EFFECT OF COMPLEXED TRACE MINERAL SUPPLEMENTATION ON JOINT HEALTH IN YOUNG, EXERCISING HORSES

A Thesis

by

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ABSTRACT

Osteoarthritis has been named one of the major causes of lameness in horses, and remains one of the main reasons for performance loss. Nutritional interventions to prevent joint disease have not been investigated at length, and very little emphasis has been placed on the effects of trace mineral supplementation on joint health. To test the hypothesis that complexed trace minerals (CTM; Zn, Mn, Cu amino acid complexes and Co glucoheptonate) would benefit articular cartilage, sixteen Quarter Horse yearlings (9.1±0.17 mo) entering a submaximal exercise training program were balanced by age, sex, BW, and farm of origin, and randomly assigned to either CTM (n = 8) or inorganic (n = 8) dietary Cu, Zn, Mn, and Co for 12 wk. Horses had received their respective diets for 12 wk prior to trial initiation. Synovial fluid samples were collected at wk 0, 8, and 12 of exercise, and analyzed for concentrations of carboxypropeptide of type II collagen (CPII), and collagenase cleavage neoepitope of type II collagen (C2C), and chondroitin sulfate-846 (CS-846). Treatment differences were detected using PROC MIXED in SAS (v9.4) with diet, time, and diet × time interaction included as fixed effects and horse (diet) as a random effect. At wk 12, CPII was higher ($P \leq 0.0001$), and C2C ($P < 0.0001$) and CS-846 ($P = 0.005$) were lower than at wk 0, but none were affected by diet in this study. The ratio of CPII:C2C, or synthesis to degradation, increased from wk 0 to 8 ($P < 0.0001$) in all horses but continued increasing to wk 12 ($P = 0.015$) in CTM horses. Dietary Cu, Zn, Mn, and Co source appears to enhance cartilage synthesis relative to degradation during low-intensity exercise training in young horses. Dietary CTM may lead to improved joint cartilage maintenance as the horse progresses through its performance career.

DEDICATION

I dedicate this thesis to my mama, my role model, and best friend. You are my hero.

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CHAPTER I

INTRODUCTION

Orthopedic diseases are one of the main sources of performance loss in horses due to the poor regenerative capacity of articular cartilage (van Weeren et al., 2008). Osteoarthritis (OA) is a major cause of lameness characterized by a loss of balance between cartilage synthesis and degradation (Wedekind et al., 2015). While OA is normally associated with aging, it is thought to result from cartilage damage occurring in young horses undergoing training (Kahn et al., 2017). The role of nutrition in preventing joint disease has been inconclusive, with little emphasis placed on the effects of trace mineral supplementation on joint health (Wedekind et al., 2015).

Trace minerals only account for about 0.01% of an organism's total mass, yet their presence is essential for many physiological functions (Smart et al., 1981). While needed for normal body processes, dietary mineral absorption and utilization can be affected by many factors, making bioavailability an important consideration when meeting an animal's nutritional requirements (Georgievskii et al., 1979; Forbes and Erdman, 1983). Organic minerals consisting of the metal ion attached to an organic ligand (Yenice et al., 2015) are believed to be more bioavailable; however, the efficacy of organic mineral sources has long been debated, largely due to the difficulty of quantifying mineral absorption. The organic form of trace minerals such as Zn, Cu, Mn, and Co may be beneficial for joint health, as these minerals are required for collagen synthesis and cross-linking, development of the articular cartilage extracellular matrix, and tissue repair (Studzinski et al., 2006; Wedekind et al., 2015; Kinobe, 2016).

The objective of this study was to determine the effects of organic trace mineral supplementation on joint homeostasis after submaximal exercise-induced loading in yearling

Quarter Horses. We hypothesized that the improved bioavailability of organic Cu, Zn, Mn, and Co would enhance cartilage synthesis and repair mechanisms in young horses undergoing exercise stress.

CHAPTER II

REVIEW OF THE LITERATURE

Introduction to Minerals

Minerals are defined as inorganic elements that are found in the Earth's crust (Herdt and Hoff, 2011). Minerals can be classified into two categories, based on their requirement levels: macrominerals or microminerals. Macrominerals are those that are required in relatively large amounts by the body (expressed in g/kg or as a percentage), and include calcium, phosphorus, magnesium, potassium, sodium, chlorine, and sulfur (NRC, 2007). Microminerals, or trace minerals, are found in very small concentrations in the body (expressed in ppm or mg/kg) and include copper, cobalt, iodine, iron, manganese, selenium, and zinc (NRC, 2007). While trace minerals make up less than 0.01% of an organism's total mass, many are considered essential and their presence is critical for many physiological functions, including, but not limited to, acid-base balance, formation of structural components in the body, enzyme formation, immune system function, and as co-factors in many metabolic reactions (Smart et al., 1981; Ott and Johnson, 2001; NRC, 2007; Rabiee et al., 2010).

Trace mineral content in feeds is determined by mineral availability and composition of soil (Smart et al., 1981; NRC, 2007). Mineral availability has to do with the "effective concentration" of the minerals in the soil (Smart et al., 1981). This concentration is influenced by pH levels, moisture, and the presence of other elements and microbes in the soil. Soil composition is determined by the parent rock, erosion, and use of pesticides and fertilizers. (Smart et al., 1981). At the plant-level, mineral uptake and content is affected by many environmental factors such as soil type, soil acidity, mineral abundance, moisture, temperature, and season (Herrick, 1993). The mineral that is then available to the animal once the plant has

been harvested depends on the age of the plant (a more mature plant shows declining levels of trace minerals) and the way in which the plant was handled and processed (Smart et al., 1981; NRC, 2007). While all animals depend on plants as their primary source of minerals (Ashmead, 1993), forages do not typically provide adequate levels of trace minerals due to mineral depletion in many soils (Herrick, 1993). For this reason, many performance animal diets are supplemented with grain concentrates which provide trace minerals in an effort to fill the missing gaps in their diet (Herrick, 1993; Yenice et al., 2015).

A mineral is considered essential based on six characteristics: 1) It is present in all healthy tissue; 2) Its concentration in the body is generally constant from one animal to the next; 3) Its withdrawal from the body results in reproducible abnormalities in both structure and function; 4) Its addition prevents or reverses these abnormalities; 5) Deficiency-induced abnormalities are associated with specific biochemical changes; and 6) These changes can be prevented or cured with addition of the mineral (Herrick, 1993). Of the sixteen nutritionally essential trace elements (Herdt and Hoff, 2011), seven have been identified as having a daily requirement level for horses (NRC, 2007). Of these seven, Copper (Cu), Cobalt (Co), Manganese (Mn), and Zinc (Zn) have shown conflicting data across species, which is the reason they are the primary focus of the current study. These trace minerals are all essential elements, and are all cations with a charge of 2^+ (Georgievskii et al., 1979). They are all transition metals and are known for forming highly stable covalent bonds with ligands (Georgievskii et al., 1979).

Bioavailability and Factors Affecting Absorption of Minerals

Bioavailability refers to the efficiency in which a nutrient can be absorbed, enter circulation, and subsequently be used and/or stored by the body (Forbes and Erdman, 1983; Fairweather-Tait and Hurrell, 1996; Kienzle and Zorn, 2006). For minerals, absorption from the

gastrointestinal tract is the limiting step in achieving high bioavailability (Kienzle and Zorn, 2006). Minerals are primarily absorbed through the wall of the small intestine, and are regulated via homeostatic mechanisms (Fairweather-Tait and Hurrell, 1996). When the amount absorbed is greater than the animal's immediate needs, the extra minerals are stored, often in the liver, or excreted (Fairweather-Tait and Hurrell, 1996). Animals that retain more minerals than they are losing or excreting are in a positive mineral balance (NRC, 2007). This state is expected during growth as the tissues accumulate minerals, but once an animal reaches maturity and normalizes, a "zero balance" is more likely (NRC, 2007).

Upon absorption, independent of an animal's status and age, minerals are incorporated into one of three pools: the functional, transport, or storage pools (Herdt and Hoff, 2011). When dietary mineral intake is inadequate, the storage pool will be depleted first, followed by the transport pool (Herdt and Hoff, 2011). Functional deficiencies and disease states are only seen once both the storage and transport pools have been depleted (Herdt and Hoff, 2011). A functional trace mineral deficiency can be classified into one of two categories; a primary deficiency is due to inadequate intake levels in the diet, while a secondary deficiency results from consumption of another substance that antagonizes the absorption or utilization of the mineral so that it cannot perform its function in the body (Arthington, 2003).

The animal body operates largely through regulation of homeostasis, or working to maintain a constant internal environment, and minerals are no different (Georgievskii et al., 1979). Minerals are absorbed through intestinal nutrient transporters, which are regulated by the diet itself (Diamond and Karasov, 1987; Ferraris and Diamond, 1989). Maintenance of proteins in the body requires significant amounts of energy, and because transporters are proteins, the body will downregulate transporters if they are too energetically expensive. In other words, if

transporters are not actively bringing metabolizable calories or essential nutrients into the body, they will be downregulated (Diamond and Karasov, 1987; Ferraris and Diamond, 1989). Essential nutrients that the body needs to obtain from the diet on a daily basis, but that do not provide caloric value, such as vitamins and trace minerals, can be achieved at relatively low levels, so these transporters will be downregulated to save energy and also to prevent toxicities (Ferraris and Diamond, 1989). During a time of deficiency, absorption rates will be upregulated due to an increase in the mineral's V_{max} or increased transporter affinity for that specific mineral (Ferraris and Diamond, 1989). This results in elevated absorption of a mineral when the animal is deficient in that mineral, and decreased absorption during excessive intake (Kienzle and Zorn, 2006). Overall, the body's homeostatic mechanisms operate to up or downregulate absorption of minerals depending on if they are in low or high supply (Fairweather-Tait and Hurrell, 1996).

Each individual animal's mineral status is affected by its species, age, health, and nutritional status. On a broad level, each species is different, so extrapolating data concerning mineral absorption rates across species must be performed cautiously. The differences in gastrointestinal tract structure will greatly influence nutrient digestion and absorption between livestock species. Where digesta will initially undergo fermentation in the rumen of a ruminant animal, it will first be subject to enzymatic digestion in the stomach and small intestine of the horse. Furthermore, monogastric animals and ruminants have differing stomach acidity and digestive tract pH levels, in addition to different passage times through the small intestine (Kienzle and Zorn, 2006). While mineral transport systems may be similar in function, they often differ in capacity, and may be regulated at different levels due to differing toxicity levels between species (Kienzle and Zorn, 2006). This is of importance because while there has been a large number of studies concerning ruminants and mineral supplementation, these may not be

applicable to horses. For example, the trace mineral molybdenum has the ability to bind to Cu and make it unavailable for absorption (Smart et al., 1981). While this is a critical interaction in ruminants, horses do not seem to be negatively affected by molybdenum (Kienzle and Zorn, 2006).

The age of the animal also plays a key role in nutrient absorption. Mineral concentrations in the body undergo the greatest amount of change during the growth period (Georgievskii et al., 1979). Concentrations of calcium, potassium, phosphorus, magnesium, Zn, and Mn are higher in adults as compared to young animals, but sodium, chlorine, iron, Cu, iodine, and molybdenum decrease with age (Georgievskii et al., 1979). Cobalt concentrations do not undergo great fluctuation throughout an animal's life (Georgievskii et al., 1979). For example, foals with a not-yet fully developed hindgut, small stores of trace minerals, and a diet that does not include large amounts of forage will have different absorptive rates than aged horses (Kienzle and Zorn, 2006). In the same manner, a pregnant mare with a higher demand for minerals or an exercising horse that is losing large amounts of minerals through sweat will have higher requirements, so the body will upregulate its transporters, leading to higher bioavailability (Kienzle and Zorn, 2006).

Interactions between different minerals and between other dietary substrates is also an important consideration when discussing mineral bioavailability. Certain sugars and fibers may bind to metals within the gastrointestinal tract, which affects mineral absorption (Osorio et al., 2016). Plants contain tannins and other polyphenols, which act to protect the plant against pathogens and rot. Most tannins, however, contain *o*-dihydroxyphenyl chelating functional groups, which can form complexes with many metal ions, and thus decrease absorption of some essential minerals in the digestive tract (McDonald et al., 1996). Phytates, or phytic acid, another

compound found in forages, can also bind to, and form stable insoluble complexes with, metal ions (Georgievskii et al., 1979). These insoluble phytate complexes have been shown to decrease Cu and Zn absorption (Mills, 1985). In horses and other nonruminants, large amounts of undigested fiber in the gastrointestinal tract due to forage-heavy diets can increase the binding of minerals and decrease absorption (Faulkner et al., 2017). Furthermore, many trace minerals, such as Co, Mn, and iron, share common uptake mechanisms, creating competition within the digestive tract for mineral absorption (Osorio et al., 2016). Trace mineral absorption is also heavily dependent on intake of other trace minerals. While some minerals are synergistic, meaning that they are beneficial to one another, many are antagonistic, and may inhibit absorption of another mineral (Georgievskii et al., 1979). These antagonistic relationships exist in the digestive tract as well as in the tissues themselves where the minerals are being metabolized (Georgievskii et al., 1979). The more metabolic processes in which a mineral is involved, the higher the probability of it interacting with other minerals and feedstuffs (Ashmead, 1993). It has been well established that excessive amounts of Zn will induce a Cu deficiency, as Zn directly competes for the same transporters as Cu and induces metallothionein synthesis, which binds Cu (Forbes and Erdman, 1983; Kienzle and Zorn, 2006; NRC, 2007; Herdt and Hoff, 2011). Zinc deficiency can be induced by phytate and excess calcium (Mills, 1985; Herdt and Hoff, 2011). Excess amounts of both calcium and phosphorus have been shown to inhibit manganese absorption (Forbes and Erdman, 1983). While these are just a few examples of the complexities surrounding mineral absorption, many other mineral-to-mineral and mineral-to-diet interactions exist, which remains one of the largest challenges to conducting mineral research.

Mineral Sources and Forms

The source, or way in which the mineral is packaged, also affects absorption and utilization of trace minerals. Traditionally, minerals have been added to animal feeds as inorganic salts (Spears, 1996). Minerals in a salt form are classified as carbonates, oxides, or sulfates (Ashmead, 1993). When the salt form of the mineral is ingested, it becomes ionized in the stomach due to the acidic environment (Ashmead, 1993). As the mineral moves distally through the digestive tract, it loses its solubility due to increasing pH (Ashmead, 1993). For any metal ion to be absorbed, it must be soluble in the small intestine (Ashmead, 1993). In general, the more alkaline the lumen, the lower the rate of mineral absorption, due to the decrease in solubility (Ashmead, 1993). At this point, the metal will bind to an anion or ligand in the digestive tract and form very stable, but highly insoluble complexes, making it largely unavailable for absorption (Ashmead, 1993).

An organic mineral consists of the metal ion attached to an organic molecule or ligand, which acts as a carrier (Yenice et al., 2015). Natural compounds which can serve as ligands include carbohydrates, amino acids, proteins, peptides, and nucleic acids (Georgievskii et al., 1979). Trace minerals that are naturally found in forages are present in an organic, or chelated, form (Spears, 1996). Organic minerals can be classified as amino acid chelates, amino acid complexes, metal proteinates, and polysaccharide complexes, which are all similar in function but differ slightly in structure (Ashmead, 1993; Spears, 1996). Chelation occurs when a metal ion, or mineral, becomes bound to 2 or more atoms of the same ligand (Georgievskii et al., 1979). “Chelate” comes from the Greek word for “claw;” the ligand serves as the claw that holds the cation so that it cannot react with other substrates (Herrick, 1993). Organic minerals are assumed to be more bioavailable because they are more similar to forms naturally occurring in

the body than inorganic minerals (Spears, 1996), and chelates are the most available form of the organic minerals (Georgievskii et al., 1979). An amino acid chelate has been defined by the Association of American Feed Control Officials as, "The product resulting from the reaction of a metal ion from a soluble salt with amino acids with a mole ratio of one mole of metal to one to three (preferably two) moles of amino acids to form coordinate covalent bonds." AAFCO states that the average weight of the hydrolyzed amino acids should be approximately 150 Daltons and not exceed 800 Daltons (Ashmead, 1993; Spears, 1996). Amino acid complexes and metal proteinates are similar but are more loosely defined, so information concerning their stability, molar ratios, and molecular weights are not detailed (Ashmead, 1993). A metal chelate has a heterocyclic ring structure (Spears, 1996) and is produced from the attraction that results between a positively charged mineral or cation, and any two or more sites of high electronegativity in a ligand (Herrick, 1993). For transition metals specifically, the covalent, or "coordinate" bond between the metal ion and the ligand occurs when the ligand can contribute two electrons at the same time to the electron shells of the metals (Herrick, 1993). All amino acids are very effective ligands due to their metal-binding abilities, and act as transfer molecules when part of a chelate (Herrick, 1993). When the metal ion is bound to amino acids, it is not as affected by other dietary components, so the mineral can be absorbed at higher rates (Ashmead, 1993).

The stability and size of these complexed minerals is very important to their efficacy. The stability should be high enough for the chelate to stay intact, but not so high that the ligand fails to release the ion after absorption (Herrick, 1993). After all, higher bioavailability has more to do with how much of the mineral is available for potential use at the tissue level, rather than just absorption rates (Ammerman, 1995). The appropriate molecular weight is critical to an effective

chelate. A high weight complex has less chance of making it through the digestive tract for absorption (Ashmead, 1993). An amino acid chelate (AAC) is characterized by a small molecular weight, which makes its digestion comparable to a di- or tri-peptide, meaning that they will be primarily absorbed out of the jejunum of the small intestine (Ashmead, 1993).

One of the challenges associated with mineral nutrition is accurately measuring mineral absorption and retention rates. Mineral absorption has typically been measured as the difference between what was fed and what is excreted in the feces. Mineral retention is usually measured as the difference between what was fed and what is excreted in the feces and urine. These measures of "apparent absorption" are of limited value for measuring calcium, phosphorus, Zn, Mn, and Cu because the digestive tract is a major pathway of excretion for these particular minerals (Ammerman, 1995). To measure true mineral absorption, one must consider the so-called "endogenous element," which consists of mineral that has fulfilled its role in the body and has been deposited back into the digestive tract (Georgievskii et al., 1979; NRC, 2007). This endogenous element provides a more valid estimate of the mineral that was presented to the body for metabolic purposes (Ammerman, 1995). Furthermore, mineral balance studies have not been completed in all classes and ages of horses, so the current mineral requirements for horses are more appropriately called "recommendations," and have been estimated by observing intake levels fed without producing negative effects, such as a deficiency or toxicity (NRC, 2007).

When measuring minerals in the body, the highest concentration of minerals, and thus the most important organs to study, are found in the liver, blood, skeletal muscles, and bone (Georgievskii et al., 1979). While these locations have a large concentration of minerals, a direct measurement of trace minerals in the blood and tissues is still limited, as minerals may not be present in the blood, and inflammation and stress can affect mineral concentrations in serum

(Herdt and Hoff, 2011). In addition, concentrations of Cu, Mn, and Zn in blood serum are generally low and mostly unresponsive to diet changes, further substantiating the need for a different method of measuring these minerals (Faulkner et al., 2017).

Copper

Copper (Cu) is an essential mineral that serves as a catalyst for many Cu-dependent enzyme systems. These enzymes include cytochrome *c* oxidase, which plays a role in ATP production in the mitochondrial electron transport system (Tomlinson et al., 2008), tyrosinase, which is responsible for melanin synthesis, and lysyl oxidase, which is important for cartilage synthesis and connective tissue formation (Ott and Asquith, 1995; Ott and Johnson, 2001; NRC, 2007; Herdt and Hoff, 2011). Copper is also an important component of cytosolic superoxide dismutase (SOD), an antioxidant that converts free radicals into nontoxic hydrogen peroxide (Wagner et al., 2010). One study found that after 60 d of a Cu-deficient diet, steers showed a 25% reduction in copper-zinc SOD (SOD1) activity and lambs showed a 22% reduction in copper-zinc SOD activity after only 30 days (Wagner et al., 2010).

Copper is absorbed in the small intestine. Newly absorbed Cu binds to albumin, amino acids, or transcuprein in the plasma. It is then transported to the liver, where it is incorporated into ceruloplasmin, metallothionein, or large proteins excreted in bile (Cymbaluk and Smart, 1993; Fairweather-Tait and Hurrell, 1996). Ceruloplasmin (Cp) acts as the primary Cu transport protein (Arthington, 2003) and storage reservoir for other tissues (Studzinski et al., 2006) and interacts with ceruloplasmin receptors in tissues throughout the body (Cymbaluk and Smart, 1993). Copper is present in all tissues (Studzinski et al., 2006), but is found in highest concentrations in the liver, brain, spleen, bones, and hair, and in moderate concentrations in the muscles, kidneys, pancreas, and heart (Georgievskii et al., 1979). The liver is the primary storage

site for Cu (estimated at containing about 15% of the total Cu body store) (Studzinski et al., 2006), and reflects long term availability of Cu to the animal (Herdt and Hoff, 2011). Excretion of endogenous Cu with bile is the main pathway in maintaining homeostasis for this element (Georgievskii et al., 1979; Studzinski et al., 2006). The liver has been estimated to only contain about 2.5-7% of the body's copper in horses under 1 year of age, and about 1.9% of the body's Cu in mature horses, but the liver is recognized as the only tissue with Cu that can be easily mobilized (Cymbaluk and Smart, 1993). In ruminants, the concentration of Cu in the liver has been correlated to the level of bioavailable Cu in the animal's diet (Kincaid, 1999). Horses have been shown to absorb 14-50% of dietary Cu in contrast to the estimated 2-5% observed in cattle (Cymbaluk and Smart, 1993). Excess iron, Zn, and calcium have been shown to interfere with Cu absorption (Forbes and Erdman, 1983; Mills, 1985; Herdt and Hoff, 2011), with Zn being one of the most important inhibitors, as it directly competes for the same transporters (Cymbaluk and Smart, 1993). In horses, Cu deficiency has been associated with osteochondritis and developmental orthopedic diseases (Hurtig et al., 1993), epiphysitis, limb deformities, and osteodysgenesis (NRC, 2007), as well as loss of hair color and impaired immune function (Herdt and Hoff, 2011). Copper toxicity is generally viewed as more of an issue for sheep than for horses and other livestock animals (Studzinski et al., 2006) due to a less effective excretory mechanism in sheep (Sommerville and Mason, 1985) .

Measuring an animal's Cu status has proven to be a difficult task. In the blood, Cu levels in both plasma and serum are influenced by many factors, such as time of reproductive cycle, infection, breed, and genetics, meaning that there are many contributors other than dietary intake alone (Kincaid, 1999). When Cu intake is less than the required level, plasma Cu levels are not consistently decreased until the liver Cu stores have been depleted (Kincaid, 1999). Measuring

Cu status by evaluating levels of enzymatic SOD1 has also proven not to be useful, as levels of the enzyme do not appear to decline with deficient intake until after plasma Cu and Cp levels are reduced (Kincaid, 1999).

A high percentage of circulating Cu is bound to Cp, an acute phase protein that is secreted in response to tissue inflammation and stress (Arthington, 2003; Hambidge, 2003). Therefore, the level of Cu in the blood is directly dependent on the amount of Cp which is present, so during times of physiological stress, plasma Cu content increases by way of increased Cp (Cymbaluk and Smart, 1993; Hambidge, 2003). A normal acute phase reaction lasts 24 to 48 hours, but Cu deficiency may lead to chronic inflammation due to changes in the negative feedback pathways that involve anti-inflammatory responses (Arthington, 2003). Copper found in the blood is representative of the transport pool and is potentially mobilized during inflammation, making blood a poor measurement of true Cu status (Herdt and Hoff, 2011). More importantly, a lapse in the amount of time between decreases in plasma Cu and Cp and the development of physiological changes associated with deficiency exists, so blood is not effective for measuring Cu status (Mills, 1987).

This explains then why some studies investigating different sources of Cu have seen no changes in bioavailability when looking at plasma Cu and Cp concentrations (Spears, 1996). A higher Cu retention rate for steers fed Cu-lysine, the chelated form, versus Cu-sulfate was observed when absorption rates and urinary excretion of Cu were the units of measurement (Spears, 1996). Similar studies have found supplementation from Cu-lysine to be 114% compared to Cu-sulfate in poultry, 105% compared to Cu-sulfate in piglets, and 104% compared to Cu-sulfate in ruminants (Mézes et al., 2012). While it is known that Cu deficiency causes

severe developmental defects, the comparative effects of Cu availability from different mineral sources has not been well established in the horse.

Zinc

Zinc (Zn) is recognized as one of the most studied minerals in animal nutrition (Arthington, 2003), and also as the most abundant intracellular trace mineral in the body (Studzinski et al., 2006), second only to iron (Herdt and Hoff, 2011). It is present in all tissues and body fluids, with the majority (about 80-85%) being found in skeletal muscle and bones (Fairweather-Tait and Hurrell, 1996; Studzinski et al., 2006). Zinc is mainly absorbed from the small intestine through a carrier-mediated process, but small amounts are also absorbed from the stomach and large intestine (Studzinski et al., 2006). After absorption, Zn is transported in the blood by albumins and globulins, taken up by the liver, and then redistributed throughout the body (Fairweather-Tait and Hurrell, 1996; Studzinski et al., 2006). Intracellular Zn transport is accomplished primarily by metallothionein, a ligand with a high affinity for binding Zn (Studzinski et al., 2006). Endogenous Zn is eliminated from the body with pancreatic and intestinal juices in the feces and urine (Georgievskii et al., 1979; Studzinski et al., 2006). In monogastrics, Zn absorption is thought to be only about 7-15% of total intake, but relative absorption is thought to be higher in young animals (Georgievskii et al., 1979). Zinc is tightly regulated in the body, and a Zn deficiency will increase Zn absorption five-fold (Ferraris and Diamond, 1989). Phytate is the most potent inhibitor of Zn absorption (Forbes and Erdman, 1983; Mills, 1985; Fairweather-Tait and Hurrell, 1996), and an intense exercise bout completed after a 16-week training program caused a decrease in the true digestibility of Zn from 25 to 14% in horses (Hudson et al., 2001).

Zinc is a component of more than 300 enzymes in the body (Tomlinson et al., 2008; Nayeri et al., 2014). This includes carbonic anhydrase, alkaline phosphatase, carboxypeptidase (NRC, 2007), and others that are especially related to carbohydrate, protein, lipid, and nucleic acid metabolism (Nayeri et al., 2014). Zinc plays very important roles in cell division, regulating appetite, growth, and immune function (Herdt and Hoff, 2011). Zinc influences many cellular processes through its effects on gene expression as it forms the "Zinc-finger" in DNA with cysteine and histidine residues, and is also a component of SOD1, and as such plays a key role in eliminating free radicals from the body (Studzinski et al., 2006). Zinc is involved in bone mineralization and is well known for its influence on skin, hair, and hoof development (Ott and Johnson, 2001). A study in horses found the most significant difference between yearlings fed trace mineral proteinates and those receiving inorganic trace minerals was greater hoof growth, which was attributed to the more bioavailable Zn source (Ott and Johnson, 2001). Zinc deficiency manifests as decreased growth rate, parakeratosis, and alopecia (NRC, 2007), while excess Zn intake has been found to induce Cu deficiency, leading to cartilage and connective tissue defects (NRC, 2007). Monogastrics show the greatest risk of experiencing a Zn deficiency due to dietary phytate, in contrast to ruminants which benefit from rumen microbes that breakdown phytate (Herdt and Hoff, 2011).

Most of the Zn in the blood can be found in the erythrocytes in the forms of carbonic anhydrase and SOD1 (Studzinski et al., 2006). Optimal biomarkers have yet to be identified for Zn. This is due to the fact that the body is very effective at maintaining plasma, serum, and tissue concentrations of Zn even in times of deficient intake, and that in animals, physical signs of deficiency will manifest long before a significant decrease in tissue and total body Zn is detectable (Mills, 1987; Hambidge, 2003). Furthermore, only about 0.1% of the total Zn in the

body is found in plasma (Fairweather-Tait and Hurrell, 1996), and numerous factors can cause fluctuations in Zn serum and plasma levels, including age, stress, and illness (Smart et al., 1981; Kincaid, 1999).

Despite the large number of studies that have investigated sources of Zn, there have been conflicting results due to this clear lack of biomarkers. A study in lambs reported that bioavailability of Zn-methionine (Zn-Met), the chelated form, was similar to Zn-oxide based on plasma Zn levels, but that urinary excretion of Zn was lower for Zn-Met supplementation, implying higher retention rates for the organic form (Spears, 1996). Multiple cattle studies have found improved quality grades in steers, as shown by higher marbling scores and higher fat percentages, and increased milk production with decreased somatic cell count in lactating dairy cows fed Zn-Met, suggesting that Zn from these sources may be absorbed at similar rates, but are metabolized differently in the body (Spears, 1996). Similarly, a study in dairy cows reported increased benefits during reproduction, with cows being able to maintain body weights more efficiently during lactation, and increased colostrum quality as the ratio of chelated Zn to Zn-sulfate was increased (Nayeri et al., 2014). Zinc-Methionine has also been shown to positively affect the immune response in cows challenged with Infectious Bovine Rhinotracheitis virus and in calves experiencing shipping stress (Spears, 1996). Baker et al., 2005 found that mature horses had higher retention rates when fed Zn-oxide as opposed to Zn-chelate, while a study in yearlings showed higher Zn retention with the organic vs. the inorganic form (NRC, 2007). Other studies have found that, compared to Zn-sulfate, Zn-Met supplementation was 133% more bioavailable in poultry, 99% in piglets, and 109% in ruminants, and have attributed this, in part, to Zn-Met having increased protection from phytate and fiber (Mézes et al., 2012).

Manganese

Despite being one of the least abundant trace minerals in the animal body (Herdt and Hoff, 2011), Mn is present in all tissues, and especially concentrated in the bones, liver, and kidneys (Studzinski et al., 2006). It is mainly absorbed from the duodenum of the small intestine, transported by globulins and transferrin (Fairweather-Tait and Hurrell, 1996), quickly removed from the blood, and stored in the liver, bones, and hair (Georgievskii et al., 1979; Studzinski et al., 2006). Endogenous Mn is excreted mainly through the digestive tract with bile (Georgievskii et al., 1979; Studzinski et al., 2006), and this is the main way that Mn is regulated in the body, rather than through changes in the efficiency of absorption (Fairweather-Tait and Hurrell, 1996). Absorption of Mn from feed is thought to be very low and inefficient, approximately 2-5% of total intake in animals (Georgievskii et al., 1979), and less than 5% in humans (Fairweather-Tait and Hurrell, 1996). Its low absorbability is mainly associated with the formation of insoluble complexes with phytate and fiber, calcium, and phosphorus (Forbes and Erdman, 1983; Fairweather-Tait and Hurrell, 1996). In fact, phytic acid and fiber may decrease Mn absorption by up to 40% (Fly et al., 1989) and the addition of phytase to diets rich in phytate improved the absorption and utilization of Mn in growing nonruminants (Studzinski et al., 2006). High amounts of dietary iron also inhibits Mn absorption by competing for the same binding and absorption sites (Fairweather-Tait and Hurrell, 1996). Thoroughbred horses in a 16-week conditioning program and subjected to an exercise test meant to mimic the speed and endurance of a 3-day event showed a decrease in Mn true digestibility from 58 to 40% (Hudson et al., 2001; NRC, 2007).

Manganese is a component of many enzymes in the body, including arginase, which is responsible for urea formation, pyruvate carboxylase, which catalyzes the first step of

carbohydrate synthesis, and manganese-SOD (SOD2), which is an antioxidant acting to eliminate free radicals in the mitochondria (Fairweather-Tait and Hurrell, 1996). Manganese is essential for carbohydrate and lipid metabolism (Fairweather-Tait and Hurrell, 1996; Herdt and Hoff, 2011), and is well known for its importance in the synthesis of chondroitin sulfate, which is necessary for proper cartilage formation (Ott and Johnson, 2001; NRC, 2007).

The largest complication typically associated with Mn deficiency is abnormal cartilage development and bone malformation (NRC, 2007). Deficiency can also cause impaired growth and immunity, ataxia, and defective metabolic systems (Herdt and Hoff, 2011). While Mn is one of the least toxic trace minerals (Herdt and Hoff, 2011) and there have been no known toxicities in horses, large amounts of Mn do interfere with phosphorus absorption (NRC, 2007).

Measuring Mn in the body is controversial and difficult (Herdt and Hoff, 2011), and concentrations of Mn in both the plasma and serum are poor forms of measurement, due to the liver's removal of Mn from the blood (Kincaid, 1999; Faulkner et al., 2017). Manganese that is taken up by the liver is excreted through the bile, so any accumulation of Mn in the liver does not accurately reflect dietary intake (Kincaid, 1999). It is thought that dietary Mn affects the concentration of the mineral in the bones and other tissues (Kincaid, 1999). One study found relative availability of Mn from Mn-methionine (Mn-Met) to be 120% of that of Mn-sulfate based on the accumulation of Mn in the bone, kidney, and liver of lambs (Spears, 1996). A study in chicks found Mn-Met to be much more bioavailable than Mn-oxide based on Mn accumulation in the tibia (Fly et al., 1989). This same study tested the effects of a high phytate diet on sources of Mn supplementation and found that Mn-oxide bioavailability decreased by 37% with addition of fiber, while Mn-Met bioavailability decreased only 15% (Fly et al., 1989). Overall, Mn-methionine was concluded to provide 30.1% more bioavailable Mn in the basal (no

fiber added) diet and 74.4% more bioavailable Mn in the fiber-added diet than Mn-oxide (Fly et al., 1989). Other studies have found supplementation of Mn-Met to be 110% more bioavailable compared to Mn-sulfate in poultry and 120% to Mn-sulfate in piglets (Mézes et al., 2012).

Cobalt

Cobalt is best known for its role as an essential structural and functional component of vitamin B_{12}, or cobalamin (Herdt and Hoff, 2011; Kinobe, 2016). Vitamin B_{12} is not synthesized by mammalian cells, but is necessary for normal metabolic activities, such as nucleotide synthesis, fatty and amino acid metabolism, neural function, and the formation of blood components (Kinobe, 2016). In ruminants, rumen microbes can synthesize vitamin B_{12} if there are adequate levels of Co and phosphorus in their diet (Stillions et al., 1971; Abdel-Monem, 1987). A Co deficiency in ruminants may manifest as unthriftiness and decreased disease resistance (Kinobe, 2016). In the horse, a B_{12} deficiency attributable to a Co deficiency has not been reported, indicating that Co is metabolized and utilized differently between species (Kinobe, 2016). In the horse, cecal and colonic bacteria are able to use Co to synthesize and absorb vitamin B_{12} (Abdel-Monem, 1987; NRC, 2007; Kinobe, 2016). Since B_{12} synthesis takes place after the stomach and ileum, which is where Co is absorbed (Stillions et al., 1971), nonruminants require dietary Co and B_{12} (Stillions et al., 1971; Herdt and Hoff, 2011). While Co requirements can normally be met through the diet there has been some evidence that Co supplementation could have beneficial effects on horses (Kinobe, 2016). Cobalt is a component of methionine aminopeptidase-2, which contributes to protein degradation, tissue repair, and angiogenesis by removing methionine residues from proteins (Kinobe, 2016). Studies have shown that Co may be beneficial for blood restoration after worm infestation and for recovering after intense workouts or disease (Kinobe, 2016). In addition, Co ions are able to stabilize

hypoxia-inducible transcription factors (HIF), which leads to increased erythrocyte production and enhanced expression of pathways that protect against oxidative stress (Kinobe, 2016). A study in rats found that Co supplementation helped to protect muscle from exercise induced damage through its antioxidant promoting capacity (Kinobe, 2016). For these reasons, Co has been a "hot topic" in recent years for its performance-enhancing capabilities in equine athletes.

After absorption, Co ions circulate in the blood bound to albumin, macroglobulins, transferrin, lipoproteins, and haptoglobin (Kinobe, 2016). While normally not stored in large quantities, cobalt is found in highest concentrations in the liver, kidneys, bone, adrenals, and spleen (Georgievskii et al., 1979; Studzinski et al., 2006). The accumulation of Co in tissues changes over time. The liver from rats given a single oral Co administration initially took up 4-5% of the absorbed Co, but after 132 days, the highest levels were seen in the muscle and skeleton (Kinobe, 2016). While more research is needed, it is thought that in horses, Co accumulates in tissues with repeated regular administration and reaches a steady state, where rate of administration equals rate of elimination, after about 33 days (Kinobe, 2016). Cobalt is mainly excreted through the urine and via bile in the feces (Georgievskii et al., 1979; Studzinski et al., 2006). If there is a B_{12} deficiency, assimilation of Co increases, but otherwise remains low (about 15-20%) due to the low requirements for the mineral (Georgievskii et al., 1979).

Cobalt in the form of B_{12} is involved in red blood cell formation with iron and copper (NRC, 2007) and in the regulation of erythropoietin synthesis in the bone marrow (Studzinski et al., 2006), which is why it has been used as a means of enhancing aerobic performance in horses. Cobalt is an important co-factor for protein, carbohydrate, lipid, and nucleic acid metabolism (Herdt and Hoff, 2011), can act as an enzyme catalyst with Mn, and may be able to replace Zn in zinc-containing enzymes (Studzinski et al., 2006). Cobalt deficiency typically manifests as a B_{12}

deficiency; signs in cattle include decreased feed intake, reduced growth, anemia, impaired immune function, and decreased reproductive performance (Herdt and Hoff, 2011). There have been no cases of Co or B_{12} deficiency in horses, as the recommended amount should easily be met through normal consumption of feed (NRC, 2007; Kinobe, 2016). A cobalt toxicity is also not usually a problem, as the concentrations needed for toxicities are much higher than what is found in typical livestock diets (Herdt and Hoff, 2011; Kinobe, 2016). The most effective means by which to determine the body's Co reserves is to measure B_{12} concentration in the liver (Kincaid, 1999). There are normally very low concentrations of both Co and B_{12} in the plasma and serum, and levels change very quickly with intake of Co (Kincaid, 1999; Herdt and Hoff, 2011). In addition, a large portion of B_{12} in the plasma is inactive, so analyzing the active versus inactive analogues of Co is a challenge for most assays (Mills, 1987; Kincaid, 1999). Some suggest measuring accumulation of methylmalonate to detect a significant Co deficiency, but it is unclear if this accumulation occurs before or after physical manifestations of deficiency (Mills, 1987).

Cobalt supplementation has been shown to increase overall digestibility of crude fiber and cellulose (Abdel-Monem, 1987). Cobalt also plays a role in the conversion of volatile fatty acids, such as propionate, to glucose, as hepatic gluconeogenesis requires an enzyme containing vitamin B_{12} (Abdel-Monem, 1987). A source of more bioavailable Co comes from cobalt glucoheptonate (C-GH), a complex salt formed between the Co cation and the alpha hydroxyl organic acid glucoheptonic acid (Abdel-Monem, 1987). Overall dry matter digestibility for C-GH was 29.7% versus 26.7% for commercial organic Co complexes and 25.8% for Co-sulfate (Abdel-Monem, 1987).

Overall Conclusion of Organic vs. Inorganic Sources of Minerals

Organic vs. inorganic sources of minerals have been debated and results have proven inconclusive and varied between species. A study in lactating dairy cows found that organic trace mineral supplementation increased milk production by 0.93 kg/day, as well as increasing the quality of the milk in the forms of higher fat and protein yield (Rabiee et al., 2010). Yearling horses receiving proteinated Cu, Zn, and Mn showed growth parameters including weight, wither height, body length, and heartgirth gains comparable to inorganic sources, but proteinated minerals may have been beneficial for quality of hoof growth (Ott and Johnson, 2001). The overall inconclusiveness of mineral studies may be partly attributed to the challenge of measuring trace minerals in the body, as well as the many dietary interactions between trace minerals and other nutrients.

Introduction to Nutrition and Joints

In horses, orthopedic disorders, specifically joint related problems, are primarily to blame for losses in performance (van Weeren et al., 2008) and the major cause of lameness is osteoarthritis (OA) (Wedekind et al., 2015). While many studies have investigated the relationship between calcium, phosphorus, vitamin D, and improved bone matrix, there has been less focus on microminerals, which contribute to the organic matrix of the joint (Wedekind et al., 2015). As stated previously Zn and Cu are critical for proper collagen formation, as collagen synthesis is a Zn-dependent process and the crosslinking of collagen subunits is a Cu-dependent process (Wedekind et al., 2015). In addition, Mn is necessary for the proper development of the proteoglycan matrix of the extracellular matrix (ECM) (Wedekind et al., 2015). Some studies have shown improvements in joint and skeletal health as a result of feeding chelated trace minerals as opposed to inorganic forms (Wedekind et al., 2015). For these reasons, it is thought

that feeding chelated trace minerals, which are believed to be more bioavailable, would improve overall joint function and health, especially in response to the stress and inflammation associated with exercise.

Synovial Joint Structure

A synovial or diarthrodial joint is classified as a joint that has two functions: 1) To allow for movement; and 2) To transfer load between bones. The joint consists of the articular surfaces of at least two bones, the articular cartilage, the joint capsule, and ligaments (Todhunter, 1996; te Moller and van Weeren, 2017). The ends of each subchondral bone are covered in a layer of hyaline, glassy or translucent, articular cartilage. The joint capsule surrounds the synovial joint itself, and is made of a thick fibrous layer and an inner synovial membrane, which is in contact with the synovial fluid (SF). The joint capsule is lined by the synovial membrane, a vascularized, lymphatic supplied collagenous tissue, which covers all the structures in the joint except the cartilage (Sutton et al., 2009). Synovial cells, or synoviocytes, are arranged in three to four layers within the synovial membrane. Type A synoviocytes are mainly phagocytic macrophages. Type B synoviocytes are fibroblasts, which produce collagen, fibronectin, and hyaluronan for SF (Sutton et al., 2009). The synovial membrane secretes SF, which occupies the intra-articular space in healthy joints to provide nourishment and lubrication (Sutton et al., 2009). Synovial fluid is a colorless or pale-yellow ultrafiltrate of plasma, meaning that most of its ions and molecules are found in plasma except for hyaluronan, which is found in high concentrations in SF. High hyaluronan content gives SF a viscous property, which allows it to act as a shock-absorber and joint lubricant during movement (Todhunter, 1996; te Moller and van Weeren, 2017). Synovial fluid also acts as a communication medium between tissues and plays a role in bringing nutrients to the cartilage and removing waste products (Todhunter, 1996; te Moller and

van Weeren, 2017). Synovial fluid is subject to constant turnover, and since it is in direct contact with all joint tissues except bone, its composition provides accurate information on joint homeostasis at the time at which samples are collected. In addition, SF is the only joint component that can be obtained with relatively non-invasive techniques (te Moller and van Weeren, 2017).

Structure of Articular Cartilage

Adult articular cartilage (AC) is a metabolically active tissue designed to handle the compressive forces generated in the joint due to movement and load (Ray et al., 1996). Cartilage is composed of chondrocytes and the ECM of chondrocytes. The ECM is a 3-D network of fibers comprised primarily of collagen, proteoglycans (PGs), and water (Ray et al., 1996; Todhunter, 1996), with the two main components being type II collagen and aggrecan (Billinghurst et al., 2003; de Grauw et al., 2009). The survival of the ECM depends on a balance between synthesis and degradation of certain matrix molecules, such as type II collagen (Nelson et al., 1998). Type II collagen provides the AC with its tensile strength by forming a "gel framework" in which the PGs are enmeshed (Ray et al., 1996). In adult AC, most of the PGs are called aggrecan, which are defined as molecules aggregated with hyaluronan and link proteins to form macromolecular aggregates (Ray et al., 1996). Aggrecan is responsible for the compressive strength and stiffness of the AC (Ray et al., 1996; Billinghurst et al., 2003). This configuration of tightly packed hydrophilic aggregates in collagen gives the AC its unique properties of resilience and strength, allowing for shock absorption and resistance against compression, while also forming an obstacle for rapid transport of macromolecules through the ECM (van Weeren et al., 2008).

Type II collagen is synthesized by chondrocytes and initially secreted as a procollagen molecule with propeptide extensions. These extensions allow for correct alignment of

procollagen molecules during fibril formation. The procollagen molecule is secreted from the cell into the ECM (Nelson et al., 1998), where it forms fibrils through the action of propeptidases, which are responsible for the cleavage of these extensions, and converts procollagen to collagen. The released propeptide extensions are the basis of the CPII marker (discussed later) in SF (Ray et al., 1996). The basic collagen fibril then, is composed of approximately 50-100 collagen molecules (Todhunter, 1996). Following fibril formation, collagen molecules within the fibril undergo an extensive cross-linking process, which is mediated by the enzyme lysyl oxidase, for which Cu is a necessary component (Ray et al., 1996). Collagen fibrils have a high breaking strength due to the formation of covalent crosslinks between adjacent molecules (Todhunter, 1996; Brama et al., 2000). Type II collagen is classified as a homotrimer, meaning that it is composed of three identical alpha chains which form a triple helix structure (Todhunter, 1996; Billinghurst et al., 1997). The triple helix fibril is stabilized by intermolecular crosslinks (Billinghurst et al., 1997).

Proteoglycan monomers are primarily composed of side chains of highly charged glycosaminoglycans (GAGs). Sulfated GAG side chains are bound to a core protein to form a single proteoglycan monomer (McIlwraith, 2005). The most prominent GAGs produced by chondrocytes are keratin sulfate (KS) and chondroitin sulfate (CS). Aggrecan consists of a core protein to which CS and KS chains are attached (Chu et al., 2002). Hylauronan is also produced by chondrocytes in AC, but the hyaluronan found in SF is produced by synoviocytes. In normal AC, the PGs face constant turnover, which is a balance between chondrocyte synthesis and extracellular proteinase breakdown. This turnover process reaches an equilibrium constant under normal conditions, and large PG fragments can be found in the SF after turnover (Ray et al., 1996). It is estimated that the PG components of the ECM can be completely replaced after 300-

1800 days, but the collagen network has been estimated to turnover every 120 years in dogs and 350 years in man (van Weeren et al., 2008). The extended turnover time of collagen is most likely due to its lack of vascular supply and the heavily crosslinked network that is relatively resistant to enzyme degradation (van Weeren et al., 2008).

Collagenases belong to a family of Zn-dependent enzymes called matrix metalloproteinases (MMPs), and are able to initiate the cleavage of collagen's triple helix (Billinghurst et al., 1997). Three specific collagenases responsible for the primary cleavage of the triple helix are collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13) (Chu et al., 2002). Collagen is cleaved at a single site within each alpha chain, resulting in a ¾ length and ¼ length fragment (Chu et al., 2002). Once the fragments are cleaved, they are even more susceptible to further degradation by collagenases and other proteinases (Billinghurst et al., 1997). These three collagenases mostly originate in synovial cells and chondrocytes (Chu et al., 2002). While chondrocytes express and secrete MMP-1 and MMP-13 in both normal and OA AC, these enzymes are upregulated by interleukin-1 (IL-1). The increased collagenase expression in OA is the cause of elevated type II collagen degradation in that disease (Billinghurst et al., 1997).

In adult synovial joints, the AC is avascular and is made of four layers. The tangential or superficial zone has the highest cell density of chondrocytes that are aligned parallel to the articular surface. The intermediate or transitional zone contains larger and more rounded chondrocytes. The radial zone contains chondrocytes that are aligned perpendicularly to the articular surface. Finally, zone four, the deepest layer, or the calcified cartilage zone contains chondrocytes that are cemented in a matrix of mineralized cartilage (Ray et al., 1996; Todhunter, 1996). Chondrocytes are responsible for the synthesis and regulation of the cartilage matrix, and

while the exact mechanism for cartilage turnover is unclear, it is known that dynamic load and cytokines produced by synovial lining cells, such as interleukins and tumor necrosis factor alpha (TNF-α), accelerate turnover (Todhunter, 1996).

Joint Development

Multiple studies have investigated the role of exercise and loading on joint development. Since different parts of the same joint experience varying degrees of pressure and load during movement, AC from different areas of the joint show differing collagen characteristics (Brama et al., 2000). For example, an area that is subjected to intermittent high stress loading shows higher collagen and lower GAG content than areas subjected to more constant, lower loads (Brama et al., 2002). It is believed that a foal is born with a "blank" joint, or a joint that is uniform in regards to PG content, collagen content, and crosslinking (Brama et al., 2002; van Weeren et al., 2008). During early life, exercise plays a vital role in development of the AC extracellular matrix and collagen content and crosslinks increase significantly. Collagen turnover is not as slow in foals as it is in mature horses (Brama et al., 2000). Functional adaptation to weight bearing as a result of exercise appears to take place early in life, and the resulting heterogeneity in joint composition is essential for the different stressors that the joint and AC will face throughout life (Brama et al., 2000; Brama et al., 2002; van Weeren et al., 2008). While bone constantly undergoes remodeling throughout life, the development of the collagen network seems to be a once in a lifetime process (van Weeren et al., 2008). Studies have shown that regional differences in AC seem to develop within the first five months of a foal's life, and withholding exercise results in irreversible delays in AC heterogeneity (Brama et al., 2002). Therefore, regular joint loading and exercise during growth is essential for the proper development and strengthening of the collagen fibril network in AC.

Biomarkers

A biomarker can be defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (te Moller and van Weeren, 2017). Biomarkers can be used to more closely investigate joint processes, differentiate between affected vs. non-affected joints and determine the degree of degradation in the AC, monitor the joint's response to therapy, or give accurate prognoses (Frisbie et al., 2008). Biomarkers are typically normal products or by-products of metabolic processes that occur in the tissue of interest (McIlwraith, 2005). Synovial fluid biomarkers of cartilage matrix turnover can indirectly reflect AC damage, whether it is inflammatory or traumatic in origin (de Grauw et al., 2009). Of the many factors that influence SF levels of cartilage matrix turnover markers, local inflammation may be the most important (de Grauw et al., 2009). It has been shown that inflammation and cytokines have an inhibitory effect on cartilage matrix synthesis (Poole, 1996) and these pro-inflammatory mediators accelerate cartilage degradation (Barrachina et al., 2017). Cytokines cause proliferation and enhanced secretion of enzymes by chondrocytes, which leads to excessive degradation of cartilage (Chu et al., 2002). For example, IL-1 stimulates the catabolic process in AC through its stimulation of PGE_2, aggrecanase, and MMPs by chondrocytes, which degrade the ECM (Todhunter, 1996; McIlwraith, 2005).

Carboxypropeptide of Type II Collagen

Carboxypropeptide of Type II collagen (CPII) is a biomarker that indicates the rate of collagen synthesis (Ray et al., 1996). In joints with OA, CPII levels are increased, but not in the areas where the destruction is occurring. Additionally, CPII levels are higher in growing and injured joints (Ray et al., 1996). The increase in CPII after joint injury is usually associated with

a reparative response to mend the damage to collagen (de Grauw et al., 2009). The increase in CPII in OA is seen especially in the middle and deep zones of AC, even though the most pronounced type II collagen degradation is usually seen in the superficial zone of AC in OA (Nelson et al., 1998). This suggests that the cartilage that is still intact after OA attempts a reparative response by increasing the synthesis of type II procollagen (Nelson et al., 1998). In horses with osteochondral fragmentation, studies have shown increased concentrations of CPII in serum, leading to the conclusion that joint trauma and injury results in increased type II procollagen synthesis (McIlwraith, 2005). In a study which induced acute inflammation in weanlings using a lipopolysaccharide (LPS) injection model, levels of synovial CPII increased with increasing levels of LPS, indicating the joint's attempt to repair damage in response to insult (Lucia et al., 2013).

Collagenase Cleavage Neoepitope of Type II Collagen

The degradation of Type II collagen is mediated by matrix metalloproteinases, or collagenases, that cleave the collagen triple helix into ¾ and ¼ length fragments (Trumble et al., 2009), exposing the epitopes previously hidden by the helix (Ray et al., 1996). An epitope is the "area on the surface of an antigenic molecule against which an immune response is directed," and is typically made of a few monosaccharides or amino acid residues (Ray et al., 1996). In specific markers found in SF, epitopes are found on fragments of PGs that have been turned over from AC (Ray et al., 1996). One such epitope, collagenase cleavage neoepitope of Type II collagen, known as C2C, is exposed at the carboxy terminus of the ¾ length cleavage product, and the degradation products are subsequently released from the cartilage into the synovial fluid (Trumble et al., 2009). While there have been conflicting results concerning increased concentrations of C2C in equine SF after exercise, it is known with certainty that injury and joint

inflammation cause an increase in the amount of C2C products in the synovial fluid, and there seems to be a positive correlation between C2C concentrations and injury severity (Trumble et al., 2009). In osteoarthritic cartilage, there are higher amounts of C2C than in normal AC (Billinghurst et al., 1997).

Chondroitin Sulfate-846

Chondroitin sulfate-846 (CS-846) is a marker of aggrecan synthesis. It has been shown to be released in close association with newly synthesized aggrecan molecules (Laverty et al., 2000). This particular epitope is normally found in fetal and OA cartilage, but is mostly absent in healthy adult human, equine, and canine AC (Chu et al., 2002; McIlwraith, 2005). It is most dramatically increased in OA cartilage when the disease progresses to phase II and the cartilage becomes very degenerative (Chu et al., 2002). Studies have shown that there is an early increase in the release of aggrecan fragments from cartilage in response to injury or inflammation, and this inflammation induced enhancement of aggrecan turnover is fast and short lived (de Grauw et al., 2009). The CS-846 epitope is increased in equine joint fluids after trauma or injury and in osteoarthritis (Poole, 1996; Chu et al., 2002; McIlwraith, 2005).

Inflammation

Inflammation can broadly be defined as the biological response for restoring homeostasis. A disruption in homeostasis can occur for many reasons, but may be due to tissue malfunction, infection, injury, or some other imbalance (Medzhitov, 2008; Ashley et al., 2012). One of the trademarks of inflammation is that it is a tissue-destroying process and self-damage is unavoidable (Ashley et al., 2012). Inflammation has two functions: 1) to quickly destroy or isolate the source of disturbance; and 2) to restore tissue homeostasis (Ashley et al., 2012). Inflammation is a tightly regulated cascade response and when inflammatory stimuli are not

present, the inflammatory response (IR) is actively suppressed by regulatory gene products in order to prevent excessive collateral damage (Ashley et al., 2012). An inflammatory response resulting from an infection or injury is of the highest magnitude. A more common reason for the initiation of an IR is a tissue malfunction, but this may be of lower magnitude (Medzhitov, 2008). The nature and degree of the tissue malfunction will influence whether or not the IR can be detected using common biomarkers, such as TNF-α or IL-1β (Medzhitov, 2008).

Any cell in the body is in one of four states at any given time. During normal, healthy conditions, a cell will be in a basal state. When the cell must adapt to an abnormal condition due to any environmental changes, such as temperature, osmolarity, oxygen content, etc., the cell enters a stressed state. If the change in the parameter is greater than the stress response can handle, the cell enters an apoptotic state. If the change is even greater, the cell becomes necrotic (Medzhitov, 2008). Transitions between the cell states are not graded and occur in an all-or-none fashion; however, tissue states are graded depending on the degree of the problem at the time and how many cells are in each state (Medzhitov, 2008). The state of cells and tissues is monitored mainly by tissue-resident macrophages, which make up 10-15% of most tissues (Medzhitov, 2008). Inflammation plays a general physiological role in maintaining tissue homeostasis, monitoring tissue malfunction, and promoting adaptation to abnormal conditions that tissues cannot resolve by themselves (Medzhitov, 2008).

Cytokines are “hormone-like proteins” that mediate inflammatory responses through their effects on other cells and are often used as markers of inflammation (Lamprecht et al., 2009). Cytokines can exert their effects through endocrine, paracrine, or autocrine pathways (Price et al., 1992). It has been shown that stimuli such as exercise, energy crisis, oxidative stress, and

increased amounts of stress hormones modulate cytokine production, which is why they are studied on a frequent basis (Lamprecht et al., 2009).

In adult cartilage, cytokines influence how chondrocytes proliferate and respond to their environment (Price et al., 1992). For example, IL-1β, one of the earliest mediators of the immune system, is expressed by synoviocytes, chondrocytes, and macrophages, and acts to increase PGE_2, and MMP production by synovial cells and chondrocytes, which leads to the degradation of cartilage (Sutton et al., 2009). In addition, IL-1β inhibits type II collagen and proteoglycan synthesis (Price et al., 1992), while also stimulating other cells to produce pro-inflammatory cytokines, further contributing to an inflammatory environment (Sutton et al., 2009).

Interleukin-6, expressed by synoviocytes and chondrocytes (Sutton et al., 2009), is thought to be one of the most sensitive markers of fatigue, muscle damage, and metabolic turnover (Lamprecht et al., 2009). Interleukin-6 inhibits proteoglycan synthesis and chondrocyte proliferation, while also increasing MMP and aggrecanase activity, leading to proteoglycan catabolism (Sutton et al., 2009).

In contrast, IL-10, a cytokine known for its anti-inflammatory roles, has been shown to decrease TNF-α and IL-1β production, which also inhibits the release of PGE_2. Interleukin-10 is expressed by synoviocytes, chondrocytes, and macrophages (Sutton et al., 2009).

Sources of Cell Stress

As stated previously, stress occurs when an animal is required to make an abnormal or extreme adjustment in its behavior or physiology to cope with an environmental effect. In horses, stress can be divided into two categories: 1) psychological stress, which can be quantified by

measuring heart rate, cortisol, and endorphins; and 2) physical or physiologic stress, which is indicative of trauma and/or disease (Leadon and Hodgson, 2014).

Exercise is an acute physical stress placed on the systems of the body that strive to maintain homeostasis (McKeever and Lehnhard, 2014). In fact, the coordinated response of multiple organ systems to support the increased demand due to exercise stress is why training is beneficial, with the end result and goal of training being to enhance physiological function (McKeever and Lehnhard, 2014). One adaptation to training evident in both humans and horses is a reduction in the inflammatory response that appears post-exercise. Typically, post-exercise inflammation is associated with the production of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. The increased expression of these cytokines post-exercise has been associated with a multitude of physiologic disruptions ranging from muscle soreness to debilitating soft tissue, bone, or joint damage (Horohov et al., 2012).

Exercise also influences joint homeostasis and can up or downregulate anabolic and catabolic processes (te Moller and van Weeren, 2017). Specifically, exercise and joint loading can change the composition of AC through alterations in chondrocyte metabolism (Dykgraaf et al., 2008). For example, inflammation of soft tissues due to trauma from repeated activities can contribute to degradative or catabolic processes in the joint by releasing inflammatory mediators and cytokines into the SF (te Moller and van Weeren, 2017). On the other hand, exercise causes changes in joint angle which increases blood flow and alters intra-articular pressure. This is beneficial for stimulating fluid exchange and promoting SF turnover. Similarly, changes in various factors in the synovial fluid during exercise may lead to changes in glycosaminoglycan synthesis by chondrocytes (Patterson and Firth, 2014). In contrast, if a joint is overloaded and develops effusion, blood flow will decrease, negatively affecting SF turnover. Studies have

shown significant increases in CS-846 and CPII in the synovial fluid as a result of exercise (Frisbie et al., 2008). While maintenance of normal articular cartilage composition, structure, and function depends on repetitive joint use (Billinghurst et al., 2003), there does appear to be a fine line between beneficial and detrimental effects of exercise on joint homeostasis (te Moller and van Weeren, 2017).

Conclusion

Chronic stress from exercise training likely has an effect on the horse's inflammatory response, in addition to cartilage breakdown and formation. Organic minerals might be beneficial for horses experiencing these stresses by providing more bioavailable substrate for the pathways involved in cartilage formation. In addition, microminerals are necessary for their known roles in the immune system, metabolic function, and performance, which may alter the inflammatory response.

Quantifying trace mineral absorption is notoriously challenging. Investigating effects of the minerals in target tissues such as the joint or in the circulation may be one option to examine mineral utilization within the body using relatively non-invasive techniques. The effects of different mineral sources are particularly interesting in the young, growing horse, which is already subject to great developmental changes, in addition to the burden of undergoing a training program. Modifying dietary components in order to positively affect growth and the many stressors associated with early training would be beneficial for industry practices. Organic mineral sources may be an avenue for achieving this goal.

CHAPTER III

MATERIALS AND METHODS

Horses

Sixteen Quarter Horse yearlings (n=7 fillies, n=9 colts) with a starting age of 9.1 ± 0.17 mo and BW of 300.80 ± 5.44 kg were used in this 12-wk trial. Horses originated from two sources: 7 yearlings had been raised at the Texas A&M University Dick Freeman Arena (College Station, TX) and 9 yearlings were leased from Birdsong Farms (Hearne, TX) for the duration of the trial. Yearlings had no previous history of lameness or forced exercise. Horses were group-housed by gender at Texas A&M University's Freeman Arena in approximately 0.5-hectare pastures. Although horses were housed on pastures, the study was conducted in the months of January through April when Bermudagrass forage in pastures was dormant. Grazing was not prevented, but due to the low amount of grass in the pasture and the size of the pasture itself, it was assumed to play an insignificant nutritional role. Furthermore, estimates of hay consumption (1.23% BW; DM basis) and documentation of concentrate intake (1.25% BW; DM basis) indicated that pasture forage was likely a minor component of total daily DM intake based on NRC (2007) estimates of 2.5% BW DM for yearling horses. Horses were hand walked into 3 x 3 m stalls twice daily to be individually fed their grain concentrate meal. All care, procedures, and handling of animals was reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University (AUP# 2016-0294).

Dietary Treatments

Horses were individually fed 1 of 2 custom-formulated concentrates (Cargill Inc., Minneapolis, MN) at 1.25% BW (DM basis), split equally into AM and PM meals at 0600 and 1700 h, respectively. Refusals were recorded daily with an average daily refusal rate of 0.31 kg

per horse for the duration of the study. Yearlings were grouped by BW, gender, age, and farm of origin, and randomly assigned to 1 of 2 treatment groups for 12 wk: Zn, Mn, Cu amino acid complexes and Co glucoheptonate (CTM; n=3 fillies, n=5 colts; Zinpro 4-Plex C, Zinpro Corporation, Eden Prairie, MN) or iso-levels of inorganic Zn, Mn, Cu, and Co (INORG; n=4 fillies, n=4 colts). Horses also received coastal Bermudagrass hay (*Cynodon dactylon*) ad libitum in their pastures all sourced from the same cutting and field. Individual hay intake was estimated from disappearance of known quantities offered and was assumed to be equal among all horses in the group. This resulted in an average hay intake of 1.23% BW (DM basis) for each horse. All horses had received their respective treatment diets for 12 wk prior to the start of this study.

Diets were formulated to meet or slightly exceed requirements for growing horses in light work. Nutrient analysis was performed on all feedstuffs before the initiation of the trial by Cargill's NIR Central Laboratory (Elk River, MN). Guaranteed analysis of feedstuffs is provided in Table 1.

Table 1. Nutrient composition of feeds offered to yearling horses.

		Concentrate	
Nutrient[1]	**Bermudagrass hay**	**CTM**[2]	**INORG**[2]
DE, Mcal/kg	1.97	2.76	2.80
Crude fat, %	2.26	6.20	7.05
CP, %	12.51	21.00	20.62
NDF, %	65.99	35.60	35.66
ADF, %	35.51	19.80	18.95
Ca, %	0.38	1.46	1.28
P, %	0.26	1.26	1.01
Zn, ppm	29.19	230.18	323.02
Cu, ppm	7.91	60.63	50.65
Mn, ppm	187.05	208.85	275.75
Co, ppm	0.00	10.11	11.25
Se, ppm	0.39	0.81	0.97

[1]Values presented on a 100% DM basis.
[2]CTM = Zn, Mn, Cu amino acid complexes and Co glucoheptonate; INORG = iso-levels of inorganic Zn, Mn, Cu, and Co.

Horses were weighed weekly on a platform livestock scale calibrated using 226.8 kg of applied weight and accurate to ± 0.45 kg (Bastrop Scale Company Inc., Bastrop, TX), and concentrate intake was adjusted accordingly. Body condition score was also evaluated weekly by two independent investigators using the 1 to 9 scale described by Henneke et al. (1983) on the same day BW was measured. Horses were fed to maintain a BCS between 5 and 6.

Submaximal Exercise Training

Beginning after wk 0 collections, horses were enrolled in a submaximal exercise training program for 30 min/d, 5 d/wk on an 8-horse Panel Walker, measuring approximately 21 meters in diameter (Priefert Manufacturing, Mount Pleasant, TX). Gait speed was increased gradually over the course of the study and reached final speeds by wk 9. Final gait speeds (walk = 1.12 m/s, trot = 2.95 m/s, and canter = 5.36 m/s) remained constant from wk 9 to 12. The 30-minute exercise program consisted of 15 min of walking, 10 min of trotting, and 5 min of canter. Horses started the exercise bout in different directions each day and were reversed midway through the bout each day in an effort to induce equal loading on all four limbs.

Sample Collection

Synovial fluid samples were collected at wk 8 and 12 of the study. Week 0 samples were obtained from the final collection of a previous study in which the horses were enrolled (Millican et al., 2018). Sampling occurred at 0600 prior to receiving any concentrate.

The carpal arthrocentesis procedure to collect synovial fluid was performed on the radiocarpal joint by a board-certified veterinarian from the Texas A&M University Large Animal Clinic (College Station, TX). Prior to the procedure, both carpal joints were clipped and aseptically prepared. Horses were sedated by intravenous administration of either 0.1-1.0 mL of detomidine hydrochloride (Dormosedan; Pfizer Animal Health, New York, NY) or 0.5 mg/kg BW of xylazine hydrochloride (XylaMed; MWI Animal Health, Boise, ID). For sampling consistency, ease of collection, and to obtain the volume of fluid required (1 to 4 mL per sample), the carpal joint was aseptically aspirated utilizing a location medial to the extensor carpi radialis tendon in the palpable depression between the radial carpal bone and the third carpal bone, to a depth of approximately 12.7 mm to avoid unnecessary contact with articular cartilage

(McIlwraith and Trotter, 1996). Synovial fluid was transferred into sterile non-additive tubes (BD Vacutainer Blood Serum Collection Tubes; Becton-Dickinson and Company, Frankling Lakes, NJ) and immediately placed on ice. Samples were then divided into small aliquots of 0.5 to 1.0 mL and stored at -80°C for later analysis.

CPII, C2C, and CS-846 Concentrations

Synovial fluid samples were analyzed for concentrations of carboxypropeptide of type II collagen (CPII), collagenase cleavage neoepitope of type II collagen (C2C), and chondroitin sulfate-846 (CS-846). Synovial CPII and C2C concentrations were measured using commercially available ELISA kits (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada) previously validated for use in horses (Frisbie et al., 2008). Standards were diluted according to manufacturer's recommendations and samples were diluted at a 1:4 dilution using buffer provided in the kit. All samples were analyzed in duplicate. The intraassay and interassay CV were 5.95% and 11.26%, respectively, for CPII and 5.10% and 4.54%, respectively, for C2C.

Synovial fluid concentrations of CS-846 were also determined using a commercial ELISA kit (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada). Standards were diluted according to manufacturer's recommendations and samples were diluted 1:400 with a buffer provided in the kit. Standards and samples were analyzed in duplicate. The intraassay and interassay CV for CS-846 were 5.02% and 8.81%, respectively.

Statistical Analysis

Differences in CPII, C2C, and CS-846, as well as the ratio of CPII:C2C, were analyzed using PROC MIXED in SAS (v 9.4; SAS Institute Inc., Cary, NC). Prior to this trial, all horses had undergone an acute LPS challenge contained to either the left or right fore radiocarpal joint, while the alternate fore radiocarpal joint served as the unchallenged control (Millican et al.,

2018). Therefore, the statistical model for this trial contained time, diet, knee (LPS or control), and all interactions as fixed effects, and horse within dietary treatment as a random effect. However, due to lack of significance, knee was removed from the model. After analyzing and adjusting for outliers, data were combined and reanalyzed using a modified model statement for variables that lacked significance in one or more fixed effects. Values that were not normally distributed were log-transformed prior to analysis. All data are expressed as least square means ± SEM. Significance was declared at $P \leq 0.05$, and trends declared at $P \leq 0.10$.

CHAPTER IV

RESULTS

Synovial Fluid Biomarkers

Concentrations of CPII increased from wk 0 to 8 ($P < 0.0002$) and remained elevated at wk 12 ($P < 0.0001$; Fig. 1). There was no influence of dietary treatment on CPII concentrations (Fig. 1).

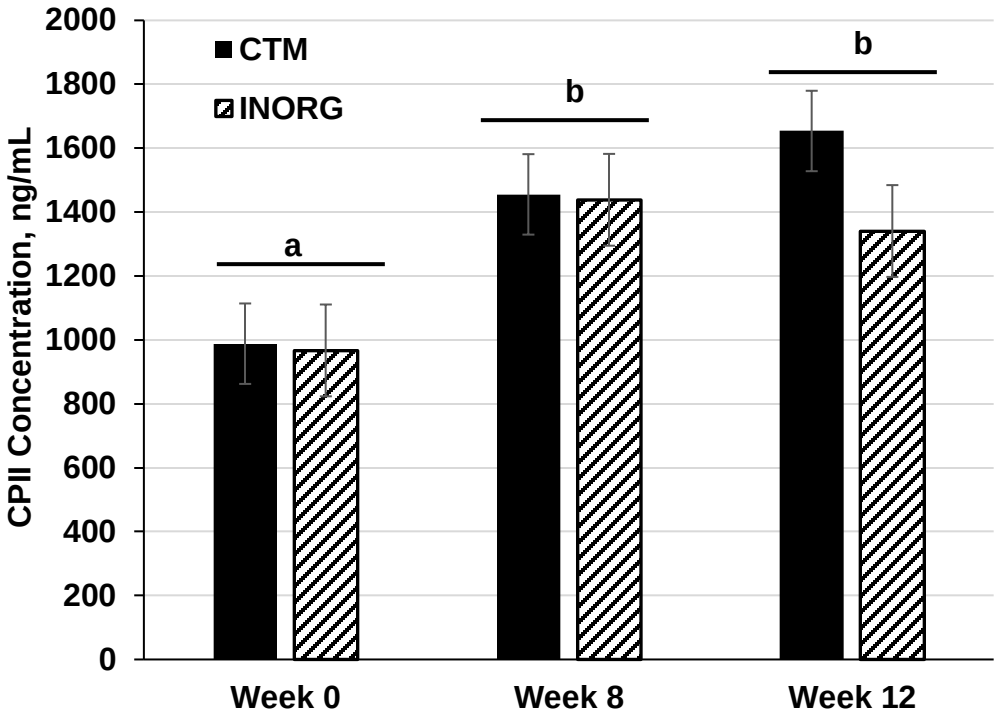

Figure 1. Synovial fluid carboxypropeptide of type II collagen (CPII) concentrations from horses receiving either complexed (CTM) or inorganic (INORG) dietary Zn, Mn, Cu, and Co and undergoing submaximal exercise training for 12 wk. Overall effects of time ($P < 0.0001$), dietary treatment ($P = 0.310$), and time × diet ($P = 0.375$). [a,b] Means with different letters differ ($P \leq 0.05$).

Conversely, the concentration of C2C decreased from wk 0 to 8 ($P < 0.0001$), and remained suppressed at wk 12 ($P < 0.0001$; Fig. 2). There was no influence of dietary treatment on SF concentrations of C2C, and no interaction between diet and time was observed (Fig. 2).

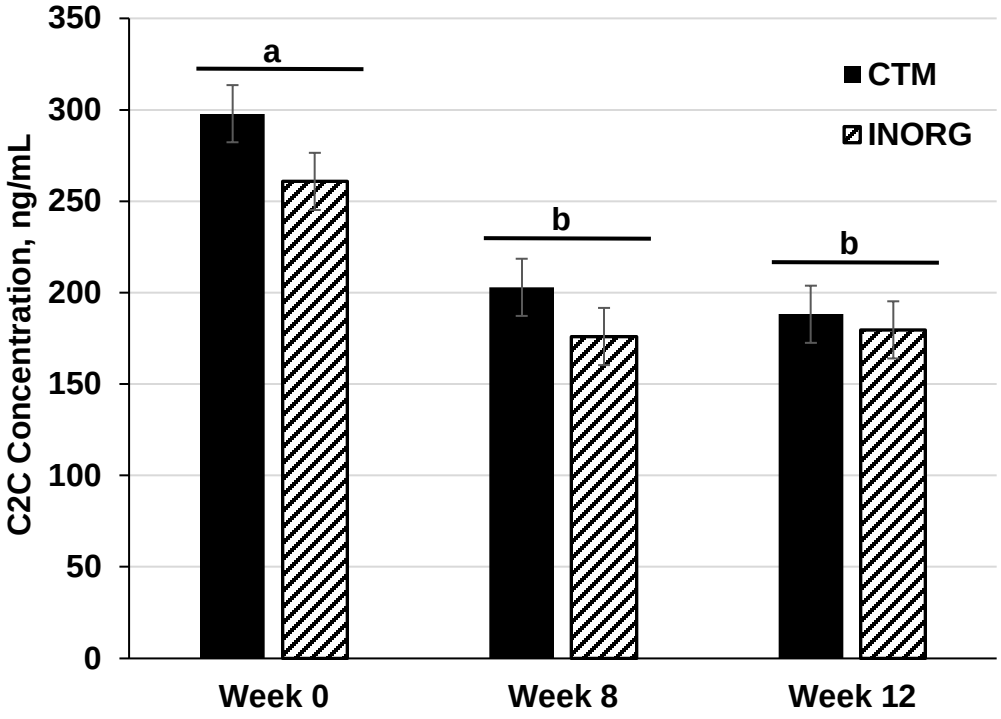

Figure 2. Synovial fluid collagenase cleavage neoepitope of type II collagen (C2C) concentrations from horses receiving either complexed (CTM) or inorganic (INORG) dietary Zn, Mn, Cu, and Co and undergoing submaximal exercise training for 12 wk. Overall effects of time ($P < 0.0001$), dietary treatment ($P = 0.144$), and time × diet ($P = 0.569$). [a,b] Means with different letters differ ($P \leq 0.05$).

The ratio of CPII:C2C showed a trend for an interaction between time and diet ($P = 0.087$; Fig. 3). The ratio increased in all horses from wk 0 to 8 ($P < 0.0001$). The ratio continued to increase in CTM to wk 12 ($P = 0.015$), while remaining unchanged between wk 8 and 12 in INORG (Fig. 3).

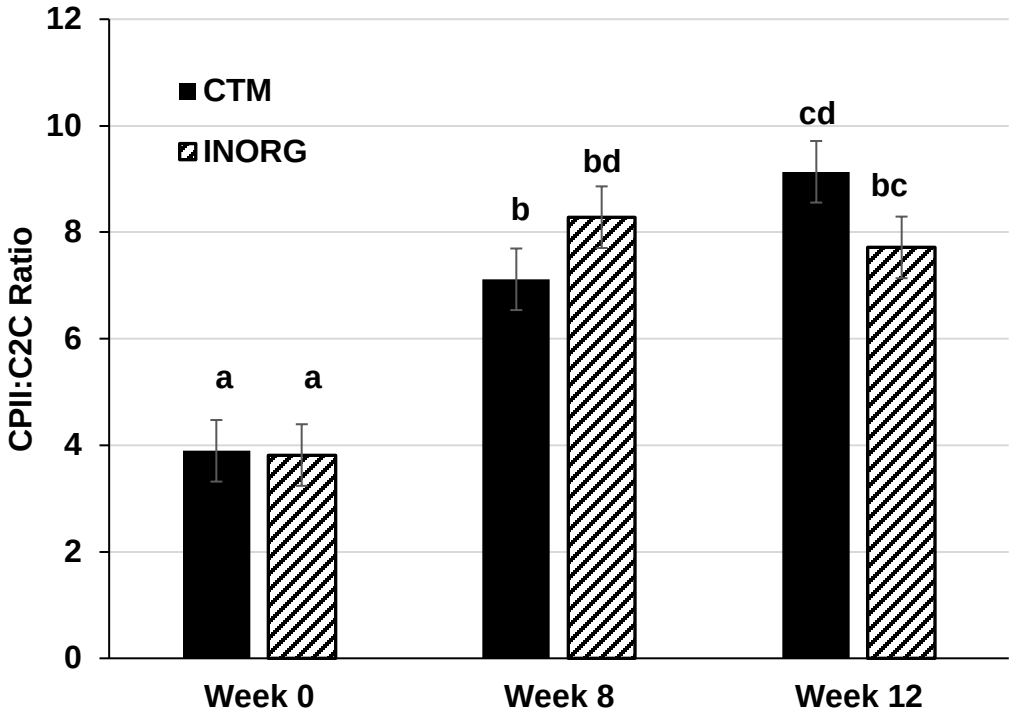

Figure 3. The ratio of carboxypropeptide of type II collagen (CPII):collagenase cleavage neoepitope of type II collagen (C2C) ratio from horses receiving either complexed (CTM) or inorganic (INORG) dietary Zn, Mn, Cu, and Co and undergoing submaximal exercise training for 12 wk. Overall effects of time ($P < 0.0001$), dietary treatment ($P = 0.819$), and time × diet ($P = 0.087$). [a,b] Means with different letters differ ($P \leq 0.05$).

There was an overall effect of time observed for CS-846 levels ($P = 0.005$). Concentration of CS-846 showed a trend for an increase from wk 0 to 8 ($P = 0.099$), but decreased from wk 8 to 12 ($P = 0.001$), resulting in CS-846 tending to be lower at wk 12 than 0 ($P = 0.098$; Fig. 4). No effect of dietary treatment was observed (Fig. 4).

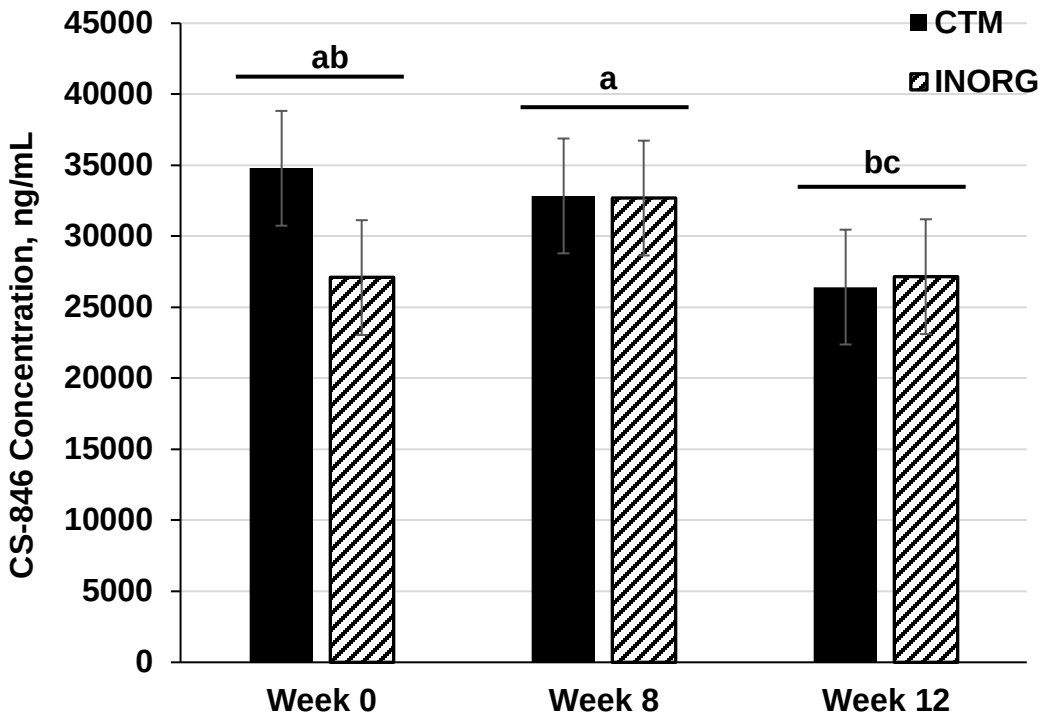

Figure 4. Synovial fluid chondroitin sulfate-846 (CS-846) concentrations from horses receiving either complexed (CTM) or inorganic (INORG) dietary Zn, Mn, Cu, and Co and undergoing submaximal exercise training for 12 wk. Overall effects of time (P = 0.005), dietary treatment (*P* = 0.431), and time × diet (*P* = 0.895). [a,b] Means with different letters differ ($P \leq 0.05$). Wk 0 values were included as a covariate for statistical analysis.

CHAPTER V

DISCUSSION

There have been limited studies investigating the role of diet and exercise in joint metabolism and homeostasis in yearling horses. Furthermore, previous research on trace mineral supplementation has provided inconclusive data, making it difficult to determine whether or not varying sources of minerals is beneficial. In the present study, the effects of organic (in the form of amino acid complexes) vs. inorganic trace mineral supplementation on cartilage metabolism was determined by analyzing concentrations of specific biomarkers known for their roles in catabolic and anabolic cartilage activity.

The finding that dietary treatment (i.e. inorganic vs. complexed trace mineral supplementation) did not result in significant differences in the joint is in agreement with reports of no differences in absorption or retention of Cu, Zn, or Mn when fed as an oxide, sulfate, or chelate in miniature horses (Wagner et al., 2005). Similarly, complexed Cu, Zn, Mn, or Co did not show consistent improvements in digestibility, mineral balance, or growth in Quarter Horse yearlings compared to an inorganic alternative (Naile et al., 2005). Ott et al. (2001) fed either inorganic or proteinated Cu, Mn, and Zn to 15 yearlings of different breeds for 16 wk and found no differences in growth or development, but did conclude that the trace mineral proteinates may be beneficial for special functions such as hoof growth. While these studies measured the effects of organic trace minerals differently, it is not unexpected that the joint would be unaffected if overall digestibility and absorption were unaffected by mineral source.

Yearlings were enrolled in a previous study prior to the initiation of the current study in which one carpal joint was injected with LPS to investigate the inflammatory response to an acute stressor (Millican et al., 2018). Since the long-term effects of an acute LPS insult in horses

has not been well documented, it was advantageous to analyze data by knee in order to determine any consequences of inducing acute inflammation in the joint. While this has not been studied in horses before, Lucia et al. (2013) reported that SF C2C concentrations were unaffected by intra-articular treatment (LPS vs. control) up to 2 wk after injection. Concentrations of CPII increased with increasing amounts of LPS, especially 6 and 24 h after injection, but returned to baseline values by 2 wk (Lucia et al., 2013). In a similar study, Kahn et al. (2017) also observed that SF C2C and CPII concentrations returned to baseline by 2 wk post-LPS injection. Following the same pattern, CS-846 levels returned to baseline concentrations by 1 wk post-LPS challenge in mature Warmblood mares (de Grauw et al., 2009). The finding that there was no difference between LPS-injected and control knees at 10 and 14 wk post-LPS challenge (wk 8 and 12 of this study) is in agreement with these results, supporting that no disruption in CPII, C2C, and CS-846 is seen up to 14-wk post-acute LPS insult in yearling Quarter Horses undergoing submaximal exercise training.

While dietary treatment did not show any distinct differences in the current study, there was an overall effect of time. In the previous study in which these horses were enrolled, SF CPII and the CPII:C2C ratio increased over 8 wk in the absence of exercise (Millican et al., 2018). Therefore, the current results suggest that submaximal exercise training did not negatively affect cartilage synthesis in young, growing horses. Concentrations of CPII increased from wk 0 to 8 and 12. When the joint is faced with injury or insult, increased CPII levels are generally associated with a reparative response to mend collagen damage (de Grauw et al., 2009). Any type of exercise or impact to the joint will result in small micro-tears in cartilage and bone, which may account for the increase in CPII seen here. Frisbie et al. (2008) also reported significant increases in CPII in synovial fluid after a 91-d high-intensity exercise program in

two-year-olds, which is in agreement with the current study. Additionally, a recent study using the established LPS model found that CPII concentrations in yearlings increased or remained the same after joint insult, regardless of injection, as compared to two- and three-year olds and mature horses, which showed decreasing CPII levels after injection (Kahn et al., 2017). This finding, in conjunction with the current study, suggest that yearlings have a greater rate of cartilage formation as compared to mature horses, which could account for the additional increase in CPII seen over time as a result of exercise. Concentrations of SF CPII have been found to increase in horses with osteochondrosis (OCD) and OA, but only in association with increased collagen degradation (Laverty et al., 2000).

Concentrations of C2C decreased over time, specifically from wk 0 to 8 and 12. Previous studies have shown that SF C2C concentrations in two-year-olds increased significantly in the final week of a 91-d strenuous exercise trial completed on a high-speed treadmill to stimulate race training (Frisbie et al., 2008). While the different results seen in our study could be attributed to a shorter exercise training period (84 d), it is more likely that the submaximal, low-intensity exercise program did not create excessive load or inflammation. Inflammation is one of the most important factors influencing SF biomarkers (de Grauw et al., 2009) and overproduction of inflammatory mediators causing an increase in MMP production leads to higher rates of collagen degradation. Trumble et al. (2008) observed no change in synovial fluid C2C concentrations in horses ranging from 14 to 20 mo of age after 5 to 6 mo of exercise training on a racetrack. Horses in the current study were younger at the start of the trial, and as cartilage turnover is more active in the young horse (Brama et al., 1998), this could explain the initial high concentrations of C2C. The absence of changes in C2C concentrations from wk 8 to 12, when the horses were 12 to 14 mo of age, is then in agreement with Trumble et al. (2008).

Previous studies have shown increasing C2C concentrations in response to induced inflammation, and have reported increased baseline levels for mature horses as compared to weanlings and yearlings (Lucia et al., 2013; Kahn et al., 2017). As the yearlings in the current trial grew older over the course of the study, C2C decreased, suggesting that submaximal exercise may be beneficial in the aging process.

The CPII:C2C ratio also increased over the course of the study, reaching a maximum of 7.0 to 9.0 ng/mL at wk 12, which is 3 to 4 times greater than values reported in normal, healthy tarsocrural SF from Warmblood yearlings (de Grauw et al., 2011). The radiocarpal joint of the forelimb likely experiences more load than the hindlimb tarsocrural joint; therefore, an increase in the ratio of synthesis to degradation was not unexpected. Kahn et al. (2017) reported a baseline yearling CPII:C2C ratio similar to the wk 0 ratio of the yearlings in the current study, but did not find a significant increase in the ratio 2 wk after LPS injection in either LPS or control knees. This indicates inflammation may not affect the ratio of synthesis to degradation in yearlings as much as submaximal exercise. Interestingly, the CPII:C2C ratio continued to increase for the CTM group at wk 12, while remaining unchanged in INORG horses. This suggests that dietary trace mineral source may enhance cartilage synthesis and joint regeneration, and perhaps differences between dietary treatments would have emerged if the study was prolonged.

The 846 epitope is found at its maximum expression in fetal and newborn articular cartilage, and progressively disappears with aging (Laverty et al., 2000; Chu et al., 2002). It has been established that high levels of CS-846 indicate aggrecan synthesis and therefore cartilage anabolic activity (Chavez et al., 2016). Concentrations of CS-846 have been shown to be elevated in joint fluid after a trauma or in OA (Poole, 1996). Frisbie et al. (2008) reported a

range of SF CS-846 concentrations in two-year olds that was similar to concentrations in the yearlings in the current study, but also found that concentrations of the epitope significantly increased as a result of high-intensity exercise. The trial performed by Frisbie et al. (2008) included weekly arthocenteses procedures, which has been identified as a confounding factor because of the associated inflammation within the joint. Glycosaminoglycan content, which includes chondroitin sulfate chains, were greater in cartilage from horses subjected to a high intensity training program as opposed to those undergoing a low intensity exercise program (Murray et al., 2001). Levels of CS-846 in the present study tended to increase from wk 0 to 8 and tended to decrease from wk 0 to 12, but overall maintained a relatively small physiological range. This further demonstrates that submaximal training at a level that likely did cause excessive joint damage or inflammation may be beneficial for joint homeostasis. In a prospective study of young racing Thoroughbreds, CS-846 levels were found to be significantly lower in horses suffering from intra-articular fragmentation 6 mo prior to injury as compared to uninjured control horses (Frisbie et al., 2010). Similarly, Laverty et al. (2000) reported significantly lower CS-846 levels in young joints affected with osteochondrosis (OCD) compared to young healthy joints, while there was no difference in concentrations between mature normal and mature OCD joints. Laverty et al. (2000) also found that CS-846 levels were significantly higher in normal joints from young horses under 2 years of age compared to normal joints of mature horses older than 2 years. The yearlings used in the current study had no previous history of lameness and were all in sound condition when the project ended, which may be a function of high CS-846 levels.

Limitations of this study include the lack of serum biomarker measurements. While synovial fluid concentrations may be a more accurate measure of what is happening at the joint

level, they may also be more variable than serum biomarkers (Wedekind et al., 2015). It could have been useful to compare serum and synovial fluid markers, as cartilage turnover products may diffuse out of the cartilage and into either synovial fluid or blood (Todhunter, 1996). Additionally, this study did not have a true, non-exercised control group, which would have allowed differentiation of results due to diet, age, or exercise training to be definitively identified.

Overall, different sources of dietary Cu, Zn, Mn, and Co did not cause significant differences in the joint, but the increase in CPII relative to C2C at wk 12 in CTM horses indicates that complexed trace minerals, in conjunction with submaximal exercise training, may be beneficial for joint health in the young performance horse. Additionally, it should be mentioned that submaximal training, even on a circle with a diameter of 21 m, did not appear to be damaging in the young horse undergoing training for the first time, as seen by the decrease in collagen degradation. Future studies are needed to further understand the role that diet and submaximal exercise play in maintaining a healthy joint environment with respect to articular cartilage. These findings could potentially be translated to the field of human medicine.

CHAPTER VI

SUMMARY

The importance of trace minerals in the body is well understood, but the best way to increase absorption has yet to be fully elucidated. Increasing a mineral's bioavailability through chelation has been extensively studied, but has not provided consistent results in support of feeding organic trace minerals. In this study, sixteen Quarter Horse yearlings were grouped by gender, age, BW, and farm source, and randomly assigned to one of two treatments: CTM or INORG trace mineral supplementation. All horses entered a submaximal exercise training program and synovial fluid samples were collected via arthrocentesis at weeks 0, 8, and 12. Joint fluid samples were analyzed for concentrations of a catabolic cartilage biomarker, C2C, and anabolic cartilage biomarkers, CPII and CS-846. After statistical analysis, it was determined that dietary treatment had no effect on cartilage metabolism but over time, C2C concentrations decreased and CPII concentrations increased. Concentrations of CS-846 tended to increase from wk 0 to 8 and tended to decrease from wk 0 to 12. The ratio of CPII:C2C increased over the course of the study in all groups, but only continued to rise at wk 12 for the CTM group, while the INORG ratio remained the same from wk 8 to 12. These results indicate that diet, in addition to submaximal exercise, causes changes in the joint environment, leading to increased cartilage synthesis and decreased cartilage degradation. While future studies investigating the effects of submaximal exercise on various ages of horses are necessary to determine whether or not these results are repeatable, it is an important finding that submaximal exercise, not intense enough to cause joint overload, may be beneficial for long term joint health.

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