3.1[b]	4.7[c]	0.3	<.0001
Cu ug/mL	0.8	0.8	0.8	0.8	0.06	0.9
Mn ng/mL	2.1	1.4	1.3	1.8	0.5	0.6
Se ng/mL	130	122	124	133	12	0.6

Washout Phase. There were observed differences in mineral consumption, fecal mineral content, mineral retention, and mineral digestibility during washout phases. However, no differences were seen in the urinary mineral content during these phases. The differences in mineral consumption correspond with the aforementioned increase in feed intake during the entirety of period 3. All data from washout phase can be referenced in Appendix C.

With exception to the two trends in the cubic contrast for NDF and DM digestibilities, there was no effect of treatment on fiber digestibility. Also, there were no differences in mineral balance for all measured variables except Co and cobalamin. Furthermore, there was no statistical relationship between any measured variable and horse or period, with the exception being an increase in mineral intake during the third washout phase

CHAPTER 3. Discussion and Conclusion

Body Weight and Condition.

Horses were initially fed at a rate of 1.6% BW in forage, with no concentrate. Although there were no statistical changes in BW or BCS on a weekly basis, two horses showed a gradual decrease of BW of over 25 kg. This was likely enhanced by extreme cold temperatures experienced during the initial stages of this project. In an effort to sustain steady body weight and body condition, hay was increased daily during the third washout period and intake per horse was aptly revised. Once the optimal amount of roughage was provided to each horse (determined by measured orts), the daily feed intake remained constant. All feed increases were accounted for and recorded. As feed intake increases, mineral intake will generally increase accordingly, considering the mineral content of the forage. This association is likely the explanation for the observed elevation in fecal mineral concentrations during the third washout period (Appendix C).

Subject Handling.

When the study began, the snow cover allowed for daily turnout of all 4 horses (with the exception of days on which total collections were performed). However, because the content of all feeds being consumed was vital to the outcome of the experiment, the horses were not allowed turnout on days when grass was accessible to avoid confounding factors in the collected data. In an effort to provide

daily exercise to the horses on non-turnout days, they were walked on a mechanical walker each morning for 30 minutes.

Fiber Digestion.

The statistical analysis of ADF and lignin showed no significance in overall digestibility regardless of treatment. However, there was a trend for significant cubic contrasts seen in the digestibility of both DM and NDF. This trend towards treatment differences between the control group and the low treatment group may suggest that the Co concentration provided in the low treatment (5.6 mg per day) may be optimal. Due to the small sample size utilized in this experiment, further research involving more horses would be necessary to prove the accuracy of this claim. The results of preliminary research led to the initial hypothesis that the increase in Co had the potential to cause a dramatic elevation in the microbial population of the hindgut, which are responsible for the breakdown of indigestible fibers (Larson, 2014). Given that these colonies of bacteria utilize Co as a prime constituent in the process of cellulose metabolism, it was expected that increasing Co would result in a higher fiber digestibility of consumed roughage. A lack of statistical correlation between the two did not fully support this theory. It is still possible that the addition of Co caused a consequent increase in the microbial population of the hindgut, but because gastrointestinal bacterial levels were not measured, conclusions regarding this conjecture cannot be made.

Serum Cobalamin Levels.

Contradictory to our findings is the abundance of literature indicating elevated levels of serum cobalamin when Co levels are adequate and/or above adequate. The results of this study illustrated an inverse relationship between serum cobalamin and concentration of Co supplement. These results contradict the findings of a 1966 study, which used Co supplements to treat pregnant sheep that expressed apparent Co deficiencies. The data yield demonstrated significantly increased vitamin B_{12} levels in the serum samples of the ewes and their lambs (Andrews and Stephenson). In another ovine study, Co-deficient lambs showed lower serum vitamin B_{12} levels than their healthy counterparts. After supplementation with oral doses of Co, treatment groups showed significant differences ($p < 0.001$) in B_{12} serum concentrations (Fisher and MacPherson, 1991).

Literature divulging these associations within bovine models seems to yield similar findings. A 2007 study evaluated the effect of Co supplementation on colostrum, milk, liver, and serum B_{12} levels of cows. The results indicate that dietary Co supplementation did increase B_{12} levels in the milk and colostrum of these cows; however, did not affect concentrations of Co in the liver, or blood serum (Kincaid and Socha, 2007). Little research of this nature has studied equine vitamin B_{12} concentrations in blood serum as a result of supplementation. However, Stillions et al. (1971) conducted a study to examine the utilization of dietary vitamin B_{12} and dietary Co by mature horses. The data from Stillions' research yielded similar

results to the ovine and bovine studies, illustrating a linear relationship between increases in B_{12} following increases in Co labeled dietary B_{12}.

All of the aforementioned studies provide contradictory results to those found in this research, leaving us with little explanation as to why we saw elevated cobalamin levels in the blood during times of little to no Co supplementation. The control group had an average concentration of 2,684 $\pm$ 25 pg/mL, while the high treatment group had an average concentration of 2,526 $\pm$ 25 pg/mL. While these differences are significant, they are relatively minor and may not have physiological significance.

Mineral Balance.

The mineral compositions of the feed, feces, and urine failed to provide evidence of any correlation between treatment and levels of Ca, P, Mg, Cu, Zn, Se, or Mn. There were drastic treatment differences, however, in the magnitude of the intake, as well as output, of Co. This outcome is to be expected considering the treatment regimens were comprised of varying concentrations of Co.

Co retention showed quite a distinctive trend in variation ($p = 0.03$). The percent of retained Co while on all three Co-supplemented treatment groups did not differ amongst each other, but showed a significant decrease when compared to that of the control treatment. Additionally, the apparent digestibility of Co while on the control diet was also negative.

Graphing the Co intake against the Co retention can reveal the intercept at which dietary intake would result in zero retention; this point would theoretically

represent minimal requirements for the animal (Hintz and Schryver, 1972). Figure 3 shows this intercept lying at 0.39 mg, which is in close proximity to the 0.46 mg (0.05 mg Co/kg of DM when average DMI was 9.3 kg) as suggested by the 2007 NRC recommendations for Co intake. This recommendation was reduced from the 1989 NRC minimum requirement of Co, which was 0.1 mg/kg of DM. The reasoning behind the reduction in minimal requirements for horses was the lower values at which Co deficiency was occurring in cattle and sheep (0.04 mg Co/kg of DM and 0.07 mg Co/kg of DM respectively). However, the R-square value may be too small to draw any concrete conclusions regarding minimum daily requirements for Co.

As illustrated in Table 15, the hay provided to all subjects on this study was not a sufficient source of Cu, Zn, or Se as per recommended by the 2007 NRC. This information may point to the fact that although forage plays a critical role in the equine diet, a complimentary concentrate may be a necessary additive to some daily feed regimens to ensure that horses are consuming adequate amounts of minerals. Horses on this study were also not provided with mineral salt licks, which often can function as a sufficient source of many minerals (My Horse University, 2012).

Table 15. Average daily mineral intake provided by hay fed throughout the study vs NRC 2007 daily-recommended requirements. Average BW of horses was 503 kg and average DMI was 9.3 kg/day.

	Provided by Hay	**NRC Recommendations**
Ca (g)	55.9	20.12
P (g)	20.1	14.1
Mg (g)	25.3	1.5
Cu (mg)	77	100
Zn (mg)	178	372
Se (mg)	0.45	0.93
Mn (mg)	668	372
Co (mg)	0.6	0.5

Percent apparent digestibility of minerals was calculated as per the suggestions of the NRC (2007). The NRC uses the following equation to determine apparent digestibility:

$$\frac{\text{(amount of mineral ingested – amount of mineral in the feces)}}{\text{amount of mineral ingested}} \times 100$$

Although very useful in estimating the digestibility of the measured variables, apparent digestibility does not account for the endogenous loss of these minerals. Therefore this measure is actually lower than the true digestibility, which accounts for the endogenous secretions. The absence of this variable is what is causing several of the values for apparent digestibility to appear negative, as seen in Cu, Zn, and Se. The endogenous secretions (unaccounted for) are the likely source of the negative numbers for these four minerals; as they were being fed below daily requirements.

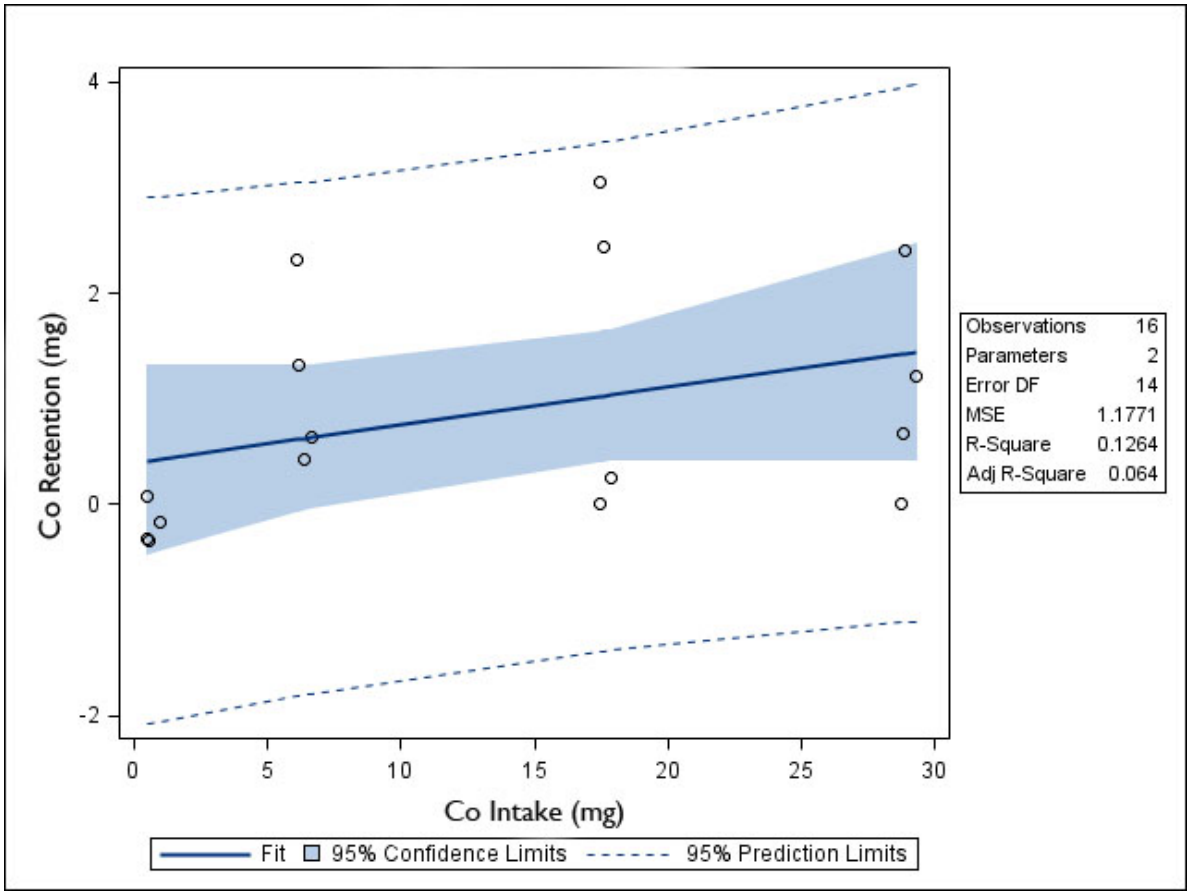

Figure 3. Linear regression of the relationship of mean Co intake (mg) to mean Co retention (mg). This regression line can also be used to determine the apparent digestibility of Co. The intercept at which mineral intake is zero (0.39 mg) can be used to estimate the baseline requirement of Co.

Cobalt Toxicity.

Although Co has not proven its effects on improving fiber digestion, it has been documented as an erythropoetic pharmaceutical and one that is often abused for performance enhancement. Due to its rather frequent abuse by athletes (human and animal), Co toxicity, and methods to quantify it, may be of concern. A recent study set out to measure the Co levels of human tissues by evaluating doping urine samples. The study found that when orally supplemented with 500 μg of Co, consequent urine levels reached 318 ng/mL (Krug et al., 2014). Common range for human ingestion is 5-40 μg/day and 0.1-2.0 ng/mL for urinary concentration. The subjects in this study did not report any adverse side effects at these concentrations. However, at extreme concentrations, Co toxicity can be a threat. In 2010, a succession of human patients with metal-on-metal (MoM) hip arthroplasties were reporting symptoms of Co toxicity, as the corrosive wear on these prostheses can become a source of increased serum metal ion levels (Savarino et al., 2006). Diagnostic data regarding animal health and welfare suggests that adequate Co intake lies at 0.1-0.5 ppm, yet becomes toxic when ingested at a rate of >400 ppm and 50-500 ppm (on a dry weight basis) in swine and poultry respectively (Puls, 1988). Neither equine deficiency nor toxicity was recognized by this text, and dietary requirements were suggested to be less than ruminants at <0.1 ppm The results of our study support these suggestions. This study did not examine values for Co toxicity. Side effects of high Co have been shown to include thyroid dysfunction, myocardial necrosis, GI illnesses, nervous system failure, and sensory deprivation (Ebert and Jelkmann, 2014). This may provide an explanation for a

recent upsurge in the unexplained deaths amongst racehorses, which have been linked to an overload of supplemental Co.

Cobalt Toxicity as it Relates to Morbidity.

In 2014, a string of reported sudden deaths amongst racehorses caused much speculation within the industry, and toxic levels of systemic Co seem to be the culprit. Co has been linked to athletic performance enhancement due to its abilities to increase erythropoesis. Similar to EPO, its ability to concertedly inhibit HIF regulatory enzymes and stabilize the involved transcription factors leads to increases in the expression of EPO genes, and thus increases RBC populations. More RBCs leads to higher oxygen carrying capacity, which could be linked to increased athletic abilities. A press release from the American Quarter Horse Association on August 5th, 2014 discussed the consideration of Co-poisoning as the main suspect of these deaths. Dr. Bruce Duncan, veterinary supervisor of the Ontario Racing Commission, stated regarding Co usage, "When administered in appropriate quantities, there is likely very little performance benefit. And when used in excess, this element can be toxic to horses." Following his claim, the California Horse Racing Board (CHRB) issued a notice warning against the overdose of Co, as it was found to be associated with myocardial pathology when administered in large doses. In an attempt to regulate the legal limits of Co administration, the Racing Medication and Testing Consortium (RMTC) proposed a threshold based on two studies currently being conducted at the University of California at Davis and Morrisville State College. However, the executive director of the RMTC stated that it is too unclear as to what

point Co becomes toxic to horses, as well as whether these post-mortem concentrations are the result of a deliberate administration. In agreement, a statement from Rutgers University supports this claim in saying that high tissue concentrations of Co are merely indicative of high doses of administered B_{12}. As vitamin B_{12} is virtually non-toxic and rapidly excreted via kidney filtration in high doses, it is not likely that there would be any associated negative side effects other than a calmer horse; which may even be undesirable for racehorses (AQHA, 2014). Ultimately, the RMTC threshold proposal was withdrawn due to a lack of statistical evidence of the concentration at which Co becomes lethal.

The Indiana Horse Racing Commission (IHRC), however, has taken a stand against the misuse of Co in horses. In 2014, the IHRC recommended Co be classified as a Class B substance to allow for serious penalties against misuse, as well as implementing a testing threshold at 25 parts per billion in tested blood serum. Our study concluded that when supplemented at a rate of 28 mg Co/day, the average blood serum concentration of Co is 4.7 parts per billion; far beneath the acceptable threshold. The IHRC began performing post-race blood tests for Co and found several samples to be as high as 353 parts per billion in some Standardbreds. One Thoroughbred was tested after a winning race at a level of 1,127 parts per billion – nearly 45 times higher than the legal threshold (Paulick, 2014). In a sworn affidavit, the accused veterinarian was quoted saying, "[Cobalt] makes them run like a beast, but you only get one or two races out of them, and then they're done," making it apparent that the risks associated with Co poisoning were recognized, and

administration of toxic concentrations still ensued. What is most troublesome is that due to fraudulent bookkeeping and manipulation of records, there was no clear indication of the amount of Co being administered to induce such high levels of blood concentration; leaving the industry in ambiguity when it comes to safe Co administration (LaMarra, 2014). However, by plotting the intake values of Co used in this study, against the subsequent serum concentrations of Co, we were able to illustrate a linear regression (Figure 3). Using the equation of the line, we can then provide a theoretical point to illustrate the estimated concentration of Co intake when serum values reach over 1,100 ppb. The equation of the line is as follows:

Y = 0.1423 (x) + 0.669

when

Y = Serum Co Concentration

X = Dietary Co Intake

Therefore, when serum Co concentrations meet the threshold set by the IHRC (25 ng/mL) the estimated dietary intake would be at 170 mg/day. Most troublesome, however, is that when serum levels reach 1,127 ng/mL, the estimated dietary intake would be at 7,900 mg/day. This is over 280 times the amount provided to the high treatment group on this study.

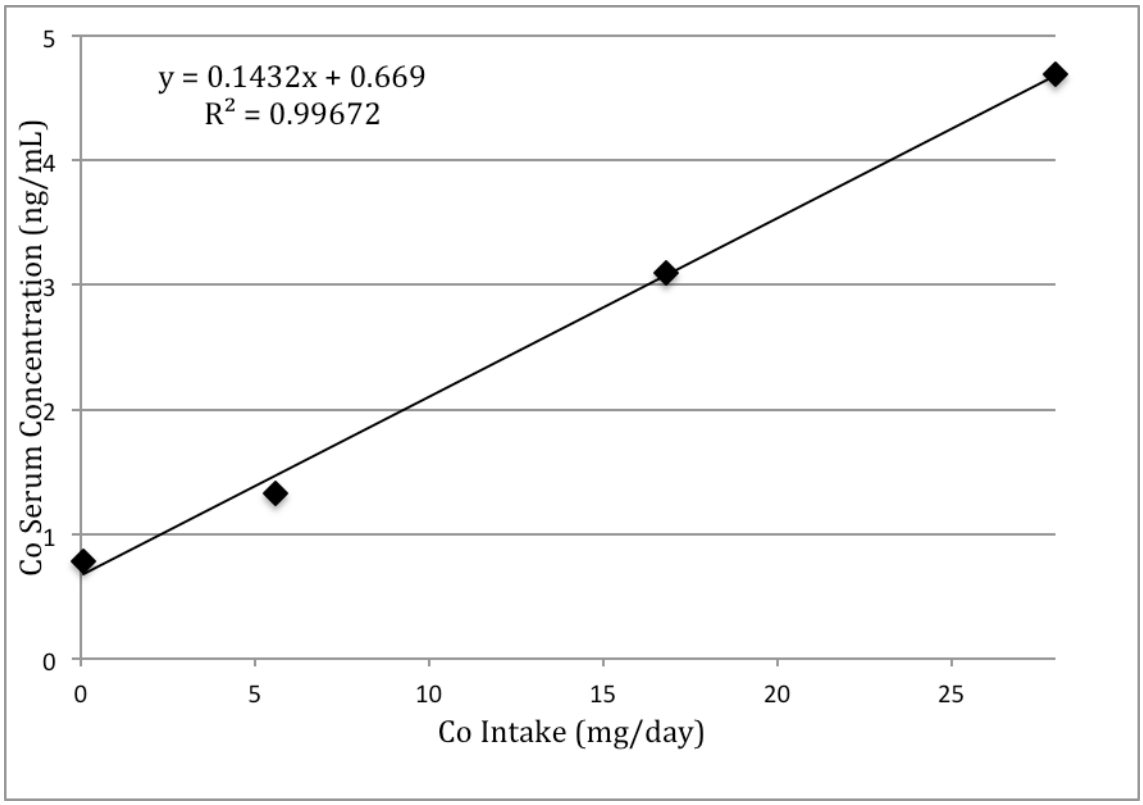

Figure 3. Serum Co concentrations (ng/mL) for each treatment tested on d 35 of each period.

Results from this study suggest the minimum daily intake of Co to be 0.04 mg Co/kg DM; optimal daily intake of Co may be higher than this, however, further research is needed to draw any further conclusion. The largest amount of Co fed on this study reached 3 mg Co/kg of DM and subjects showed no adverse affects at this concentration; nearly 90% less than the maximum tolerable concentration of 25 mg Co/kg DM as set by the 2005 NRC for other livestock species. Theoretically, this means that in order to reach potentially dangerous levels of Co (>25 ppm), horses must be provided with Co concentrations exceedingly higher than what is proposed as the daily minimum requirement (0.04 ppm) of the mineral.

Ironically, the illogical theory behind the usage of Co in equine blood doping renders it seemingly useless. Horses are flight animals – meaning that when startled or fearful, they attempt to escape danger rather than fight off their predators. Evolutionarily, the mechanisms within the equine CNS have developed accordingly to support this flight reaction. When elevated levels of adrenaline and noradrenaline are sensed in the body, it triggers a splenic contraction, which leads to an endogenous release of excess RBC stores (Kurosawa et al., 1998). With this ability to naturally increase the circulation of RBCs throughout their bodies, horses have little use for EPO or Co as means to increase erythropoesis. Therefore, the addition of Co to the equine diet in efforts to enhance performance by way of increasing RBC count is questionable (Satué et al., 2012).

A 2014 study found no significant change in EPO concentrations following the administration of a 49 mg dose of Co. However, it was also explained that equine athletes have been shown to naturally express hematocrit levels of up to 65% when exercising at VO_2 max. Therefore, if Co is in fact capable of increasing equine erythropoiesis, as anecdotal evidence claims, then its administration to a racehorse (that is potentially already reaching a hematocrit of 65%) could lead to possibly lethal cardiovascular complications (Kynch et al., 2014). This theory could provide a possible explanation for the recent upsurge in racehorse deaths following the administration of high levels of Co. Although the data provided by Kynch et al. (2014) revealed no increase in the hematocrit, EPO, or hemoglobin levels during Co supplementation, the hypothetical conjecture of potential Co induced cardiac myopathy may hold some validity in explaining cause of death.

The most commonly used pharmaceutical drugs for Co supplementation provide, at the most, about 5 mg of Co daily (Table 15). According to a 2014 study evaluating commercial Co products, when the recommended dosage of the oral Co supplement "Hematinic" was administered, consequent urinary Co levels were as high as 113 ng/mL (Ho et al., 2015). The subjects in our study received supplementation ranging from 0.0 mg up to 28 mg of Co daily. Even at concentrations as high as 28 mg/day, the highest observed urinary Co level was 33 ng/mL. Table 16 compares the intake and urinary output values for Hemantinic™ and the supplement concentration provided in the high treatment of this study, formulated by Zinpro Performance Minerals.

Note that horses on this study were provided with up to 5 times the amount of daily Co as horses in the study evaluating Hemantinic. However, the administration of Hemantinic yields peak urine concentrations nearly 3.5-fold to those found in this study. The data from such similar studies yield quite contradicting results; both studies provided Co orally, both measured urinary Co using IPC-MS, and still, the results are incredibly paradoxical. To ensure that the ingredients and concentrations listed on the label were indeed accurate with the actual supplement itself, the researchers of our study chose to analyze the content of the Co tablets, independently of the manufacturer. The Michigan State University's Diagnostic Center for Population and Animal Health found no discrepancies between Zinpro's listed ingredients and the actual contents of the tablets, making Zinpro's Co supplementation tablet a safe, effective, and precise means for altering systemic Co levels. Therefore, it may only be appropriate to consider the possibility

that the concentrations in Hemantinic may be inaccurate or it may be from a source that has much greater availability. This could hold major implications in the horse industry. Considering what we now know about the potentially lethal properties attributed to high levels of Co, it is absolutely critical that each consumer, veterinarian, and horse own be aware of exactly what is in the supplements they choose to provide their equine athletes. With a mineral that is so heavily abused in the racing industry, and so easily attainable, there is not much margin for error when animal welfare is at stake.

It is important to note, however, that the study that examined Hemantinic did not report values for total urinary output per day. Total volume of daily urine would aid tremendously in accurately comparing the Co concentrations of urine from horses supplemented with Copro 25 and Hemantinic.

Table 16. Current pharmaceuticals for Co supplementation, their ingredients, and recommended dosages (Ho et al., 2015).

Supplement	Cobalt ingredient as listed	Recommended daily dose	Co equivalent per daily dose (mg)	Peak total urinary Co level observed (ng/mL)
Injections				
Hemo-15	Cyanocobalamin 150 μg/mL	10 mL	0.99	81-530
VAM® Injection	Cobalt gluconate 0.7 mg/mL	11 mL	1.08	374-424
Oral Supplements				
Farrier's Formula	Cobalt carbonate 1.9 mg/170g	1 cup (255 g)	1.41	Not observed
Hemopar	Cyanocobalamin 800 μg/L Co sulphate monohydrate 9mg/L	60 mL	0.19	Not observed
Hematinic	Cyanocobalamin 180 mg/L Cobalt carbonate 110 mg/L	80 mL	4.99	56-113

Table 17. Co levels in Hematinic and Zinpro supplements, daily recommended dosage of Hemantinic and highest daily dose of cobalt tablets, and consequent urinary Co concentrations.

Supplement	**Cobalt ingredient as listed**	**Daily dose**	**Co equivalent per daily dose (mg)**	**Peak total urinary Co (ng/mL)**
Hemantinic	Cyanocobalamin 180 mg/L Cobalt carbonate 110 mg/L	80 mL	4.99	56-113
COPRO®	2.0% Cobalt glucoheptonate	10 tablets	28	34

Overall Conclusion.

The unpredictable results were those that exemplified an inverse correlation between Co intake and consequent serum vitamin B_{12} levels. These calculations yielded outcomes that were not only unexpected, but were also in complete opposition to the originally proposed theories. Little congruent research is available to support this finding. This difference, although significant, was minor. Also, the addition of dietary Co above the NRC recommended levels did not cause any changes in the balance of minerals Ca, P, Mg, Cu, Mn, Se, or Zn. It did, however, successfully increase Co intake, as well as Co concentration in the feces and urine – both of which were expected with varying concentrations of Co. Furthermore, with exception to the trends in cubic contrast for DM and NDF, Co supplementation did not prove to have any statistically significant effect on consequent fiber digestion as theorized in the hypothesis of this study. However, it is important to consider the implications of the small sample size utilized in this study when evaluating the lack of treatment differences. Subsequent research involving a larger sample size would be of great importance to further examine the usage of Co supplementation as a dietetic agent for increasing fiber digestion.

However unexpectedly, the results concerning the correlation between supplemental Co concentrations and subsequent urinary Co concentrations may have helped to uncover a fundamental flaw within Co abuse in the racing industry; as there is newly found evidence that points to misrepresented supplements as a possible cause of these recently observed toxic concentrations of Co. Additionally, we were able calculate estimated values of Co ingestion via blood serum Co

concentrations, which suggest that some fatalities in the racing industry may be attributed to Co overdose.

Implications for Future Research

An interesting and unanswered question in this study is the potential still for Co-induced population growth of the microbial colonies in the hindgut. A trend in increasing DM and NDF digestibility between the control and low treatment groups may suggest that 5.6 mg per day (the amount provided to the low treatment group) may be within the optimal range for maximal fiber digestion. Although we did not see any other consequential elevations in fiber digestion, as measured by ADF and lignin, we cannot rule out the remaining possibility that Co additives are capable of increasing the gastrointestinal microbial populations. Further research with the inclusion of hindgut bacterial evaluation should be conducted in order to obtain a concrete response to this remaining uncertainty.

Additionally, it is of deeper interest to pinpoint the rational behind the seemingly backwards ratio ingested Co concentrations and resultant levels of serum cobalamin. In order to gain a better perspective on these numbers, and ensure their accuracy, perhaps larger sample sizes could be utilized to alleviate any possibility of error, and more tissues could be analyzed for minerals in question.

Finally, it would be quite interesting to evaluate the hematocrit of these animals over the course of the treatments. This would have been simple to measure in this project but the concern over Co-supplementation in horses became public only after this study had ended. Since Co is known to have positive effects on erythropoesis in other species; it would be of great interest to evaluate its effect on the RBC formation in horses. Given the serious nature of the recent numbers of Co-

related equine deaths, and the limited studies evaluating the rational behind them, it would be of great importance to further research the potentially lethal concentrations of this mineral, as well as the rational behind the performance enhancement that accompanies it. Granted, due to the horse's ability to produce adequate levels of excess RBCs during excitatory events, these measurements may hold little implication.

APPENDICES

Appendix A. Fiber and Excrement

Table 18. Average daily urine output of each horse during collection periods

Horse	Period	BW (kg)	Treatment	Avg Urine Out/d (ml)
A	1	526	wash	4,400
C	1	611	wash	2,000
D	1	435	wash	5,400
H	1	484	wash	2,200
A	2	522	High	4,000
C	2	608	Control	5,100
D	2	440	Medium	4,700
H	2	482	Low	3,900
A	3	520	wash	5,600
C	3	608	wash	7,900
D	3	438	wash	9,900
H	3	480	wash	11,200
A	4	517	Low	4,350
C	4	603	High	6,800
D	4	424	control	2,200
H	4	473	medium	6,900
A	5	510	wash	6,400
C	5	598	wash	6,300

Table 18 (cont'd)

D	5	421	wash	9,000
H	5	463	wash	8,300
A	6	508	control	6,600
C	6	591	medium	6,600
D	6	422	low	10,300
H	6	462	high	6,600
A	7	505	wash	5,800
C	7	581	wash	5,500
D	7	434	wash	10,800
H	7	468	wash	4,400
A	8	504	medium	5,300
C	8	586	low	4,800
D	8	443	high	10,800
H	8	471	control	7,800

Table 19. Average daily fecal output of each horse during total collection periods

Horse	Period	BW (kg)	Treatment	Avg Fecal Output kg/d
A	1	526	wash	14.7
C	1	611	wash	15.5
D	1	435	wash	13.7
H	1	484	wash	14.2
A	2	522	High	14.8
C	2	608	Control	16.3
D	2	440	Medium	14.9
H	2	482	Low	14.5
A	3	520	wash	14.9
C	3	608	wash	16.1
D	3	438	wash	15.7
H	3	480	wash	15.4
A	4	517	Low	16.0
C	4	603	High	16.4
D	4	424	control	15.6
H	4	473	medium	15.5
A	5	510	wash	16.7
C	5	598	wash	17.9
D	5	421	wash	17.1
H	5	463	wash	18.4

Table 19 (cont'd)

A	6	508	control	19.7
C	6	591	medium	21.4
D	6	422	low	19.1
H	6	462	high	18.9
A	7	505	wash	19.1
C	7	581	wash	21.1
D	7	434	wash	19.6
H	7	468	wash	18.8
A	8	504	medium	20.0
C	8	586	low	22.0
D	8	443	high	23.5
H	8	471	control	21.2

Table 20. Average digestibility of DM, ADF, NDF, and lignin during treatment periods, reported by period.

	Period 1	Period 2	Period 3	Period 4	SEM
DM %	53	46	58	55	0.9
ADF %	48	39	54	52	1.3
NDF %	53	44	56	55	1.4
Lignin %	-6	-18	15	7	2.9

Table 21. Reagent dilution factors for colorimetric assay phosphorous standards.

Concentration (mg/dL)	15 mg/dL Phosphorous	Deionized water	MS	Elon
0.0 mg/dL	0.000 mL	0.500 mL	2.5 mL	0.25 mL
1.0 mg/dL	0.033 mL	0.467 mL	2.5 mL	0.25 mL
2.0 mg/dL	0.067 mL	0.433 mL	2.5 mL	0.25 mL
3.0 mg/dL	0.100 mL	0.400 mL	2.5 mL	0.25 mL
4.0 mg/dL	0.133 mL	0.367 mL	2.5 mL	0.25 mL

Table 22. Fiber content of forages fed by period

Period	DM%	%ADF on DM Basis	%NDF on DM Basis	% Lignin on DM Basis
Washout 1	91.8	41.7	64.1	6.2
Treatment 1	91.5	41.9	64.8	5.9
Washout 2	91.8	41.6	65.5	6.2
Treatment 2	92.2	41.1	66.4	5.3
Washout 3	92.3	40.6	63.9	5.3
Treatment 3	92.4	40.9	64.3	5.8
Washout 4	91.7	40.6	61.7	6.2
Treatment 4	92.4	40.2	60.9	6.1

Table 23. Average digestibility of DM, ADF, NDF, and lignin during treatment periods, reported by horse.

	A	C	D	H	SEM
DM (%)	57	54	50	51	0.9
ADF (%)	54	50	45	44	1.3
NDF (%)	56	54	48	50	1.4
Lignin (%)	10	4	-3	-13	2.9

Appendix B. Mineral Concentration

Table 24. Mean mineral intake as measured on d 14 of each washout phase.

	Period 1	Period 2	Period 3	Period 4	SEM	P Value
Consumed Ca (g)	47.3	45.6	63.2	72.2	3.7	0.0003
Consumed P (g)	16.7	16.7	25.8	25.6	1.4	0.0003
Consumed Mg (g)	20.6	20.9	31.3	29.3	1.7	0.0006
Consumed Cu (mg)	64.8	63.9	93.1	87.0	5.2	0.001
Consumed Zn (mg)	150	148	216	202	12	0.001
Consumed Se (mg)	.38	.38	.56	.52	.03	0.001
Consumed Mn (mg)	523	554	846	682	45	0.0007
Consumed Co (mg)	.47	.49	.58	.64	.03	0.005

Table 25. Mean urinary mineral excretion as measured on d 14 of each washout phase.

	Period 1	Period 2	Period 3	Period 4	SEM	P Value
Excreted Ca (g)	12.5	13.7	9.7	15.7	4.2	0.09
Excreted P (g)	0.02	0.03	0.02	0.01	0.004	0.2
Excreted Mg (g)	3.4	4.9	3.9	4.3	1.0	0.5
Excreted Cu (mg)	0.14	0.14	0.16	0.21	0.03	0.3
Excreted Zn (mg)	1.0	0.8	2.1	0.8	0.7	0.6
Excreted Se (mg)	0.24	0.23	0.19	0.29	0.07	0.4
Excreted Mn (mg)	0.06	0.30	0.21	0.24	0.07	0.1
Excreted Co (mg)	.004	0.01	0.01	0.01	0.002	0.1

Table 26. Minerals excreted in feces as measured on d 14 of each washout phase.

	Period 1	Period 2	Period 3	Period 4	SEM	P Value
Excreted Ca (g)	29.1	30.0	29.3	32.1	2.9	0.3
Excreted P (g)	15.8	16.7	16.7	20.4	0.9	0.002
Excreted Mg (g)	12.7	15.0	16.0	18.2	0.7	0.002
Excreted Cu (mg)	73.4	69.5	81.8	84.5	7.9	0.3
Excreted Zn (mg)	158	157	188	190	10	0.0001
Excreted Se (mg)	0.47	0.48	0.52	0.73	0.05	0.005
Excreted Mn (mg)	491	690	664	573	44	0.02
Excreted Co (mg)	0.68	0.99	0.82	0.66	0.14	0.1

Table 27. Minerals consumed vs. minerals excreted as measured on d 14 of each washout phase.

	Period 1	Period 2	Period 3	Period 4	SEM	P Value
Ca (g)	5.6	1.9	24.3	24.4	5.8	0.0005
P (g)	0.8	-0.001	9.0	5.2	1.3	0.002
Mg (g)	4.5	0.8	11.4	6.8	1.6	0.003
Cu (mg)	-8.7	-5.8	11.2	2.3	8.1	0.3
Zn (mg)	-8.1	-9.5	26.4	11.2	11.4	0.09
Se (mg)	-0.31	-0.33	-0.15	-0.49	0.07	0.02
Mn (mg)	33	-136	182	109	53	0.005
Co (mg)	-0.22	-0.51	-0.26	-0.32	0.13	0.1

Table 28. Mineral digestibility as measured on d 14 of each washout phase.

	Period 1	Period 2	Period 3	Period 4	SEM	P Value
% Ca	12.4	5.3	37.0	33.5	8.4	0.0001
% P	4.7	0.3	33.9	19.8	3.7	0.0003
% Mg	21.7	4.5	35.3	22.9	4.8	0.003
% Fe	-364	315	-207	-303	86	0.3
% Cu	-13.9	-8.4	10.7	2.5	9.7	0.27
% Zn	-5.5	-6.3	10.9	5.3	4.9	0.05
% Se	-81.9	-82.7	-29.2	-96.0	15.9	0.02
%Mn	6.7	-25.3	20.3	15.7	6.9	0.003
%Co	-47.6	-102.2	-47.1	-4.1	25.1	0.09

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