THE ROLE ALTERATIONS ON DIETARY CALCIUM AND SILICON BALANCE PLAY ON BONE AND CARTILAGE IN HORSES AND POULTRY

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ABSTRACT

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Alterations to mineral balances can affect skeletal quality as minerals are the major component in bone and can influence cartilage development. Calcium makes up a large portion of bone, and homeostasis is tightly regulated with several mechanisms to maintain stable calcium concentrations in blood. Silicon associates mainly with collagen in soft tissues but has been implicated in bone development and cartilage quality. Previous research in horses documented that furosemide negatively impacted calcium balance for 3 days but did not determine when calcium balance returned to baseline, which may have long-term implications for bone of horses that regularly receive furosemide. Silicon has positive effects on bone mineral content and density and can increase collagen synthesis in multiple species, potentially affecting osteoarthritis in mature horses and bone quality in immature broilers. The overall purpose of the studies within this dissertation was to determine the extent to which impacts on mineral balance could affect both mature bone and cartilage as well as immature, growing bone. The three hypotheses tested within this dissertation include: 1) that Ca balance would be negatively impacted for at least 3 days but would be no different than controls within 7 days of furosemide administration; 2) that Si supplementation would increase bone formation markers and cartilage turnover through increased collagen degradation and formation markers and reduce lameness severity over time and as compared to controls in mature horses; and 3) and that increasing Si concentrations would improve bone quality measures in immature broilers as compared to controls and that these effects would be most prominent at the highest supplement concentration.

Calcium balance in horses administered furosemide was more negative on d 1 than d 3 ($P < 0.05$), and fecal calcium concentrations were lower in furosemide-administered horses on d 7 as compared to d 1 ($P < 0.001$), indicating a potential mechanism to restore calcium balance. These findings corroborate previous studies on furosemide and calcium balance and provide evidence for a possible mechanism to recover net calcium losses after furosemide administration. In mature horses, silicon supplementation did not increase collagen degradation and/or synthesis markers in synovial fluid as compared to controls, indicating that cartilage turnover remained unaffected. While promising in bone and cartilage development in young animals, it may be too late for silicon supplementation to affect bone and cartilage in mature animals. In broilers, bone morphological and mechanical measures and density were unaffected by silicon supplementation, but supplementation did increase serum calcium concentrations and improved footpad dermatitis and hock burn welfare scores ($P < 0.05$). These differences demonstrate that silicon affects metabolism of other minerals and may be more beneficial for skin, rather than cartilage or bone. Overall, these studies show the difficulty of altering mature bone and cartilage composition and strength through alterations in mineral balance. Additionally, dietary silicon supplementation may not improve bone and cartilage quality but may be helpful in addressing problems associated with skin and connective tissue.

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CHAPTER 1: Literature Review

THE SKELETON

The skeleton is a unique system that serves four main purposes: structure, hematopoiesis, mineral reservoir, and locomotion (Clarke, 2008; Florencio-Silva et al., 2015). The cranium, vertebrae, and thoracic cage all protect vital organs while the length and orientation of other bones affect overall conformation of an animal. Bone marrow houses stem cells responsible for hematopoietic and immune production and function. Bone constitutes the body's largest reservoir of calcium (Ca) and phosphorus (P) as well as other minerals. Long bones and cartilage at the joints between bones allow for movement and efficient transfer of kinetic energy through attachment sites for muscles and other connective tissues. Due to its wide range of function, the bone and cartilage that make up the skeleton can be impacted by various factors, including growth, exercise, and nutrition.

BONE COMPOSITION

Bone is a heterogenous tissue. It consists of around 65% mineral, 25% organic matrix, and 10% water by weight (Burr, 2019), though estimates may vary depending on bone type, age, and health (Clarke, 2008; Boskey, 2013). Hydroxyapatite makes up the majority of the mineral phase of bone and is chemically composed of $Ca_{10}(PO_4)_6(OH)_2$ (Boskey, 2013), which forms crystalline structures within the organic matrix and provides compressive strength and hardness to the bone (Bonser and Casinos, 2003). Increases in Ca content, as a measure of increasing mineral phase, can contribute to stiffer bone (Currey and Brear, 1990) but may also create more brittle bone (Rath et al., 2000; Boskey, 2013). Due to hydroxyapatite's chemical composition, bone contains 99% of the body's Ca and 85% of its P (DiMeglio and Imel, 2019), making it,

overwhelmingly, the largest reservoir of both macrominerals in the body. However, Ca makes up between 30-40% of this mineral phase with P at 15-20% of the composition and the remainder an assortment of other minerals including magnesium (Mg), silicon (Si), and boron (B) (Jugdaohsingh et al., 2015a; Gallant and Weaver, 2019). This crystalline structure can lead to ionic substitutions at any location, including zinc oxide for Ca (Saxena et al., 2018), fluoride for hydroxyl (Qiao et al., 2017), and silicate for phosphate (Ratnayake et al., 2017). Depending on the ion, these substitutions can alter crystallinity, crystal size, and the hydroxyapatite lattice (Ratnayake et al., 2017), leading to changes in the chemical and biomechanical properties of the whole bone.

The organic matrix of bone is 90% type I collagen with the remaining 10% made of various noncollagenous proteins (Clarke, 2008; Boskey, 2013). Osteoblasts synthesize and secrete type I collagen during bone growth and repair (Raggatt and Partridge, 2010; Bellido et al., 2019), and these collagen fibers impart elasticity and strength (Martin and Ishida, 1989; Martin and Boardman, 1993). Other matrix proteins include osteopontin to promote adhesion and osteocalcin to enhance mineral binding and deposition (Burr, 2019). This matrix provides a template on which the hydroxyapatite can form, so changes to the orientation and crosslinking of these proteins can alter structural and biomechanical properties of the whole bone. As bone ages, collagen crosslinks are increased but so are collagen advanced glycation end products (Boskey, 2013), ultimately creating more fragile bone. The flexibility of the organic matrix allows bone to withstand stresses and strains, especially at high speeds, without damage (Burr, 2019).

Finally, water serves as a means for metabolic and ion exchange within bone, and water can impact biomechanical and mechanosensory functions (Boskey, 2013). Less than half of water within bone is bound to either mineral or collagen, serving either to reduce stresses or

increase stability (Burr, 2019). The remaining water flows freely throughout the tissue for signal transduction and cell nourishment. Porosity may increase water content (Boskey, 2013) and mineralization typically decreases water (Burr, 2019) by changing the amount of space available for water to fill. While greater porosity is typically associated with weaker bone (Williams et al., 2004), the reduction in water due to mineralization generates more brittle bone (Burr, 2019). Bone's combination of mineral and organic matrix enables it to serve as a mineral reservoir, provide structure and protection, and allow movement.

JOINTS

Joints in the skeleton are integral for movement. Joints are structures between bones that typically consist of a synovial membrane, articular cartilage, subchondral bone, and supportive ligaments. The synovial membrane encapsulates the joint space, providing blood supply to an otherwise avascular space while maintaining synovial fluid volume and composition (Bertone, 2008). Synovial fluid, a plasma filtrate, fills the joint space, allowing for the exchange of nutrients and metabolic products (Steel, 2008) since articular cartilage receives no blood supply. The synovium consists of an outer layer of fibrous connective tissue and an inner layer of synoviocytes responsible for producing lubricin and hyaluronic acid to maintain fluid viscosity and joint lubrication (Mathiessen and Conaghan, 2017). Movement is necessary to maintain the fluid flow into and out of the joint space (Bertone, 2008). Subchondral bone separates trabecular bone from the calcified cartilage located directly underneath the smooth articular cartilage of the joint's surface (Cucchiarini et al., 2016). Damage to the subchondral bone causes improper thickening and stiffening of the bone through disturbed signaling pathways (Cucchiarini et al., 2016), ultimately damaging the articular cartilage as well. If trabecular bone located directly

beneath the subchondral bone thickens, it will also thicken the subchondral plate, calcified cartilage, and hyaline layers of articular cartilage (Murray et al., 1999). Additionally, damage to cartilage can result in increased superficial fibrillation and deeper propagated cracks (Cucchiarini et al., 2016), weakening the cartilage's ability to withstand loading. As this damage grows into lesions, it can expose the subchondral bone to inflammation and damage as well, potentially generating osteophytes (Cucchiarini et al., 2016). Articular cartilage allows for different planes of movement, but its ultimate role is to absorb and transfer loads (Bertone, 2008). Without these structures in the skeleton, locomotion would be severely limited, if not unfeasible.

CARTILAGE COMPOSITION

Cartilage, like bone, is a composite material, and its primary components include chondrocytes and the extracellular matrix (ECM). The chondrocytes produce and repair many of the macromolecules that form the ECM, including proteoglycans (PGs), glycoproteins (GPs), and collagen (Carballo et al., 2017). Each of these pieces contribute unique characteristics to articular cartilage. Collagen fibrils weave together to form a framework over the chondrocytes, providing structure as well as tensile strength and elasticity (Carballo et al., 2017). Superficially, the first layer of these fibers are thin and packed tightly over a second layer of similar fibers arranged perpendicularly to the first (Huber et al., 2000). Underneath this superficial layer, an intermediate layer of rounded chondrocytes and less organized fibers transitions to a deep layer of columnal chondrocytes and fibers (Huber et al., 2000) with PG content increasing through the layers closer to subchondral bone (Cucchiarini et al., 2016). Hyaluronan or hyaluronic acid (HA) provide a backbone for the aggrecan complexes formed from PGs, connecting to different collagen fibrils, and help maintain viscosity for the overall joint (McIlwraith, 2005). The PGs

attached to HA and their subsequent glycosaminoglycan (GAG) sidechains are negatively charged and repel each other, attracting water and positively charged ions while lending compressive strength to the cartilage (Carballo et al., 2017). In contrast to bone, around 90% of the collagen fibrils in articular cartilage are type II collagen (Carballo et al., 2017) with no type I collagen present within the cartilage. The differences between these collagens lie in their structures with type II collagen formed from a homotrimer and type I collagen from a heterotrimer of α chains (Bella, 2016). Other collagens present in articular cartilage include types III, VI, IX, and XI which form additional fibrils in the ECM (Carballo et al., 2017). Due to the low metabolic activity of chondrocytes (Cucchiarini et al., 2016), replenishing components of the ECM occurs very slowly. Once damaged, cartilage has a very limited capacity for spontaneous repair (Hunziker et al., 2015), and this damage may predispose the joint to osteoarthritis (OA).

ASSESSING BONE AND CARTILAGE QUALITY

Methods for measuring bone and cartilage quality can be organized into two categories: noninvasive and invasive. Noninvasive assessments can be performed repeatedly on a living animal with little to no discomfort and can include blood or synovial fluid sampling for biochemical markers (Seibel, 2005; Steel, 2008; Garvican et al., 2010b), radiographs (Meakim et al., 1981; Genant et al., 2009), and computed tomography (Schmidt et al., 2003; Genant et al., 2009). Invasive assessments however usually require the bone or cartilage of interest to be taken from the animal and are irreversible in the cases of mechanical testing (Turner and Burr, 1993; Sharir et al., 2008), ashing (Kim et al., 2012; Boskey, 2013), and chemical composition analysis (Boskey, 2013; Robison and Karcher, 2019).

Biochemical Markers

Biochemical markers can be defined as products, usually proteins or protein fragments, that reflect the enzymatic activities, synthesis or degradation, of a tissue or pathway. Assays for these markers are highly specific to the targeted protein, commercially available, and typically use blood or urine to determine whole-body status, making them an easy choice for longitudinal studies. However, it may be difficult to ascertain site-specific activities when sampling from serum or plasma. Collagen synthesis and degradation markers can be determined from both serum and synovial fluid, but markers in serum may be inflated due to the contribution of other tissues (McIlwraith, 2005). Depending on the assay, bone biomarkers may be measured in plasma, serum, or urine (Seibel, 2005). Common biomarkers used in judging bone and cartilage quality are listed in Table 1.1.

As mentioned above, these markers are specific for either synthesis or degradation of cartilage or bone. Osteocalcin (OC) is a major, noncollagenous, extracellular matrix protein expressed by osteoblasts and osteocytes (Bellido et al., 2019) and is frequently used as a marker for osteoblast activity (Zoch et al., 2016). It is also the most strongly associated with bone quality when used in conjunction with other measures (Nielsen and Spooner, 2008; Bailey et al., 2017; Poundarik et al., 2018). Type I collagen makes up the majority of the organic matrix in bone and shares a common amino acid sequence in the triple helix with type II collagen (Garvican et al., 2010b). As collagen degrades, portions containing this sequence are released into the blood or synovial fluid. This particular marker (C1,2C) can serve as a marker for bone and collagen degradation in serum or plasma, but determining whether cartilage or bone degradation becomes difficult.

Biomarker	Abbreviation	Tissue	Indicates	Origin	Sample
Osteocalcin	OC	Bone	Formation	Expressed by osteoblasts	Serum
Bone-specific Alkaline Phosphatase	BAP	Bone	Formation	Expressed by osteoblasts	Serum
Procollagen type I/type II N-propeptide	PINP/PIINP	Bone, cartilage	Formation	Cleaved from N-terminal of procollagen	Serum, synovial fluid
Pyridinoline/ Deoxypyrodinoline	PYD/DPD	Bone	Degradation	Released from organic matrix during resorption	Serum, urine
N-terminal cross-linking telopeptide of type I collagen	NTX-I	Bone	Degradation	Cleaved from N-terminal of collagen	Urine, serum
C-terminal cross-linking telopeptide of type I collagen/type II collagen	CTX-I/ CTX-II	Bone, cartilage	Degradation	Cleaved from C-terminal of collagen	Urine, serum
Tartrate-resistant acid phosphatase	TRAP	Bone	Degradation	Expressed by osteoclasts	Serum
Procollagen type II C-propeptide	CPII	Cartilage	Formation	Cleaved from C-terminal of procollagen	Serum, synovial fluid
Chondroitin sulfate 846 epitope	CS846	Cartilage	Formation	Formed as major glycosaminoglycan in aggrecan synthesis	Serum, synovial fluid
Collagen type I/type II fragments	C1,2C/C2C	Cartilage	Degradation	Cleaved by collagenases from mature collagen	Serum, synovial fluid
Cartilage oligomeric matrix protien	COMP	Cartilage	Degradation	Released from cartilage extracellular matrix	Serum, synovial fluid, urine

Table 1.1. Common biomarkers used to determine bone and cartilage metabolism and quality. Adapted from McIlwraith (2005), Garvican et al. (2010a, 2010b), and Allen and Burr, (2019).

However, the typical structure of articular cartilage does not contain type I collagen (McIlwraith, 2005), so cross-reactivity within synovial fluid using this assay is negligible. Likely, C1,2C concentrations within synovial fluid samples reflect the degradation of the type II collagen within articular cartilage. Type II collagen synthesis can be determined by concentrations of the carboxypropeptide of type II collagen (CPII) which is cleaved during tropocollagen processing (Garvican et al., 2010b). Both CPII and C1,2C appear to be the most sensitive to changes in cartilage following exercise or trauma (Frisbie et al., 2008; Catterall et al., 2010; Nicholson et al., 2010), making them among the most common biomarkers to assess cartilage quality.

Radiography

Radiography is frequently used as an inexpensive and quick method to estimate bone mineral content and density as well as produce images of injury and OA. Radiographic absorptiometry, photodensitometry, or radiographic bone aluminum equivalencies (RBAEs) uses aluminum stepwedges of increasing thicknesses in the same radiograph as the bone to estimate bone mineral content (Meakim et al., 1981; Genant et al., 2009). Additional methods include dual X-ray absorptiometry (DXA) that use a radionucleotide source instead of a stepwedge (Genant et al., 2009). Radiographic methods expose the subject to relatively low doses of radiation but can also be less accurate in areas surrounded by lots of soft tissue (Genant et al., 2009) and provide only two-dimensional imaging and area density rather than volumetric. With RBAEs, inflated values have been generated from processed images from digital radiographs (Pagan et al., 2017), indicating the necessity for unprocessed images to ensure accurate results (Emmert et al., 2019). In joints with OA, radiographs can show formation of osteophytes, deformation of subchondral bone, and narrowing of joint space but have difficulty showing

cartilage lesions (de Lasalle et al., 2016). Scoring systems judging the progression of OA based on subchondral bone lysis, fragmentation, and osteophyte formation have been developed (Frisbie et al., 2002; de Lasalle et al., 2016). Though not a direct measure, cartilage quality declines as OA progresses, indicating an inverse relationship with the presence of these bony developments. Radiography continues to be a frequent method for assessing bone, estimating mineral content, and imaging OA.

Computed Tomography

Rather than generate only two-dimensional images, computed tomography (CT) produces three-dimensional scans which can be used to create computer models and observe changes within the bone. Similar to radiographs, CT scans use a phantom of Ca hydroxyapatite of different densities in each scan as a comparison and have trouble imaging soft tissue like cartilage directly. Unlike radiography, CT scans can be used to calculate the volumetric density of individual cortices within bone, are better at imaging bone covered by lots of soft tissue, and can detect morphological changes like cortical thinning (Bloomfield et al., 2002; Genant et al., 2009). MicroCT has better spatial resolution than quantitative CT (QCT) or peripheral QCT (pCT) and can detect changes in the microstructure of trabecular and cortical bone comparable to those detected by histomorphometry (Schmidt et al., 2003). Stress-strain indices may also be generated from CT scans (Romano et al., 2009; Romano et al., 2015), allowing for mechanical testing without destroying the sample. Computed tomography can have difficulty detecting changes in cartilage but is better at imaging osteophytes than radiography (de Lasalle et al., 2016), so use of a contrast may be necessary to determine cartilage quality (Nelson et al., 2019; Stewart et al., 2019). Contrast attenuation on CT may indicate GAG content and is positively correlated with compressive modulus in cartilage (Nelson et al., 2019), both of which are

important in cartilage quality. Use of contrast with CT can also be more sensitive than radiography at determining the early stages of OA and cartilage degradation (Stewart et al., 2019). However, these scans produce higher doses of radiation than radiography, are highly dependent on appropriate scan settings, and are not as widely available as digital radiography (Genant et al., 2009); additionally, CT in horses may require general anesthesia. Despite these limitations, CT remains a widely accepted choice to calculate bone mineral density and morphological changes.

Mechanical Testing

Mechanical testing involves subjecting a bone to loads in various ways to test certain structural and biomechanical capacities. These tests may include tension, compression, and torsion loading, but the most common testing modes in long bones are shear and bending (Turner and Burr, 1993; Wallace, 2019), accomplished through three- and four-point bending, respectively. For both tests, bone rests on two posts, and force is applied through either one (three-point) or two (four-point) upper posts until fracture or deformation. This force and subsequent displacement of the bone from a neutral axis are measured and used to calculate stress, strain, and the modulus of elasticity or Young's Modulus (Turner and Burr, 1993). Young's modulus represents the rigidity of the bone along the linear portion of a stress-strain or force-displacement curve (Rath et al., 2000). Typically, morphological measures such as outer diameter are needed to make these calculations (Turner and Burr, 1993). Demineralized bone will have low values for Young's modulus, indicating a very slight slope on the stress-strain curve, while deproteinated bone will have a very steep slope and high Young's modulus (Wallace, 2019). Other factors that can contribute to changes in bone mechanics include orientation of type I collagen fibers within bone (Martin and Ishida, 1989; Martin and Boardman,

1993) as well as bone mass and architecture (Wallace, 2019). In fact, nearly 88% of variation in Young's modulus may be accounted for by amount of mineralization and collagen fiber orientation (Williams et al., 2004). Mechanical testing reveals these materials properties which inform how changes in structure, density, and other morphometrical factors may have real life implications. Unfortunately, mechanical testing requires bone to be *ex vivo* and renders the sample unable to be used for further testing.

Ash and Chemical Composition

A direct measure of bone mineral content is ash determination. Ash content of bone can be useful for determining stage of mineralization and mineral loss (Williams et al., 2004). Additionally, ash serves as an indicator of compressive strength (Rath et al., 2000) as increases in ash correlate to increases in breaking strength (Lewis et al., 2009; Shim et al., 2012). However, ash alone may be a poor indicator of ultimate strength (Martin and Ishida, 1989; Martin and Boardman, 1993) as it does not account for density or organization of hydroxyapatite (Kim et al., 2012; Boskey, 2013), but it still remains a popular, relatively low cost and low technology measure of bone quality. Like mechanical testing, ashing requires bone to be *ex vivo*, and afterwards the sample cannot be used for further testing aside from chemical composition analyses. Chemical composition of bone changes with age (Jugdaohsingh et al., 2015a) as well as disease (Boskey, 2013) with Ca, P, other minerals, and subsequent ratios altered due to mineral localization or metabolism. As mentioned above, other ions may be substituted at several locations in hydroxyapatite, ultimately changing the crystal structure, so determining the mineral composition may provide insights into the hydroxyapatite at a molecular level. While no method mentioned previously provides a complete picture of bone on its own, these methods may be used together to assess bone and cartilage quality.

FACTORS AFFECTING BONE AND CARTILAGE QUALITY

Due to its heterogenous composition, many factors affect skeletal quality, including growth, mechanical loading, and nutrition. These factors have several underlying considerations and may influence one another, making their relationships complicated. Nutrition can affect growth while exercise can influence nutritional effects on bone and cartilage or vice versa. Even differences in type of loading or nutritional intervention can change how bone and cartilage are affected, such as the difference between high (Hiney et al., 2004) and low intensity exercise (Spooner et al., 2008) or among various levels of energy, protein, and minerals (Venäläinen et al., 2006; Swift et al., 2012; Bradbury et al., 2017). Regardless of intervention, growth, mechanical loading, and nutrition appear to be the most important for both short- and long-term bone and cartilage quality.

Growth

The period of growth is the most important time for bone and cartilage. Any alterations during growth, such as nutrient restriction or excess (Williams et al., 2004; Romano et al., 2009; Shim et al., 2012), have long-term implications for bone and cartilage quality, even after nutritional correction (Romano et al., 2010; Jones et al., 2011). Most bones grow through endochondral ossification which is the process of mineralizing the bone's cartilage template through coordination of osteoblasts, osteoclasts, and chondrocytes (Allen and Burr, 2019). During growth, the process of modeling is responsible for altering bone size and shape. Modeling primarily is bone formation by osteoblasts or resorption by osteoclasts at a given location with these processes separate from one another rather than sequential, resulting in a net increase in bone mass (Allen and Burr, 2019). Remodeling couples these processes on the same surface to address bone healing or strengthening and, unlike modeling, results in a net-zero or

slight decrease in bone mass (Allen and Burr, 2019). Collagen and PG content increases from immature to mature articular cartilage as chondrocytes synthesize the ECM (Oinas et al., 2018). Altering growth rate can change bone properties and may affect cartilage as well. High growth rates are associated with weaker, more porous bones while slower growth rates yield stronger, denser bone (Williams et al., 2004; Shim et al., 2012). However, growth restriction during late gestation and early postnatal followed by compensatory growth can reduce bone mineral content (BMC), strength, and cortical thicknesses (Lanham et al., 2008; Romano et al., 2009) while delaying epiphyseal cartilage calcification (Jones et al., 2011).

Although slowed growth does not appear to reduce articular cartilage defects (Nakano et al., 1979; Athanasiou et al., 2000), a high growth rate is associated with increased incidences of joint lesions (Barneveld and Van Weeren, 2010; van Grevenhof et al., 2012). In bone, fast growth increases synthesis of the organic matrix to adjust for load capacity, but without similar increases in mineral apposition, bone remains porous and improperly mineralized (Williams et al., 2004). In cartilage, growth rate may influence glycosaminoglycan, water, and collagen content as well as post-translational collagen modification (Brama et al., 1999; Oinas et al., 2018). Changes to the underlying subchondral and trabecular bone can also lead to abnormal thickening of calcified cartilage (Murray et al., 1999; van der Harst et al., 2005). While growth obviously affects the overall morphology of bone and cartilage through maturation, changes in growth rate can also alter bone and cartilage quality with potential short- and long-term health impacts.

Mechanical Loading

Since both bone and cartilage respond to mechanical stimuli, mechanical loading through exercise provides a means to manipulate this response. A foundational study by Rubin and

Lanyon (1984) demonstrated that a minimum of four mechanical loading cycles can reduce mineral loss while 36 cycles appears to improve new bone formation. Since then, other studies have shown exercise to prevent mineral loss that occurs with aging and disuse (Inman et al., 1999; Bloomfield et al., 2002; Marques et al., 2012) as well as exercise's ability to increase cortical thicknesses and alter bone shape to improve strength (Wallace et al., 2007; Hollinski et al., 2018; Logan et al., 2019). While the exact cellular mechanoreceptor has yet to be identified, osteocytes appear to be the main cell involved in mechanosensing, either through membrane deformation or changes in fluid flow (Yavropoulou and Yovos, 2016; Robling et al., 2019) caused by strain from movement and exercise. Osteocyte ablation via a transgenic mouse model using a diphtheria toxin receptor results in cortical porosity and fragile bones, but mineral loss through disuse in these mice is blunted (Tatsumi et al., 2007).

Regardless of mechanism, osteoblasts and osteoclasts are recruited for modeling or remodeling as necessary in the bone to increase mineralization and strength or repair microdamage (Raggatt and Partridge, 2010). Markers of bone turnover tend to increase in exercised animals as compared to controls (Jackson et al., 2003; Gardinier et al., 2018), but these results can be mixed (Logan et al., 2019). Exercise intensity matters as low intensity, even with weights (Nielsen et al., 2002) or at long distances (Joo et al., 2003; Spooner et al., 2008), will not alter biochemical markers, bone mineralization, or strength. However, medium intensities will improve biochemical markers, morphometry, and strength over nonexercised controls (Wallace et al., 2007; Hamann et al., 2012; Zhang et al., 2017), but intensities above this threshold, especially in excess, do not appear to have increasing benefits (Zhang et al., 2017; Hollinski et al., 2018; Logan et al., 2019). Age also influences response to mechanical loading, as younger bone tends to respond to exercise with more favorable ratios of bone metabolism markers and

increases in density and strength (Biewener and Bertram, 1994; Barneveld and Van Weeren, 2010) than adult bone (Gardinier et al., 2018).

Mechanical loading can also induce changes in cartilage. Loading causes deformation of the ECM and chondrocytes, ultimately stimulating these cells to synthesize more components of the ECM and increase hydration of cartilage for compressive strength (Huber et al., 2000). An excessive single load or trauma can overwhelm and tear the ECM, creating a positive inflammatory feedback loop leading to greater cartilage degradation (Cucchiarini et al., 2016; Mathiessen and Conaghan, 2017). Still, movement is necessary to maintain synovial fluid flow and nutrient exchange between the synovial membrane and surrounding capillaries (Bertone, 2008), but immobilization of a joint can result in decreased PG synthesis and weaker cartilage (Huber et al., 2000; Jørgensen et al., 2017). As in bone, repetitive overloading can result in cartilage destruction through collagen matrix breakdown, causing PG loss, and increased catabolic pathways (Jørgensen et al., 2017). This cartilage deterioration from insufficient or overloading can contribute to the development of OA (Jørgensen et al., 2017). Although too much volume and/or intensity may contribute to injuries or microdamage, moderate mechanical loading proves beneficial to bone and cartilage quality compared to inactivity.

Nutrition

Nutrition can be manipulated in many ways to affect bone and cartilage and may be interrelated to mechanical loading and growth. Dietary energy restriction can increase marrow adiposity, despite overall body fat reduction, and reduce BMC and cortical thickness in growing and adult animals (Devlin et al., 2010; Swift et al., 2012). Protein restriction alone can reduce osteocalcin and bone formation as well as increase porosity in adult rats (Ammann et al., 2000; Bourrin et al., 2000a; Bourrin et al., 2000b). These effects on bone from energy and protein

restriction in growing animals usually stem from altering growth rate. Fast-growing animals actually improve bone quality with protein and energy restriction (Williams et al., 2004; Van Wyhe et al., 2014), but when normal growth is impaired through undernutrition, bone quality declines in the short- and long-term (Jones et al., 2011; Romano et al., 2015). However, cartilage appears unaffected by energy restriction (Athanasiou et al., 2000), but as mentioned above, quality can be reduced if growth rate is too high.

As the major component in bone, minerals and their availability in the diet can alter bone quality; others play important roles in cartilage synthesis and maintenance. While alterations to Ca in the diet have the greatest impact on bone due to its major role in hydroxyapatite, other minerals can also cause changes in bone quality and mineral metabolism. As the second most abundant mineral in bone, P intake can alter Ca metabolism as high intakes are associated with decreased plasma Ca and absorption with increased excretion (Schryver et al., 1971), eventually leading to nutritional secondary hyperparathyroidism triggered by the increased plasma P concentrations (Mendoza et al., 2017). Low P intakes can reduce bone mineralization and density (Sørensen et al., 2019; Walters et al., 2019). Magnesium is the third most abundant mineral in bone and necessary for appropriate hydroxyapatite crystal formation (Gallant and Weaver, 2019). High Mg intakes do not appear to affect bone (Farsinejad-Marj et al., 2016), but low Mg in the diet could reduce bone mineral content and density (Tu et al., 2017). Changes to these minerals affect other tissues as well, but evidence suggests that Ca and Si are the two most promising minerals to affect bone and cartilage.

Calcium

Around 30-40% of bone ash consists of Ca, making it the most abundant and most important mineral in bone. Within hydroxyapatite, Ca is arranged hexagonally, and around these

six sites, phosphate and hydroxyls are arranged in columns to provide stability (Ratnayake et al., 2017). However, this mineral serves other vital roles in muscle contraction, neural transmission, and cell signaling, making homeostasis tightly regulated. As noted earlier, bone contains 99% of the body's Ca pool, but around 1% of the body's Ca exists in body fluids with the major portion in extracellular fluid and the rest in plasma (DiMeglio and Imel, 2019). The large intestine and kidneys are the major sites for absorption, excretion, and regulation (Schryver et al., 1970b; DiMeglio and Imel, 2019). Decreasing plasma Ca triggers release of parathyroid hormone (PTH) which induces Ca release from bone resorption, reduces renal excretion, and increases calcitriol production for greater intestinal absorption (Blaine et al., 2015). Increasing plasma Ca concentrations will increase intestinal and renal excretion and may trigger release of calcitonin to reduce osteoclast activity to decrease plasma Ca (DiMeglio and Imel, 2019). However, the activation of these mechanisms may not be coupled with measurable changes to total plasma Ca (Muller et al., 2015), but rather to minor changes in ionized plasma Ca within the physiological range (Kohrt et al., 2018). This sensitivity and multiplicity of mechanisms to regulate Ca indicate its importance to many physiological functions.

Changes in Ca intake and balance can affect growing and adult animals. Low Ca intake in young animals can contribute to lower BMC, BMD, and strength (Bai et al., 2017), and deficiency can lead to bone growth abnormalities in children and increase mineral loss with aging (Gallant and Weaver, 2019). Calcium intake and source affects digestibility and absorption as greater amounts of Ca absorbed will also lead to greater excretion of Ca (Schryver et al., 1970a) which will have no added benefit to bone (Sørensen et al., 2019), and as digestibility increases, bone mineralization may improve (Maehira et al., 2009). Restricted Ca intake in adult rats can decrease tibial BMC and BMD, even when provided exercise (Swift et al., 2012). In

addition to changes in intake, alterations in Ca excretion, ultimately resulting in a negative Ca balance, can affect bone quality. Diuretics and certain diseases increase mineral, especially Ca, excretion in urine (Law et al., 2005; Alexander and Dimke, 2017), triggering PTH and subsequently causing bone mineral loss. As noted above, exercise can be beneficial to bone, but during high-intensity exercise, plasma ionized Ca can decrease, stimulating PTH release and increasing bone degradation markers (Vervuert et al., 2002; Kohrt et al., 2018). As osteoclasts release Ca from bone, it becomes more porous and loses some of its compressive strength (Rath et al., 2000; Shim et al., 2012), making it weaker and potentially more prone to fracturing. Offsetting this reduction with Ca supplementation just prior to exercise can blunt PTH release (Kohrt et al., 2018), indicating the priority of the body to maintain plasma ionized Ca through any means necessary. Due to its tight homeostatic regulation and large part in hydroxyapatite, altering Ca can have considerable impacts on bone.

Silicon

Silicon plays a role in bone and cartilage development. Early studies demonstrated that basal diets deficient in Si reduced the overall growth in chicks and rats (Carlisle, 1972; Schwarz and Milne, 1972) as well as altered the long bone and skull formation in chicks (Carlisle, 1980a; Carlisle, 1980b), producing more porous, less mineralized bone. Typically, Si associates more with the organic matrix of bone and soft tissue (Carlisle, 1976; Jugdaohsingh et al., 2015b), and declining Si concentrations in connective tissue may also be an indicator of decreasing collagen content (Jugdaohsingh et al., 2015c). Through its association with type I collagen, it contributes to early calcification of bone's organic matrix by providing a low solubility matrix to attract and contain other ions, such as Ca, at the organic-inorganic interface (Matsko et al., 2011). While deficiency proves harmful, supplementation may be beneficial. Silicon supplementation in calves

has increased hydroxyproline content, an early marker for collagen formation, in skin (Calomme and Vanden Berghe, 1997) since Si stimulates type I collagen synthesis (Reffitt et al., 2003; Dong et al., 2016). In humans, higher Si intake has been associated with greater bone mineral density (Sripanyakorn et al., 2005; Jugdaohsingh, 2007; Arora and Arora, 2017). During both normal conditions and Ca deficiency, Si supplementation inhibits bone resorption (Kim et al., 2009) by reducing osteoclast formation and activity (Mladenović et al., 2014) as well as increasing osteoblastogenesis (Maehira et al., 2009) and osteoblast activity (Reffitt et al., 2003; Shie et al., 2011; Kim et al., 2013). These direct effects on bone cells and collagen synthesis could assist in cartilage repair and bone strength and demonstrate the importance of Si within the diet.

Along with these direct effects on bone and collagen, Si can also alter mineral metabolism. These effects can be beneficial when it comes to binding metals like aluminum to generate aluminosilicates to prevent absorption and harmful accumulation in tissues (Nielsen, 2014). The effects of Si on serum Ca concentrations in supplemented animals are mixed with some studies showing greater concentrations (Najda et al., 1993; Calomme and Vanden Berghe, 1997), but more recent studies show no difference or decreased Ca concentrations with Si supplementation (Kayongo-Male and Julson, 2008; O'Connor et al., 2008). Magnesium retention (Kim et al., 2014) and serum concentrations (Najda et al., 1993; Kayongo-Male and Julson, 2008) can also be reduced with Si supplementation. However, these alterations in serum or plasma minerals do not always translate into changes in mineral concentrations in bone or soft tissue. Silicon in the diet increases Ca concentration in bone above amounts in a deficient diet (Seaborn and Nielsen, 1994; Seaborn and Nielsen, 2002), but other studies supplementing Si on top of an adequate diet show no changes in the Ca concentration of bones (Kayongo-Male and

Julson, 2008; Sgavioli et al., 2016). However, sex may influence the effects of Si supplementation, as these previous studies used male subjects whereas a study showed no difference in the tibia Ca in male rats but an increase in tibia Ca with supplementation in female rats (Jugdaohsingh et al., 2015b). Increases in Ca in bone may also be influenced by both Ca and Si sources (Maehira et al., 2009) which affects its absorption and retention (Sripanyakorn et al., 2009). By altering both mineral metabolism and collagen synthesis, Si supplementation may increase bone density (Kim et al., 2009; Jugdaohsingh et al., 2015b) and strength (Sgavioli et al., 2016; Scholey et al., 2018; Nakhon et al., 2019). All of these direct and indirect effects play a central role in improving and maintaining bone and cartilage quality during growth and later life, demonstrating Si's importance as a micromineral.

SKELETAL ISSUES IN THE EQUINE INDUSTRY

Lameness is often cited as the number one cause of training failure and economic loss through diminished performance in the equine industry as over 65% of stables in the United States have at least one horse with a lameness problem (United States Department of Agriculture, 2017). Lameness describes an abnormal gait or change in gait that is most likely due to pain but can also stem from neuromotor dysfunction or mechanical restriction (American Association of Equine Practitioners, 1991). As the most common reason for lameness, pain can have a variety of sources from a soft tissue or bony musculoskeletal injury to OA and can be devastating for competition and racehorses. Finding methods to lessen or prevent, if possible, injury and OA could limit economic losses and improve welfare.

Musculoskeletal Injuries

From competition to pleasure to racehorses, musculoskeletal injuries can be career-ending, if not fatal. In the United States, limb injuries account for nearly 20% of lamenesses (United States Department of Agriculture, 2017). Fracture is the most common nonfatal injury in racehorses at 49.7% (Hill et al., 2015) as well as the most common cause of fatal injuries on the track in the United States and Great Britain (Stover, 2003; Sarrafian et al., 2012; Rosanowski et al., 2017). In a survey to determine risk factors and their relationship for catastrophic musculoskeletal injury, over 35% horses have experienced lameness within three months of breakdown (Hitchens et al., 2018), and previous injury continues to be a risk factor for fatal injuries (Hitchens et al., 2019). Whether previous injury increases future catastrophic injury risk through long lay-up periods for healing, changes in training approach, or simply from a pre-existing condition is unknown (Hitchens et al., 2019). Over 300 risk factors have been identified for catastrophic musculoskeletal injury (Hitchens et al., 2018; Hitchens et al., 2019), making identifying and addressing the root of these problems difficult, but improving bone quality and strength may help lessen the incidence of these injuries.

To mitigate these risk factors, nutritional and exercise interventions have been proposed. High-speed exercise can increase bone strength in growing animals (Logan et al., 2019), but too much during a short period of time can contribute to injury (Hitchens et al., 2019). This cumulative effect may be due to increased microdamage which has not been repaired, leading to weaknesses in bone, or due to the timeline and phases of remodeling. After activation, there is a period of mineral and matrix resorption by osteoclasts, followed by a short time of reversal before the osteoblasts can synthesize and mineralize the new matrix (Raggatt and Partridge, 2010); this process of resorption, reversal, and regeneration may take 90 days to a year to fully

complete (Allen and Burr, 2019). During this time, the bone is temporarily weaker as it has lost both compressive and tensile strength, making it more prone to injury in the short-term but ultimately better as it repairs and becomes able to withstand greater mechanical loads.

Exercise appears to have the greatest effect on bone, but altering mineral balance may also impact these effects. Silicon supplementation in the form of sodium zeolite A has increased distances accumulated during training before bony or soft tissue injury (Nielsen et al., 1993), likely due to reduced resorption thereby maintaining mineral content during periods of stalling (Lang et al., 2001). High Ca intake during training can help Ca retention and subsequently sustain or increase RBAE values as compared to controls (Nielsen et al., 1998). Low Ca intake in young, growing ponies can result in removal of Ca from bone to maintain homeostasis (Schryver et al., 1970a). While most commercial feeds provide adequate Ca, it seems that low intake is no longer a problem. However, furosemide, the most common medication used in racing in the United States, affects Ca balance (Pagan et al., 2013; Pagan et al., 2014), and its use has been recently debated due to its potential to negatively impact bone.

Furosemide

Although most countries have banned its use on race day, furosemide remains a controversial race-day medication in the United States with some tracks banning its use for two year-olds in 2020 and for stakes races in 2021. Furosemide is given to prevent or reduce the severity of exercise-induced pulmonary hemorrhage, but efficacy is mixed (Sullivan et al., 2015; Knych et al., 2018). After injection, furosemide acts in the thick ascending limb of the loop of Henle in the nephron where it inhibits the Na+-K+-2Cl- cotransporter type 2 (NKCC2) where it competes with chloride for binding sites, reducing the absorption of Na+, K+, and Cl- ions across the lumen (Kim, 2004). This inhibition delivers more ions to the distal tubule and subsequently

increases urine production by changing the transepithelial gradient and limiting water reabsorption in the distal convoluted tubule and collecting duct (Hinchcliff and Muir, 1991). This diuresis reduces plasma volume and increases fluid losses from extracellular fluid (Hinchcliff et al., 1991; Forro and Lindinger, 2006), consequently decreasing blood pressure as a proposed mechanism for furosemide's effects on exercise-induced pulmonary hemorrhage. While furosemide does not act directly on a Ca transporter, this gradient shift impairs Ca reabsorption through specific pores in the lumen (Alexander and Dimke, 2017), causing increased Ca excretion (Muller et al., 2015) and negatively affecting mineral balance (Pagan et al., 2013; Pagan et al., 2014). This impact on Ca balance persists for up to 72 hours post-administration (Pagan et al., 2013; Pagan et al., 2014), but no study has determined when Ca balance is restored, indicating that frequent administration may have a cumulative effect and trigger mineral loss.

Though frequent administration results in adaption and subsequently diuretic resistance in the nephron (Kim, 2004), long-term effects have not been thoroughly studied in horses. One study demonstrated the potential for weekly administration over two months to result in diuretic resistance in horses but did not show any bone responses to treatment (Pritchard et al., 2019). However, this past study used RBAEs from processed digital radiographs, and the authors noted the artificially inflated values which could have obscured results. Regardless of these results and more likely due to increased public scrutiny and pressure, many tracks in the United States are moving to ban furosemide use on race day as an attempt to reduce the number of catastrophic musculoskeletal injuries.

Osteoarthritis

Horses are considered a natural model for spontaneous OA and OA from overuse and, as models, can help researchers determine treatments and recommendations (McIlwraith et al.,

2013; Cucchiarini et al., 2016). Unsurprisingly then, OA is the number one cause of lameness in horses in the United States with nearly one-fifth of horses with chronic or recurring lamenesses identified as having OA (United States Department of Agriculture, 2017). Degraded articular cartilage is a hallmark of late-stage OA, but this disorder can also induce changes in the synovium and subchondral bone (Cucchiarini et al., 2016). Osteoarthritis decreases blood flow to the synovial membrane which generates lactate and acidosis within the synovial fluid (Bertone, 2008), affecting the clearance and delivery of metabolites from and to the joint. Within the synovial fluid of OA-affected joints, CPII and C1,2C concentrations increase (Frisbie et al., 2008), indicating greater chondrocyte metabolic activity. Although this activity would hopefully be associated with ECM remodeling, it can indicate early OA as ECM degradation outpaces synthesis (Goldring and Goldring, 2016) due to higher concentrations of pro-inflammatory cytokines prompting synoviocyte production of proteolytic enzymes (Mathiessen and Conaghan, 2017). Initially, this increased chondrocyte activity accompanies the development of rough collagen fibrils on what should be the smooth hyaline surface, subchondral bone remodeling, and swelling of the cartilage (Goldring and Goldring, 2016). As the disorder progresses, the collagen matrix deteriorates, the ECM loses PGs and cartilage lesions and/or osteophytes may form (Cucchiarini et al., 2016), causing even more pain. Proteoglycan losses with aging (Plat et al., 1998; Jørgensen et al., 2017; Oinas et al., 2018), damage (Cleary et al., 2010; Nicholson et al., 2010), or progression of OA (Cucchiarini et al., 2016; Goldring and Goldring, 2016) reduces the water content via GAG loss in articular cartilage and subsequently its compressive strength, predisposing it to even more damage. Subchondral bone and calcified cartilage thicken in OA joints due to increased bone remodeling from unusual loading (Murray et al., 1999), causing

greater ash content with fewer pyrdinoline cross-links (van der Harst et al., 2005); ultimately, this process reduces the ability to transfer mechanical loads from the joint without damage.

Due to the limited capacity for repair in cartilage, interventions can have very mixed results. Many supplements exist on the market to alleviate OA pain, improve joint function, and potentially prevent or stall OA progression, but there is limited evidence of their efficacy (Liu et al., 2018). Some supplements, including resveratrol, glucosamine, and chondroitin sulfate, may improve joint function, but results are mixed (McIlwraith, 2013; Watts et al., 2016). Other treatments include hyaluronan coupled with corticosteroid injections directly to the joint (Niemelä et al., 2018), extracorporeal shock wave therapy (Kawcak et al., 2011), and intramuscular polysulfated GAG injections (Kawcak et al., 2011). These treatments aim to reduce inflammation within the joint while providing components essential for ECM and synovial fluid function, but all failed to improve biomarkers or outcomes above placebo or control. Even surgery may fail to improve cartilage lesions in OA (Hunziker et al., 2015). It appears that appropriate mechanical loading (exercise) may be best for cartilage adaptation (Huber et al., 2000), but due to OA's multifactorial nature including nutrition, stimulating higher collagen production and greater cartilage quality may help to reduce the severity or progression of OA.

SKELETAL ISSUES IN THE BROILER INDUSTRY

Most modern commercial broiler chickens have been genetically selected for rapid growth, muscular gain, and high feed efficiency - creating birds that begin life at 40 g a day after hatching but weigh as much as 3 kg or more at six to seven weeks old. Due to the focus on rapid muscle growth, these birds can experience health problems ranging from pulmonary

hypertension to sudden death syndrome (Julian, 1998). Additionally, there is a high muscle-to-bone ratio in broilers that can strain the legs, leading to leg deformities, altered cartilage formation, and fractures (Knowles et al., 2008) which affect between 5.5 and 48.8% of broilers worldwide (Hartcher and Lum, 2019). For many years, tibial dyschondroplasia (TD) presented a large problem to the broiler industry as it increased mortality and pain and decreased productivity of birds (Hartcher and Lum, 2019). This condition caused cartilage malformation along the tibial epiphyseal plate (Tablante et al., 2003) that worsened in genetic strains with higher growth rates but has mostly been eliminated in recent years (Hartcher and Lum, 2019). However, growth rate still impacts bone quality; faster growing strains have worse bone quality by increasing bone porosity (Williams et al., 2004) and reduced ash and breaking strength per kg bodyweight (Williams et al., 2004; Shim et al., 2012) compared to slower growing strains. When growth in these faster strains is controlled through limited feed or reduced metabolizable energy, tibial ash, density, Ca, and breaking strength improve (Williams et al., 2004; Venäläinen et al., 2006; Shim et al., 2012). However, due to production foci, skeletal issues persist in the broiler industry.

To combat these issues, environmental and nutritional interventions have been proposed. Changing aspects of broilers' environment could increase mechanical loading to improve BMD and strength through adding perches (Tablante et al., 2003), reducing stocking density (Buijs et al., 2012), or increasing the photoperiod (Lewis et al., 2009). Perches could provide mechanical loading through repeated impact of climbing on and off (Tablante et al., 2003), and altering the photoperiod would influence overall movement as broilers tend to be more active during light periods (Lewis et al., 2009). Additionally, reducing stocking density would provide more room for birds to move and might encourage them to be more active and provide more opportunities

for loading (Buijs et al., 2012). However, reducing stocking density or providing environmental enrichment such as perches have not convincingly improved leg health (Riber et al., 2018; Pedersen et al., 2020). Longer photoperiods can increase tibial ash and breaking strength, but peak bone strength is achieved at 12 hours, and light periods longer than 12 hours did not increase strength (Lewis et al., 2009). Nutritional interventions have had more success than mechanical and environmental changes. As mentioned previously, slightly reducing feed intake or metabolizable energy to slow growth can allow for better bone quality in fast growing animals. Varying levels as well as sources of Ca, P, and other minerals (Onyango et al., 2003; Venäläinen et al., 2006; Bradbury et al., 2017; Güz et al., 2019) have improved bone quality in fast growing strains. In early studies, Carlisle (1976; 1980b) demonstrated the importance of Si inclusion in chick diets for the proper formation of long bones and articular cartilage. Follow-up studies in broilers show improved bone strength and ash with varying levels of Si supplementation or the inclusion of ingredients containing high Si concentrations (Sgavioli et al., 2016; Scholey et al., 2018; Nakhon et al., 2019). These nutritional interventions have an added benefit of easier implementation within the broiler industry and may provide greater improvements than environmental enrichment alone.

SUMMARY

The skeleton is a diverse system that serves multiple purposes from protection to reservoir to locomotion and presents researchers with distinctive problems. Both bone and articular cartilage are heterogenous tissues with complex interactions among cells, extracellular matrices, and minerals which growth, loading, and nutrition can affect. Measuring bone and cartilage quality and adaptations to these factors can be achieved noninvasively through

biomarkers, radiography, and computed tomography or invasively through ashing, chemical analyses, and mechanical testing. Each method may differ based on species and age but may provide different insights on how to address skeletal issues. Growth and loading both play important roles in determining or improving bone quality, but nutrition may affect the impact of both of these factors and may be the easiest to manipulate. Changes in mineral amounts and availability, specifically Ca and Si, can affect skeletal quality without affecting growth or loading. In horses and poultry, determining the role Ca and Si play in both contributing to and possibly preventing skeletal injuries and disorders has large economic and welfare implications.

OBJECTIVES AND HYPOTHESES

The overall purpose of these studies was to determine the extent to which impacts on mineral balance could affect both mature bone and cartilage as well as immature, growing bone. In order, the study objectives and hypotheses are as follows:

1. To build on previous studies, the objective for the first study was to determine when Ca balance returns to baseline values after furosemide administration to indicate potential long-term influences on bone. We hypothesized that Ca balance would be negatively impacted for at least three days but would be no different than controls within seven days of furosemide administration.
2. The second study's objective was to determine the effect of a feeding a bioavailable Si supplement on bone and collagen markers in blood and synovial fluid as well as objective and subjective lameness in mature horses. We hypothesized that Si supplementation would increase bone formation markers and cartilage turnover through increased collagen

degradation and formation markers and reduce lameness severity over time and as compared to controls.

3. The objective of the final study was to establish if supplementing bioavailable Si at two different concentrations could affect bone morphology, mineralization, and strength in immature broilers when given at an early age. We hypothesized that increasing Si concentrations would improve bone quality measures as compared to controls and that these effects would be most prominent at the highest supplement concentration.

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CHAPTER 2: Furosemide administration results in a transient alteration in calcium balance in mature horses

INTRODUCTION

Currently, furosemide is the most common race day medication in North America (Soma and Uboh, 1998; Gross et al., 1999; Sullivan and Hinchcliff, 2015). While veterinarians and trainers use the diuretic to reduce the incidence or lessen the severity of exercise-induced pulmonary hemorrhage (EIPH), its efficacy is mixed (Pascoe et al., 1985; Hinchcliff et al., 2009; Sullivan et al., 2015b; Knych et al., 2018). Additionally, administration can enhance performance (Sweeney et al., 1990; Hinchcliff et al., 1993; Zawadzkas et al., 2006), but whether this benefit is from the reduced EIPH severity or short-term weight loss (Hinchcliff et al., 1993; Pritchard et al., 2017) is unclear.

One proposed mechanism for furosemide to prevent EIPH is its reduction in overall plasma and blood volume (Hinchcliff et al., 1991a) which reduces systolic pulmonary arterial pressure (Manohar et al., 1998), thereby lessening the severity and potential occurrence of EIPH. As an loop diuretic, furosemide competitively inhibits the Na^+-K^+-$2Cl^-$ type 2 cotransporter (NKCC2) by binding the Cl^- sites, disrupting the transepithelial gradient, and increasing excretion of water and other ions, including calcium (Kim, 2004; Alexander and Dimke, 2017). In horses, calcium (Ca) increases in the urine after furosemide administration (Tobin et al., 1978; Freestone et al., 1988). While this Ca loss slows as the horse metabolizes the drug, the losses are so great that furosemide negatively impacts Ca balance for at least 72-h post-administration (Pagan et al., 2013; Pagan et al., 2014). However, these previous studies did not determine when Ca balance returned to baseline nor did the researchers propose a potential mechanism to restore Ca balance after furosemide-induced losses. If Ca balance remains negatively impacted with frequent administration of furosemide due to racing or training schedules, this result could

decrease bone mineral content, predisposing horses to catastrophic skeletal failures. Net Ca losses through urine may be offset by increased Ca retention or absorption in the intestine (Schryver et al., 1970b) to restore Ca balance, but this effect has not been documented after furosemide administration.

As Ca is crucial to bone and muscle contraction, short- and long-term effects of furosemide on this mineral should be better understood, especially as the drug's use comes into question in racing jurisdictions across North America. To build on previous studies, the objective for the current study was to determine when Ca balance returns to baseline values after furosemide administration. The current study hypothesized that Ca balance will be negatively impacted for at least three days but would be no different than controls within seven days of furosemide administration.

MATERIALS AND METHODS

Horses

The Institutional Animal Care and Use Committee (IACUC) at Michigan State University (MSU) approved all procedures prior to study initiation. Ten mature Standardbred geldings were obtained from the MSU Horse Teaching and Research Center and striated by age and weight, pair-matched, and then randomly assigned within a pair to either control (CON, n=5) or treatment (FUR, n=5) for the first collection period. Horses switched groups in the second collection period for a crossover design and were stalled during both total collection periods. Prior to the start of the study, horses had a 14-d backgrounding on a winter pasture with free choice access to hay and water and returned to pasture for 14 d between total collections for washout. Both collection periods took place during Michigan winter where average daily temperatures were below 0° C in an unheated barn without heated buckets.

Total collection and furosemide protocol

At the start of each period, horses were weighed, body condition scored (Henneke et al., 1983), and brought into stalls from pasture (d 0). They were then fitted with total collection harnesses (Equisan Marketing, Melbourne, Australia) and allowed a 24-h adaptation prior to beginning the 7-d total collection. Harnesses were emptied every 8 h, and fecal and urine sampling began after furosemide administration. Treatment horses received one administration of furosemide (1 mg/kg, IV; Salix®, Merck Animal Health, Madison, NJ) based on the bodyweights recorded on d 0. Furosemide injections marked the beginning of d 1, and samples collected in the 24-h period after were considered d 1. Total volume of urine and total feces were measured, recorded, and mixed, and a 10% sample from each day was kept. All samples were frozen at –20° C immediately after collection. Throughout this time, horses had free choice access to water and were fed hay at 2% BW per d, divided into two feedings. Hay samples from each day and period were pooled for later digestions and Ca analysis. Each morning, orts from the previous 24 h were collected, weighed, and subtracted from total fed to determine dry matter (DM) and Ca intake.

Calcium analysis

For calcium analysis, urine was acidified with 12 M HCl (EMD Chemicals, Inc., Gibbstown, NJ) at a rate of 20 μL/mL of urine to ensure all precipitate was in solution (O'Connor-Robison and Nielsen, 2006). Fecal and hay samples were oven-dried for 24-h (Thelco, Precision Scientific, Winchester, VA), ground to 1 mm particles (Cyclotec 1093 Sample Mill; Foss, Eden Prairie, MN), and microwave digested (MARS5, CEM Corp., Matthews, NC) with nitric acid (70% trace-metal grade; Fisher Scientific, Pittsburgh, PA) in duplicate as described by Shaw et al. (2002) and (Lavin et al., 2013). Calcium concentrations in hay, feces,

and urine were determined by atomic absorption spectroscopy (AA-7000, Shimadzu, Columbia, MD) with each urine sample and microwave digest run in duplicate. Fecal and feed samples were diluted at 100x with lanthanum chloride in double deionized H_2O, and urine samples were diluted at 600x with lanthanum chloride in physiological saline. All samples were run against the same inorganic standard (Calcium AA Standard, Ricca Chemical, Arlington, TX) with peach leaves (Standard Reference Material® 1547 Peach Leaves, National Institute of Standards and Technology, Gaithersburg, MD) serving as controls and diluted in double deionized water or physiological saline according to sample type. An acceptable coefficient of variation (CV) between duplicates was set at 5%, and any duplicates or samples above 5% CV were rerun or redigested as necessary.

Calculations

Calcium concentrations and total output and intake were calculated for fecal and feed samples using the following equation:

$$Fecal\ or\ Forage\ Concentration\ (g/kg) = \frac{AA\ Ca \times 25\ \times 100}{Sample\ Weight\ (g)} \times \%DM$$

Where AA Ca was the average Ca concentration generated by the atomic absorption spectrophotometer (AA) from two technical replicates of one sample.

$$Total\ fecal\ or\ forage\ Ca\ (g) = Concentration \times Output\ or\ Intake\ DM(kg)$$

In urine, calcium excretion was determined by:

$$Urinary\ Concentration\ (\mu g/ml) = AA\ Ca \times Dilution$$

$$Total\ urinary\ Ca\ (g) = \frac{Concentration \times total\ urine\ output\ (ml)}{1{,}000{,}000}$$

Total Ca excretion was calculated by adding total urinary and fecal Ca together. Calcium balance was determined by:

$$Ca\ Balance = Intake - Total\ Excretion$$

Statistical analysis

Data were tested for normality, and residuals were normally distributed. All variables were analyzed using a mixed-model ANOVA with repeated measures (SAS Inst. Ver 9.4, Inc., Cary, NC). Fixed effects included day, treatment, and day by treatment interaction, with day as a repeated measure. Horse, period, and horse by period were initially tested for significance as fixed effects, and when found to be nonsignificant, they were incorporated into the model as random effects along with day by period. Tukey's *post hoc* analysis was used for multiple comparisons among days or day by treatment interactions if significance was found. Significance was set at $P < 0.05$.

RESULTS

Body condition scores and bodyweight were not different between periods or groups ($P > 0.10$). Mean Ca concentration in the hay diet was 2.9 ± 0.1 g/kg DM. Dry matter and Ca intake were not different between groups ($P > 0.10$) but differed by day ($P = 0.03$ and $P = 0.03$, respectively; Table 2.1) with horses consuming more DM and Ca on d 7 than d 1 or 3 ($P < 0.05$ for all comparisons).

Total Dry Matter and Calcium Consumed					
		Day			
Group		1	3	5	7
CON	**Dry Matter Consumed (kg)**	8.7 ± 0.4^{a}	9.0 ± 0.4^{ab}	8.9 ± 0.4^{ab}	9.1 ± 0.4^{b}
	Ca Consumed (g)	25.0 ± 2.1^{a}	25.9 ± 2.1^{ab}	25.7 ± 2.1^{ab}	26.3 ± 2.1^{b}
FUR	**Dry Matter Consumed (kg)**	8.9 ± 0.4^{ab}	8.6 ± 0.4^{a}	8.9 ± 0.4^{ab}	9.3 ± 0.4^{b}
	Ca Consumed (g)	25.6 ± 2.1^{ab}	24.9 ± 2.1^{a}	25.8 ± 2.1^{ab}	26.8 ± 2.1^{b}

Table 2.1. Total dry matter and calcium consumed, as well as fecal output (mean ± SE), on a dry matter basis in control (CON, n=10) and furosemide-administered (FUR, n=10) horses over seven days. [ab]Values within a row lacking a common superscript differ ($P < 0.05$).

Neither total fecal output nor feces %DM were different between groups or across days ($P > 0.10$; Table 1.2). However, total feces produced on a DM basis increased in later days ($P = 0.02$). Though total fecal Ca did not differ between groups or among days ($P > 0.10$; Table 1.2), fecal Ca concentration decreased from d 1 to d 7 ($P < 0.001$). While the interaction failed to reach significance ($P = 0.07$), fecal Ca concentrations remained the same from d 1 to d 7 in CON (6.3 ± 1.3 g/kg and 5.5 ± 1.3 g/kg, respectively; $P > 0.10$) but were higher in FUR on d 1 as compared to d 7 (7.3 ± 1.3 g/kg and 4.8 ± 1.3 g/kg, respectively; $P < 0.001$).

Total Fecal Output and Calcium					
		Day			
	Group	1	3	5	7
Fecal Output (kg)	CON	19.9 ± 1.4	21.5 ± 1.4	21.4 ± 1.4	21.4 ± 1.4
	FUR	19.6 ± 1.5	20.8 ± 1.4	21.9 ± 1.4	20.9 ± 1.4
Fecal Output, DMB (kg)	CON	4.3 ± 0.2^{a}	4.8 ± 0.2^{ab}	4.9 ± 0.2^{b}	5.0 ± 0.2^{b}
	FUR	4.6 ± 0.2^{a}	4.7 ± 0.2^{ab}	4.9 ± 0.2^{b}	4.7 ± 0.2^{b}
Fecal Ca Concentration (g/kg)	CON	6.3 ± 1.3	5.9 ± 1.3	5.3 ± 1.3	5.5 ± 1.3
	FUR	7.3 ± 1.3^{a}	5.4 ± 1.3^{b}	5.2 ± 1.3^{b}	4.8 ± 1.3^{b}
Total Fecal Ca (g)	CON	28.2 ± 6.4	29.2 ± 6.4	27.3 ± 6.4	27.9 ± 6.4
	FUR	29.9 ± 6.4	29.7 ± 6.4	25.1 ± 6.4	22.8 ± 6.4

Table 2.2. Total fecal output as sampled and on a dry matter basis (DMB) as well as fecal calcium concentration and total fecal calcium (mean ± SE) from control (CON, n=10) and furosemide-administered (FUR, n=10) horses over seven days. There were no differences between groups, but ^{ab}values within a row lacking a common superscript differ ($P < 0.05$).

A treatment by day interaction ($P < 0.001$) occurred for urine output with FUR urinating over twice as much as CON on d 1 after administration (7,527 ± 524 ml and 3,213 ± 524 ml, respectively). After this day, urine outputs were similar between treatments ($P > 0.10$; Figure 2.1). Urinary Ca concentrations only differed between groups on d 1 (CON: 2.1 ± 0.4 µg/ml and FUR: 3.4 ± 0.4 µg/ml, $P = 0.04$; Table 2.3). At all other days, CON and FUR urinary Ca concentrations were similar ($P > 0.10$). When urine output and urinary Ca concentrations were multiplied together to produce total urine Ca, a treatment by day interaction was observed ($P = 0.002$) with FUR losing more urinary Ca than CON on d 1 (9.3 ± 1.0 g and 4.2 ± 1.0 g, respectively; $P < 0.001$). These differences between groups in urine Ca losses disappeared by d 3 ($P = 0.3$; Figure 2.2).

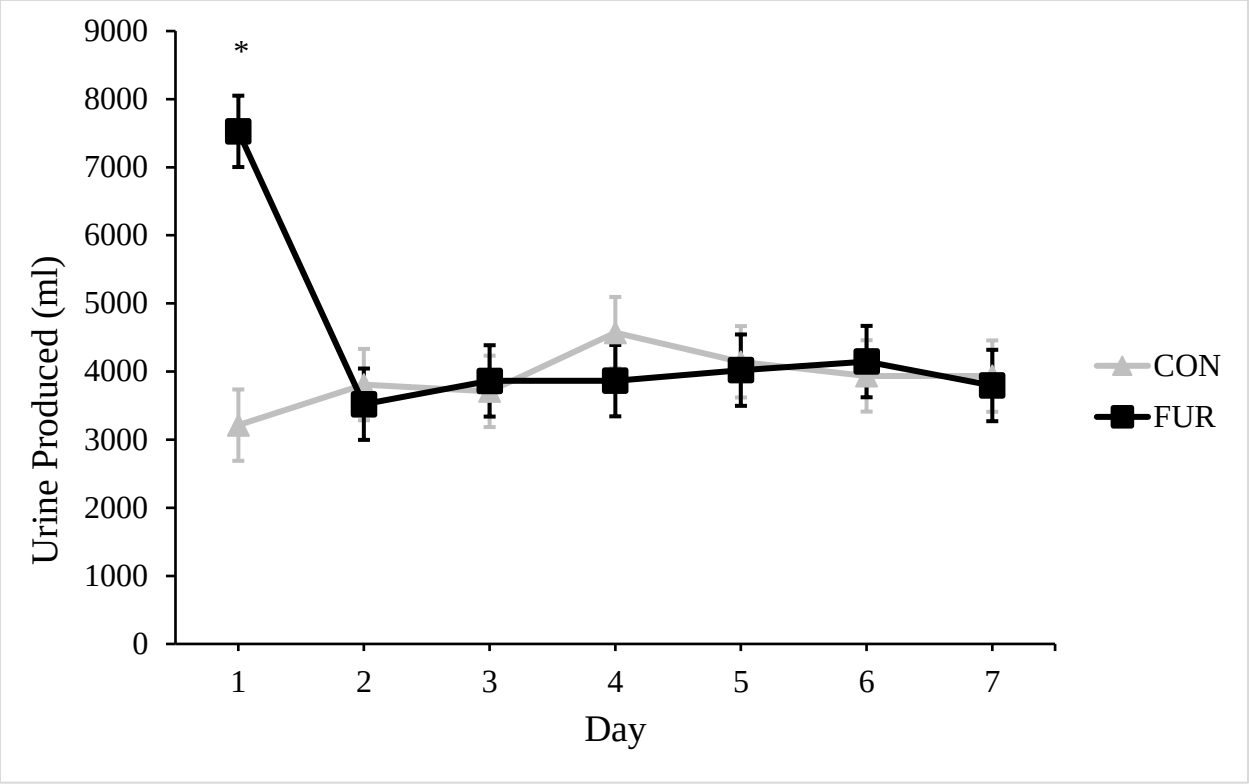

Figure 2.1. Mean (± SE) urine produced each day by furosemide-administered (FUR, n=10) and control (CON, n=10) group horses. *Indicates difference between treatments ($P < 0.05$).

Total Urine Output and Calcium					
		Day			
	Group	1	3	5	7
Urinary Ca Concentration (µg/ml)	CON	2.1 ± 0.4^{x}	1.7 ± 0.4	2.3 ± 0.4	2.2 ± 0.4
	FUR	3.4 ± 0.4^{ay}	2.3 ± 0.4^{b}	2.5 ± 0.4^{b}	2.5 ± 0.4^{b}
Total Urinary Ca (g)	CON	4.2 ± 1.0^{x}	2.6 ± 1.0	4.2 ± 1.0	4.3 ± 1.0
	FUR	9.3 ± 1.0^{ay}	3.7 ± 1.0^{b}	3.6 ± 1.0^{b}	4.7 ± 1.0^{b}

Table 2.3. Urinary calcium concentrations and losses (mean ± SE) on a dry matter basis in control (CON, n=10) and furosemide-administered (FUR, n=10) horses over seven days. ^{ab}Values within a row lacking a common superscript differ ($P < 0.05$). ^{xy}Values within a column lacking a common superscript differ ($P < 0.05$).

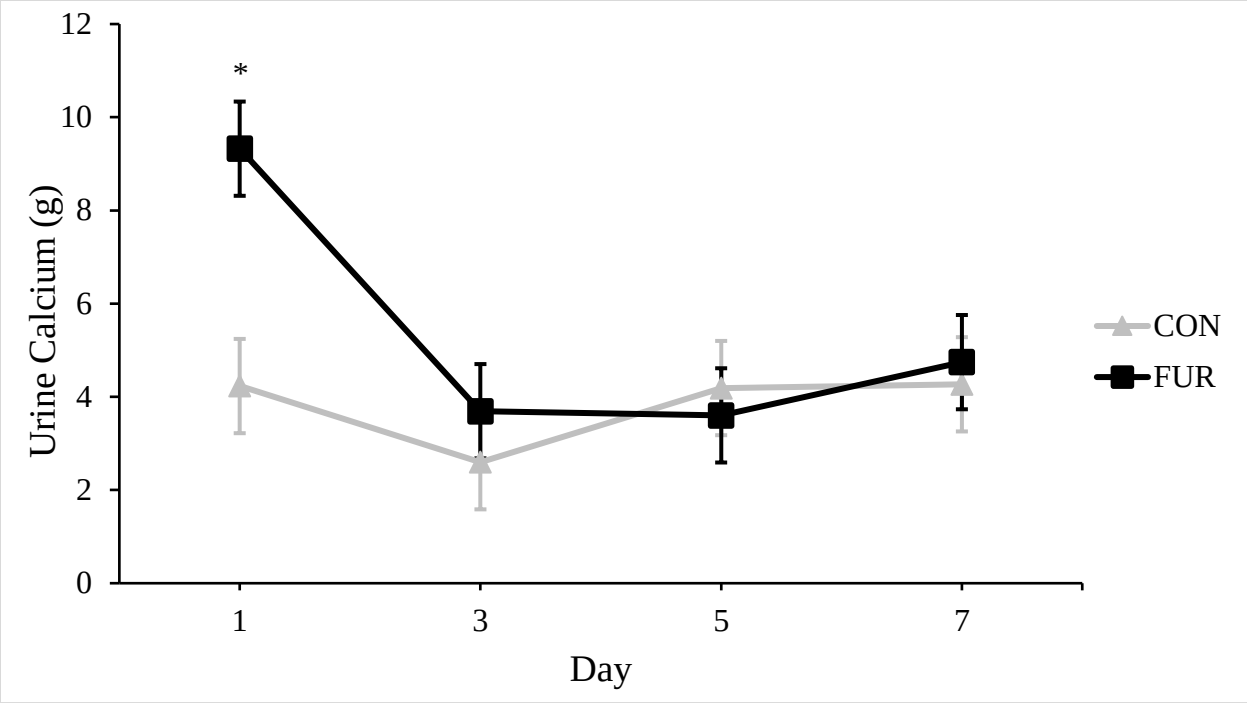

Figure 2.2. Total urine calcium (mean ± SE) excreted each day by furosemide-administered (FUR, n=10) and control (CON, n=10) group horses. *Indicates difference between treatments ($P < 0.05$).

After adding total fecal and urine Ca together, total Ca excreted showed a treatment by day interaction ($P = 0.03$, Table 2.4). Losses remained stable across days in CON ($P > 0.10$ for all day comparisons), but FUR horses lost more total Ca on d 1 than d 3, 5, or 7 ($P < 0.01$ for all comparisons; Table 2.4). Calcium balance followed a similar pattern to total Ca losses with differences observed by day ($P = 0.02$). Overall, CON Ca balance remained similar throughout the collection periods ($P > 0.10$ for all day comparisons), but FUR Ca balance was more negative on d 1 than any other day in the period ($P < 0.05$ for all comparisons).

		Total Daily Calcium Loss and Balance			
		Day			
	Group	1	3	5	7
Total Ca Loss (g)	CON	33.4 ± 5.1	31.8 ± 5.1	31.5 ± 5.1	32.2 ± 5.1
	FUR	39.2 ± 5.1[a]	28.8 ± 5.1[b]	28.7 ± 5.1[b]	27.6 ± 5.1[b]
Ca Balance (g)	CON	-7.4 ± 6.4	-6.6 ± 6.4	-5.8 ± 6.4	-4.5 ± 6.4
	FUR	-10.9 ± 6.4[a]	-3.9 ± 6.4[b]	-4.3 ± 6.4[b]	-2.3 ± 6.4[b]

Table 2.4. Daily calcium losses and balance (mean ± SE) for control (CON, n=10) and furosemide-administered (FUR, n=10) horses. [abc]Days in the same row lacking a common superscript differ ($P < 0.05$).

DISCUSSION

Due to furosemide's role in increased urinary Ca excretion and widespread use in the horse racing industry, it is unsurprising that critics of the industry have tied furosemide use to fatal breakdowns. However, based on the results presented from this study, furosemide likely did not play a role in these tragedies. The current study determined that one dose of furosemide only negatively affected Ca balance for three days after administration. As soon as three days post-administration, Ca balance in furosemide-administered horses returned to baseline values as demonstrated by control horses, which confirmed the study's hypothesis. While total fecal Ca did not change over time, fecal Ca concentrations decreased in the days following furosemide administration, indicating increased retention to restore Ca balance. The combination of these results indicates that furosemide likely does not contribute to a long-term negative Ca balance that would compromise bone strength.

Because amount of Ca consumed influences the amount of Ca excreted in manure and urine (Schryver et al., 1970a; Nielsen et al., 1998), it was important to ensure that intakes were not different between groups. The greater amount of DM and Ca consumed later in the collection periods can be explained by feeding adjustments. If a horse routinely had no orts, hay was

increased to ensure that horses would always have some hay. Though amount of hay was based on 2% BW, body condition scores ranged from 4 to 8, suggesting that an individual horse's BW may have differed from their BW at ideal condition. Anecdotally, the horses in the study that frequently had no orts, even between feedings, were those with lower BCS, so their hay was increased as needed over the course of the collection period. Previous research found a correlation between BW, dominance rank, and aggression around food (Houpt et al., 1978), so herd dynamics may have played a part in BCS as more aggressive horses could have limited hay feeder access from others while housed on pasture. When horses were brought in for total collections and placed into individual stalls, less aggressive horses had ample opportunity to finish the hay provided. Since fecal outputs mirrored DM intake, it follows that an increased amount fed would also increase fecal outputs, and this likely explains the increased outputs at the end of the collection periods. Though manure produced was different among days, total fecal Ca remained stable throughout the trial, which was expected since furosemide acts renally.

The treatment by day interaction matched previous studies (Tobin et al., 1978; Hinchcliff et al., 1991; Hinchcliff and Muir, 1991), but urine volume may have been lower in the current study as compared to older studies. A likely reason for low urine volume was ambient temperature and its influence on water intake. Cymbaluk (1990) found that urine output was directly related to their water intake and that free water intake was related to ambient temperature as cold-housed colts drank less water and produced less urine than their warm-housed counterparts. Additionally, horses housed in a cold environment drink less near-freezing water than hot or heated water (Kristula and McDonnell, 1994). While water consumption was not recorded, these conditions likely led to less water consumed, and subsequently less urine produced, than if conditions were warmer.

Although urine volume might initially appear lower than previous studies due to weather, urine volume response to IV furosemide in previous studies varied, and many studies used BW losses corrected to exclude fecal losses as a measure for urine output. A study by Hinchcliff et al. (1993) found furosemide administration decreased BW 8.8 to 10.2 ± 2.0 kg after adjusting for manure losses. A later study by the same group testing fluid administration after furosemide but before exercise found BW losses ranging from 5.9 ± 0.9 to 11.5 ± 0.8 kg (Hinchcliff and Mckeever, 1998). Horses in an earlier study by Tobin et al. (1978) produced 10.5 liters of urine at the end of four hours after furosemide administration, and even more studies have found an average 2-4% BW loss with furosemide administration (Freestone et al., 1988; Warren et al., 1999; Butudom et al., 2004; Forro and Lindinger, 2006; Zawadzkas et al., 2006; Pritchard et al., 2017). As BW for horses in the current study averaged around 530 kg, post-furosemide administration expected BW losses should be around 10.6 to 21.2 kg or around 10 to 20 l of urine. Assuming reduced water consumption and therefore reduced urine production with cold weather, the 7.5 l of urine produced by FUR on d 1 is within the realm of losses and volumes reported by previous studies.

As expected, furosemide administration increased urinary Ca excretion as demonstrated by previous studies in horses and other species (Freestone et al., 1988; Rubin et al., 1995; Pagan et al., 2013). Though furosemide acts specifically on NKCC2 which does not transport Ca, it changes the transepithelial gradient in the lumen from between +5 and +10 mV to a more neutral charge by blocking the uptake of Cl^- (Alexander and Dimke, 2017). While the basolateral space remains neutrally charged, a reduction in the lumen-positive gradient decreases the diffusion of Ca across the membrane (Suki, 2017), meaning the subject receiving furosemide excretes more Ca in urine. With increased ion delivery downstream of NKCC2, mRNA for specific ion

transporters becomes upregulated in a solute-dependent manner after a single furosemide dose (Lee et al., 2007). The change in the transepithelial gradient and subsequent response to correct it may explain the dramatic excretion of Ca in FUR on d 1 which corrects to CON values by d 3. While the current study was not mechanistic in design, knowledge of these mechanisms may facilitate understanding of the impacts and results presented by the current study.

Additionally, urinary Ca values may seem higher than previously reported. Calculating from mEq/l, Freestone et al. (1988) reported urinary Ca losses around 2.6 g after furosemide administration. In unpublished data shared in a personal communication from Pagan et al. (2013, 2014), total urinary Ca excretion was 6.1 ± 0.6 g 24 h post-furosemide administration and remained above this value 72 h post-administration (6.9 ± 0.5 g). Differences in values from the current study and previous research may come from differences in handling methods and analysis. Freestone et al. (1988) used a fluorescence-based calcium analyzer which can produce values much lower than those reported on the same samples with an atomic absorption spectrophotometer (Rodgerson and Moran, 1968). Values from Pagan et al. (2013, 2014) were closer to those in the current study, but urine may not have been acidified at the same rate which can influence analysis (O'Connor-Robison and Nielsen, 2006). However, pre-administration values matched those reported for CON in this study (4.3 ± 0.1 g), so the acidification rate used in the current study likely ensured that more of the urinary Ca precipitate post-administration was in solution and produced larger values.

When fecal and urine Ca were added together, total Ca losses differed by day. As seen in Table 2, CON total Ca losses did not differ across days, but FUR lost more Ca on d 1 than later in the collection period. As feces is the main route of Ca excretion, fecal Ca losses are much greater than urinary Ca, and treatment differences in urinary Ca were lost when examining total

Ca. However, FUR horses still had greater total Ca losses on d 1 as compared to the rest of the days with CON values remaining unchanged over the collection period. This difference indicated that furosemide nearly doubled urinary Ca in 24 h post-administration, increasing urine Ca to around 24% of total losses when typical urine Ca might only be about 12.5%.

Ultimately, total Ca losses influenced Ca balance more than urinary Ca losses as results for Ca balance parallel total Ca excretion. Overall, Ca balance appeared negative across both groups but ultimately was no different from a net zero balance. This balance was likely because of the low Ca in the hay fed for the study which was about 2.9 ± 0.1 g/kg DM or 0.3% DM, even less than the NRC estimate of 0.47% for mature cool season grass hay (National Research Council, 2007). While this hay was low in Ca, it still met the horses' Ca requirements as they were all mature and sedentary prior to and during the study. Despite changes to amount of hay fed, CON Ca balance remained stable and right around zero with no differences among days, but FUR Ca balance was more negative on d 1 than later in the collection period which agreed with results from Pagan et al. (2013, 2014). As mentioned previously, furosemide administration increases mRNA expression for ion-specific transporters. However, it may take time to appropriately translate the number and activity of these transporters in the nephron with some studies taking up to three days to negate the diuretic effects (Lee et al., 2007). This timing of transporters may explain why it took at least three days to restore Ca balance in the current study and previous research. Until transporters or metabolism of furosemide restores the transepithelial gradient, Ca absorption is impaired, making it difficult to restore Ca balance.

However, this study showed decreased fecal Ca concentrations following furosemide administration which likely restored Ca balance relatively quickly and offset initial net losses through urine. The mechanism for this result is unknown in the current study but likely has to do

with mechanisms to maintain Ca homeostasis. Decreases in blood Ca through nutrition or exercise can cause the release of parathyroid hormone (PTH) to restore blood Ca concentrations (McKeever, 2011; Toribio, 2011). While PTH acts mainly to increase bone resorption and to decrease renal Ca excretion, it also increases renal activation of Vitamin D which improves Ca absorption in the intestine (Toribio, 2011) which the major site of Ca absorption in horses (Schryver et al., 1970b). While changes in urinary Ca concentrations from d 1 can be explained by furosemide's mechanism of action, the changes in fecal Ca concentrations cannot but may be attributable to the actions of PTH and Vitamin D. Since urinary Ca concentrations remained similar after d 1, PTH's renal activation of Vitamin D was likely the main mechanism to restore Ca losses. However, without blood analysis for either of these calciotropic hormones, this idea remains speculative.

The findings of this study support previous work by some of the authors into the effects of frequent furosemide administration on bone (Pritchard et al., 2019). Briefly, the previous study administered furosemide weekly over 56 d and measured bone mineral content using radiographic bone aluminum equivalencies every 28 d as well as bone metabolism markers every 7 d in treatment and control horses. There were no changes in bone mineral content, formation, nor degradation markers attributable to treatment. Increased fecal Ca absorption in the days following furosemide administration in the current study explain the lack of treatment differences in the previous study. Furthermore, these two studies together provide evidence that frequent furosemide administration likely does not contribute to bone mineral loss.

CONCLUSIONS

A single furosemide administration doubles urinary Ca and urine output over control values, increasing total Ca output and creating a temporary negative Ca balance. While these

impacts occur after a single dose, values return to baseline relatively quickly due to potential increased Ca absorption in the intestine as evidenced by decrease fecal Ca concentrations in the days following furosemide administration. The mechanisms for restoring Ca losses after furosemide should be further investigated, but this study demonstrates that furosemide only negatively impacts Ca balance for less than three days after administration. Therefore, administering furosemide prior to racing or intense training bouts likely does not contribute to decreased bone mineral content.

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LITERATURE CITED

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CHAPTER 3: Dietary silicon supplementation may not affect bone and cartilage in mature, sedentary horses

INTRODUCTION

In the United States, over 65% of stables had one or more resident equids with a lameness problem at any time, and less than half of these horses recovered and remained sound (United States Department of Agriculture, 2017). Unfortunately, these pervasive lamenesses can lead to interrupted training and economic losses as 16.3% of horses retire, become a companion animal, are given away, or euthanized (United States Department of Agriculture, 2017). As over 20% of horses were identified as having a chronic joint problem like arthritis (United States Department of Agriculture, 2017), prevention and management of equine osteoarthritis (OA) is key to maintaining a horse's soundness, value, and welfare.

Osteoarthritis is characterized by a loss of articular cartilage, often with associated bony and soft tissue changes, leading to pain and decreased function (Schlueter and Orth, 2004; McIlwraith et al., 2013). Articular cartilage loss results from an imbalanced turnover of collagen demonstrated by increased markers of collagen degradation found in synovial fluid collected from OA joints (Frisbie et al., 2008; Nicholson et al., 2010; McIlwraith et al., 2018). Additionally, aging results in a loss of polysulfated glycosaminoglycans in healthy articular cartilage (Plat et al., 1998), further reducing collagen synthesis within the joint. Improving collagen health prior to OA onset and increasing collagen repair through higher rates of degradation and synthesis within OA-affected joints could improve health- and welfare-related outcomes in horses with OA.

Silicon (Si) plays a crucial role in long bone development (Carlisle, 1980b; Carlisle, 1981) and mineralization (Kim et al., 2013; Kim et al., 2014; Arora and Arora, 2017) through decreased bone resorption and increased osteoblast differentiation (Maehira et al., 2009;

Mladenović et al., 2014; Dong et al., 2016). Through these effects and its role in increasing collagen synthesis (Calomme and Vanden Berghe, 1997; Reffitt et al., 2003; Dong et al., 2016), Si may influence the development and progression of OA, thereby reducing lameness. Additionally, Si may affect the absorption and use of other minerals, such as calcium (Ca) and boron (B), important to bone mineralization (Kim et al., 2014; Jugdaohsingh et al., 2015a). Changes in the availability of these minerals may impact bone health.

Previous research found reduced carboxy-terminal pyridinoline cross-linked telopeptide region of type I collagen, a bone resorption marker, in Si-supplemented young horses compared to controls (Lang et al., 2001). Additionally, Si supplementation increased distance trained prior to injury in racehorses (Nielsen et al., 1993). Unfortunately, previous equine research used sodium zeolite A (Frey et al., 1992; Nielsen et al., 1993; Lang et al., 2001) which contained large amounts of aluminum, up to 130,000 mg/kg (O'Connor et al., 2008), posing potential toxicity concerns and absorption interference with other minerals. Since these studies, new Si supplements have been developed, including a purportedly bioavailable silica-collagen peptide source. The objective of the current study was to determine the effect of feeding a bioavailable Si supplement on markers of collagen degradation and formation and osteoblast activity as well as objective and subjective lameness in mature horses. This study evaluated the hypothesis that Si supplementation would increase bone formation markers and cartilage turnover through increased collagen degradation and formation markers and reduce lameness severity as compared to controls.

MATERIALS AND METHODS

Horses and Diet

Ten mature Standardbred geldings were striated by age and bodyweight (BW), pair-matched based on lameness degree and limb, and randomly assigned to either a Si treated (SIL) or control (CON) group. Horses were group-housed on summer pasture for 84 days (12 weeks) but individually fed from assigned feeders to ensure no cross-contamination of Si other than what was present in the environment. For the duration of the study, SIL horses received a bioavailable Si-collagen supplement at the rate of 0.3 g supplement/(100 kg BW·d), based on manufacturer's recommendations (Privi Life Sciences Pvt Ltd, Mumbai, Maharashtra, India). After every daily meal, feeders were checked for refusals, and any feed refusals were weighed and recorded. Horses were weighed on d 0 and every 7 d during the study to ensure appropriate dosage, and total amount supplemented was adjusted as needed due to weight gain or loss. All horses received 500 g of textured feed (Meco 15, Mason Elevator Co., Mason, MI; 15% CP, 3% fat, 5.5% fiber) once per day and had free-choice access to grass and water.

Lameness exams and Lameness Locator

A lameness examination of each horse was conducted on d 0, 42, and 84 using a traditional scoring system and a Lameness Locator system (Equinosis, LLC, St. Louis, MO). A baseline for each horse was conducted on a straight line on a flat, hard surface, and distal and proximal flexion tests of all four legs were completed and measured by the system on the same line. A veterinarian who is a Diplomate of both the American College of Veterinary Surgeons-Large Animal and the American College of Veterinary Sports Medicine and Rehabilitation (Equine) and blinded to treatment group scored overall lameness and lameness after flexions using the American Association of Equine Practitioners (AAEP, 1991) scale in 0.5 increments

ranging from 0 (sound) to 5 (non-weight bearing). The Lameness Locator system served as an objective observer.

The Lameness Locator consisted of three inertial sensor devices, two accelerometers and a gyroscope, which were positioned between the ears on the horse's poll, over the pelvis along the dorsal midline, and on the dorsal side of the front right pastern (Keegan et al., 2011). The devices were placed by the same person every time to ensure consistency and remained in place throughout each trial. Each horse was then trotted in-hand on a hard, flat surface for a minimum of 15 strides. The variables of interest included the front vector sum and the differences in maximum and minimum pelvic height (HDmax and HDmin). The front vector sums (FVS) were defined as the difference between the maximum and minimum positions of the head, and the threshold for a significant front limb lameness was considered at FVS $\geq$ 8.5 mm (Keegan et al., 2013). The maximum head difference was generated by the maximum height of the head between left and right portions of the stride compared with that of the left forelimb with the minimum head differences generated the same way by the differences at the minimum height. Similarly, HDmax and HDmin were generated by the differences in the maximum and minimum heights of the pelvis, respectively, during the stance phase of the right hind compared with the left hind limb. Thresholds for hind limb lameness detection using HDmax and HDmin were set at > 3 mm for both (Keegan et al., 2013). Once this was complete, the devices were removed from the horse, and feedback from the Lameness Locators was saved for later analysis.

Radiographs

Digital radiographs (Eklin Model EDR3 Mark III, Carlsbad, CA; HF8015+dlp, MinXray Inc., Northbrook, IL) were taken of the left carpal (LMC and LRC) and left metacarpophalangeal (LFF) joints as references as well as any other joint suspected of arthritis (including right carpal,

metacarpophalangeal, and both tarsal and metatarsophalangeal joints) prior to the start of the study. The same joints were radiographed again on d 42 and 84 to look for evidence of arthritic changes. Dorsopalmar, mediolateral, dorsolateral-palmaromedial oblique, and dorsomedial-palmarolateral oblique views (Smallwood et al., 1985) were taken of suspected OA joints and compiled into files per joint per horse per day. Any identifying information for date or horse was removed from the digital radiographic images prior to assignment to a blinded reviewer, the same veterinarian who assessed lameness, for scoring. The reviewer scored each set of radiographs using a scale of 0 to 3 in 0.5 increments with 0 being no evidence of arthritis and 3 being evidence of severe arthritis (Frisbie et al., 2002).

Blood Samples

Immediately after feeding, blood samples were collected via jugular venipuncture into vacutainer serum separation tubes (BD Vacutainer™: SST™, Becton, Dickinson and Company, Franklin Lakes, NJ) on d 0 and every 7 d until the end of the study on d 84 for serum osteocalcin (OC) analysis. On d 0, 42, and 84, additional blood samples were collected into acid-washed, K_2EDTA treated tubes for plasma silicon, boron, and calcium analysis. Serum and plasma samples were centrifuged and placed into microcentrifuge tubes before freezing at -20° C. Serum samples were analyzed using an OC enzyme-linked immunosorbent assay (Microvue™ Osteocalcin EIA, Quidel, San Diego, CA). Plasma Si, B, and Ca and Si concentration within the supplement were determined by inductively coupled plasma mass spectrometry (Agilent 7900 Inductively Coupled Plasma Mass Spectrometer, Agilent, Santa Clara, CA) at a certified laboratory (Michigan State University Veterinary Diagnostic Laboratory, Lansing, MI).

Synovial Fluid Samples

Synovial fluid samples were collected aseptically on d 0, 42, and 84 from the LFF, LMC, and LRC joints to serve as reference joints and from any other joint suspected of OA based on radiographic and lameness evaluations. These samples were divided into cryotubes and placed immediately on dry ice until transferred to the -80° C storage location. Synovial fluid was digested using 50 units/mL of hyaluronidase at 37° C for 40 min to reduce viscosity (O'Connor-Robison et al., 2014). Synovial fluid was analyzed for procollagen type II C-propeptides (CPII) and triple helix fragments of both types I and II collagen (C1,2C) as markers of collagen formation and degradation, respectively, using commercial assays (Ibex Pharmaceuticals, Montreal, Quebec). Only reference joints were included for CPII analysis while all joints sampled were included for C1,2C analysis. Differences between treatments were examined at each sampling point, as well as for changes from baseline.

Statistical Analysis

Residuals of data were tested for normality and log transformed as necessary. Absolute values of the variables generated from the Lameness Locator were used for analysis. Continuous data from biomarker, mineral, and Lameness Locator analyses were analyzed using the mixed model procedure (Proc Mixed) in SAS 9.4 (SAS Institute, Cary, NC). Data from radiographic and lameness scores were treated as continuous and analyzed using the generalized linear mixed model procedure (Proc Glimmix). Fixed effects for each analysis included treatment, day or week, and their interactions. For OC and plasma minerals, horse was included as a random effect with week used as a repeated measure for OC only. Lameness scores and radiographic evaluations included the trial or leg within horse as a random effect. Lameness Locator data were divided into straight line and flexion trials with data analyzed separately by limb flexion.

Concentrations of CPII and C1,2C were analyzed by each separate joint. Since non-reference joints were not pair-matched, concentrations of C1,2C were pooled to examine overall effect of treatment and joint was set as a random effect. Tukey's *post hoc* analysis was used for comparisons among days or between groups for significant effects. Significance was set at $P < 0.05$, and due to the small number of subjects per group (n=5), trends were considered at $0.05 \leq P < 0.10$.

RESULTS

Horses and Diet

Mean age and BW for the groups were 13 ± 1 yr and 541 ± 8 kg for SIL and 10 ± 2 yr; 542 ± 4 kg for CON. Bodyweight remained similar between groups, but BW increased in both groups from d 0 to d 84 ($P < 0.01$). The supplement contained 32.5 mg Si/g while the textured feed had 0.004 mg Si/g. Silicon intake in CON was 1.8 mg/d from feed compared to 54.5 ± 2.1 mg/d in SIL from feed and 1.5 g supplement. There were no feed refusals over the course of the study.

Subjective and Objective Lameness Evaluations

Subjective lameness scores in most limbs remained unaffected by treatment and day. However, there was a tendency ($P = 0.06$) for SIL to score lower (0.3 ± 0.3) than CON (1.1 ± 0.3) in the left front throughout the study. When examined as a change from baseline, there was no difference between the group.

From the Lameness Locator straight-line trials, there was no difference between treatments or among days for any of the variables (Table 3.1). During all flexion trials, FVS was unaffected by treatment and day. Both HDMin and HDMax values experienced a day by treatment interaction ($P = 0.003$). In HDMin, CON values remained similar over the course of

the study, but SIL values decreased from d 0 to d 84 ($P = 0.02$). For HDMax, SIL values were similar over time, but CON values decreased from d 0 to d 84 ($P = 0.003$).

Variable	Group	Day		
		0	42	84
FVS	CON	20.7 ± 3.2	19.5 ± 3.4	21.1 ± 3.2
	SIL	13.7 ± 3.2	21.0 ± 3.6	12.5 ± 3.2
HDMin	CON	6.2 ± 0.9	5.4 ± 1.0	4.8 ± 0.9
	SIL	5.2 ± 0.9	3.5 ± 1.0	3.3 ± 0.9
HDMax	CON	5.0 ± 1.1	4.9 ± 1.2	2.8 ± 1.1
	SIL	4.5 ± 1.1	6.1 ± 1.3	4.2 ± 1.1

Table 3.1. Mean (± SE) mm of front vector sums (FVS), minimum difference in pelvic level (HDMin), and maximum difference in pelvic level (HDMax) generated from a Lameness Locator system in straight line trials without joint flexions in control (CON, n=5) and silicon-supplemented (SIL, n=5) horses.

Radiographs

Overall, radiographic scores were unaffected by treatment. Radiographic scores in the LMC and LRC appeared to increase over time ($P = 0.005$) with d 0 and 42 scoring lower (0.1 ± 0.2 for both) than d 84 (0.6 ± 0.2; $P < 0.05$). In all other joints, scores remained similar throughout the study.

Blood markers

Osteocalcin concentrations did not differ between groups but did increase in certain weeks throughout the study ($P < 0.001$, Figure 3.1). Plasma Ca and B concentrations were unaffected by treatment (Table 3.2). Calcium concentrations remained similar from d 0 to d 42 (116 ± 1 µg/ml and 117 ± 1 µg/ml, respectively) but decreased at d 84 below both previous sampling days (112 ± 1 µg/ml, $P < 0.05$ for both comparisons). However, B concentrations increased from d 0 to d 42 (0.094 ± 0.003 µg/ml and 0.129 ± 0.003 µg/ml, respectively; $P < 0.001$) and decreased from d 42 to d 84 (0.104 ± 0.003 µg/ml; $P < 0.001$). Interestingly, Si supplementation did not increase plasma Si concentrations as compared to controls, but plasma

Si decreased from d 0 to d 84 in all horses (1.06 ± 0.07 µg/ml and 0.59 ± 0.07 µg/ml for d 0 and 84, respectively; $P < 0.05$).

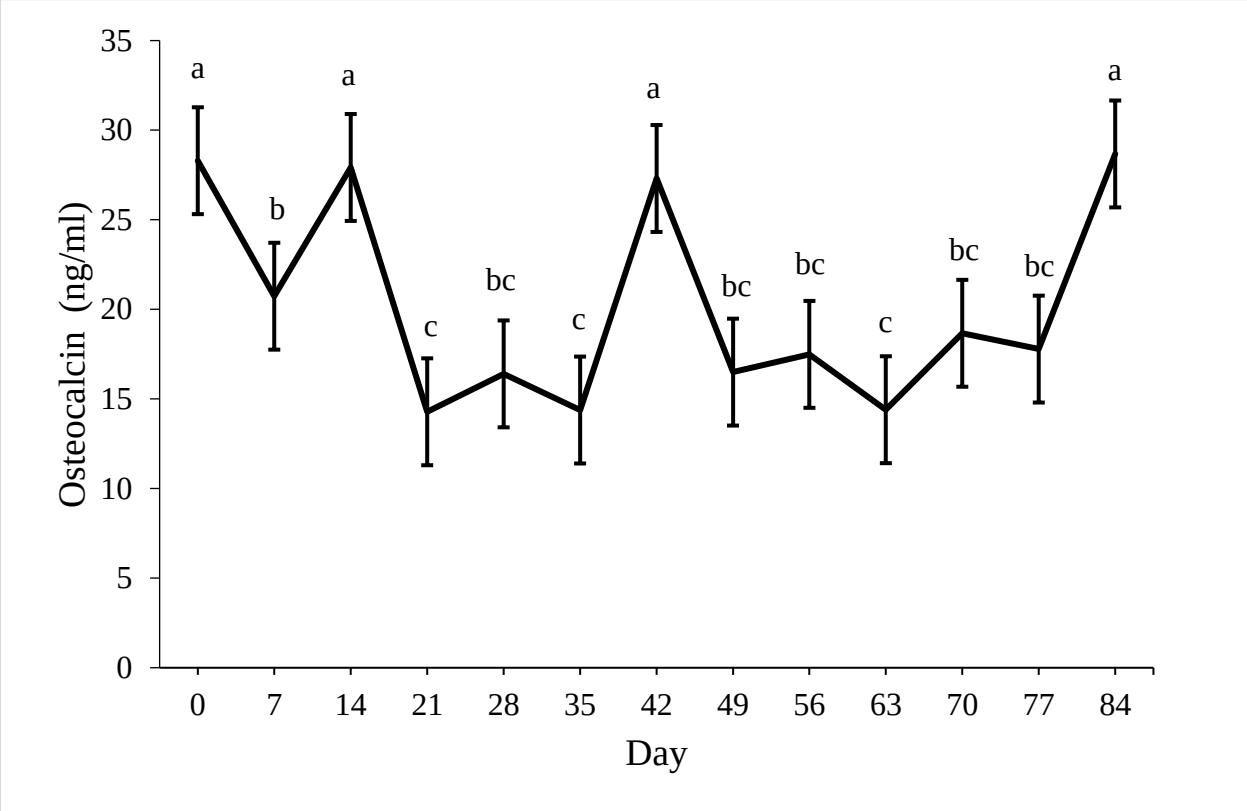

Figure 3.1. Pooled mean serum osteocalcin concentrations (± SE) collected every 7 d beginning on d 0 until d 84 from control (n=5) and supplemented (n=5) horses. [abc]Days lacking a common letter differ ($P < 0.05$).

Mineral	Group	Day		
		0	42	84
Boron	CON	0.096 ± 0.004[a]	0.132 ± 0.004[b]	0.107 ± 0.004[a]
	SIL	0.091 ± 0.004[a]	0.127 ± 0.004[b]	0.101 ± 0.004[a]
Calcium	CON	116 ± 1	116 ± 1	112 ± 1
	SIL	115 ± 1	117 ± 1	111 ± 1
Silicon	CON	1.20 ± 0.10[a]	0.82 ± 0.10[ab]	0.60 ± 0.10[b]
	SIL	0.93 ± 0.10	0.84 ± 0.10	0.57 ± 0.10

Table 3.2. Mean (± SE) plasma boron, calcium, and silicon concentrations (µg/ml) in control (CON, n=5) and silicon-supplemented horses (SIL, n=5).[ab]Values within a row lacking a common superscript differ ($P < 0.05$).

Synovial Fluid Markers

In two of the reference joints, LRC and LMC, C1,2C concentrations did not differ by group or day (Table 3.3). In LFF, overall C1,2C concentrations tended to be greater in SIL as compared to CON (0.35 ± 0.2 µg/ml and 0.30 ± 0.02 µg/ml, respectively; $P = 0.08$) but were similar at all sampling days. When analyzed as change from d 0, there were no differences between groups or days. Additionally, in non-reference joints with OA, C1,2C concentrations were similar between groups (CON: 0.33 ± 0.3 µg/ml; SIL: 0.34 ± 0.3 µg/ml). There was evidence for a day by treatment interaction ($P < 0.05$), but there was no change within groups over the course of the study or difference between groups at each sampling day in *post hoc* analysis. CPII concentrations did not show any differences between groups or among days (Table 3.4). When analyzed as a change from baseline, there were no differences between groups or days.

Joint	**Group**	**Day**		
		0	42	84
LMC	CON	0.37 ± 0.04	0.30 ± 0.04	0.32 ± 0.04
	SIL	0.42 ± 0.04	0.35 ± 0.04	0.36 ± 0.04
LRC	CON	0.36 ± 0.05*	0.27 ± 0.07	0.24 ± 0.06
	SIL	0.36 ± 0.05	0.37 ± 0.08	0.28 ± 0.07
LFF	CON	0.31 ± 0.04*	0.30 ± 0.04*	0.29 ± 0.04*
	SIL	0.36 ± 0.04	0.45 ± 0.04	0.30 ± 0.04
OA	CON	0.37 ± 0.05*	0.23 ± 0.08*	0.20 ± 0.08*
	SIL	0.26 ± 0.05*	0.31 ± 0.08*	0.24 ± 0.09*

Table 3.3. Mean (± SE) triple helix fragments of types I and II collagen **(**C1,2C) concentrations (µg/ml) as a marker of collagen degradation in synovial fluid taken from the left middle carpal (LMC), left radiocarpal (LRC), left front metacarpophalangeal (LFF), and osteoarthritic (OA) joints in control (CON, n=5) and silicon-supplemented (SIL,n=5) horses.*Indicates n=4.

Joint	Group	Day		
		0	42	84
LMC	CON	2,306 ± 812	949 ± 638	1,333 ± 638
	SIL	2,037 ± 803	1,512 ± 605	1,516 ± 605
LRC	CON	1,624 ± 363*	1,400 ± 314	1,137 ± 353
	SIL	2,069 ± 321	1,489 ± 295	1,148 ± 346
LFF	CON	1,490 ± 601*	717 ± 464*	846 ± 464*
	SIL	1,532 ± 595	1,051 ± 444	883 ± 449

Table 3.4. Mean (± SE) procollagen type II C-propeptide (CPII) concentrations (ng/ml) as a marker of collagen formation in synovial fluid taken from the left middle carpal (LMC), left radiocarpal (LRF), and left front metacarpophalangeal (LFF) joints in control (CON, n=5) and silicon-supplemented (SIL, n=5) horses. *Indicates n=4.

DISCUSSION

Lameness and OA present large problems for the equine industry. While promising in previous studies, Si supplementation in the current study did not affect lameness as subjective and objective evaluations were similar between groups. While none of the changes in the study could be attributed to treatment, OC peaked during certain weeks, and plasma mineral concentrations declined over the course of the study. For the mature horses in the current study, Si supplementation did not change whole-body or joint-specific measures of lameness and collagen metabolism.

Overall, determining the effect of Si supplementation on radiographic progression of OA and lameness proved difficult as horses in the current study presented little radiographic evidence of OA in reference joints with 71% of scores at 0.5 or below. Additionally, even after flexion, 77.5% of trials scored at 1 or below on the AAEP scale. While these horses were mature and previously raced, most were relatively sound, making differences between groups and over time difficult to observe with subjective scoring. Trained clinicians can agree on lameness scores above 1.5 around 93% of the time, but this agreement decreases to 62% when lameness is mild (Keegan et al., 2010), indicating the difficulty with using a subjective evaluation to determine

treatment differences. However, subjective evaluations are standard clinical practice, making them an important incorporation in industry-applied research. Additionally, the inclusion of an objective observer in the Lameness Locator system strengthens these subjective evaluations as this system produces similar findings to subjective evaluations (Keegan et al., 2013; Donnell et al., 2015). Previous research indicates that a FVS ≥ 8.5 mm but ≤ 20 mm will typically be scored at a 1 or below on the AAEP scale while HDMin and HDMax ranges from ≥ 3 mm to ≤ 9 mm and ≤ 4 mm, respectively, for the same score (Keegan et al., 2013). In the current study, all Lameness Locator readings stayed close to these ranges, demonstrating the accuracy and consistency of the subjective evaluations as well as the relative soundness of the research horses. While having healthy and relatively comfortable research horses is important, it did make it difficult in determining whole-body differences between treatments. To better examine the effects of Si supplementation on lameness, a minimum lameness threshold with average scores above 1.5 should be established as inclusion criteria for horses in future studies.

Activity and exercise can affect bone metabolism markers more than nutrition alone (Nielsen and Spooner, 2008), and increased bouts of free choice exercise in response to external stimuli may have influenced osteocalcin in the current study. While osteocalcin was unaffected by Si supplementation, concentrations of this marker spiked in certain weeks, potentially related to increased activity secondary to external factors. In the few days prior to sampling on d 0, horses were moved to a new pasture which served as group-housing for the current study, subsequently increasing exploration and flight response to stimuli in a new environment. The weeks of d 14 and d 42 corresponded with farm construction near the pasture and American Fourth of July celebrations, respectively, which could have spooked horses and increased their activity. While activity was not monitored in the current study, external stimuli outside of the

researchers' control may have increased free choice exercise while horses were on pasture, affecting osteocalcin concentrations in the current study.

Interestingly, plasma Si was not greater in the supplemented group as compared to controls. Previous research using sodium zeolite A as the Si source indicated that plasma Si concentrations should be higher in supplemented horses (Lang et al., 2001) and remain higher even at six to nine hours post-feeding (Frey et al., 1992). However, serum Si concentrations depend on source and renal clearance (Sripanyakorn et al., 2009). In a previous study in humans, serum Si did not peak until three hours post-consumption of a choline-stabilized orthosilicic acid source and remained relatively stable after consumption of magnesium trisilicate (Sripanyakorn et al., 2009). As plasma samples were collected within an hour after feeding, this may not have been enough time to allow Si from the supplement to become fully bioavailable. However, supplementation of orthosilicic acid in horses did not produce greater plasma Si than controls (O'Connor et al., 2008), indicating that changes in plasma Si in horses may also depend on the Si source. The source in the current study was a Si-collagen supplement which should have prevented the spontaneous formation of insoluble oligomers and silicates (Belton et al., 2012) which would have reduced absorption (Sripanyakorn et al., 2009). However, as there was no difference in plasma Si between groups and no increase in SIL over time, the source in the current study may not be as absorbable or alter plasma Si as much as previous sources.

The decrease in plasma Ca and Si concentrations in both groups likely resulted from pasture growth and declining digestibility with pasture maturation. Grasses and grains (such as oats and rice) accumulate Si and sometimes Ca more than other species as a mechanism to protect against biotic and abiotic stresses (Currie and Perry, 2007; Bauer et al., 2011; Belton et al., 2012). Silicon is usually deposited in plant cell walls and greater concentrations can be found

in wounded and older cells (Bauer et al., 2011). Additionally, increased Si concentrations in grasses are associated with reduced digestibility, providing both chemical protection of cell wall components and physical protection from chewing (Massey et al., 2008; Bauer et al., 2011). Due to the predominant cool season grasses in Michigan and their growth cycle, pasture was likely older as this study took place over the summer. With reduced growth, more Si was deposited into older cells, likely reducing the digestibility and availability of nutrients from this pasture. This dormancy of cool season grasses during the heat of the summer may have caused plasma Ca and Si to decrease over the course of the study. Unfortunately, grass samples were not collected, but given the decrease in plasma Si in both groups and group housing on pasture, this may have been the cause.

As treatment did not affect plasma B concentrations, the changes in both groups over the course of the study could be attributed to B accumulation and grass digestibility, as noted above. Grasses have low requirement for B but can concentrate it up to 800 mg/kg before reaching toxic levels (Gupta, 2016). Boron concentrations increase with plant maturity, mirroring similar increasing concentration in older bone (Jugdaohsingh et al., 2015a), and its role is less structural than Ca and Si as deficiencies interrupt protein, nucleic acid, and sugar metabolism, cellular membrane function, and seed production (Brown et al., 2002; Gupta, 2016). The peak of concentrations on d 42 and decline to d 84 could represent this B accumulation; as grasses accumulated more B and Si and matured, digestibility decreased, affecting B concentrations in the second half of this study. Since both groups follow the same pattern, the cause was likely the pasture, but this remains speculative.

Overall, collagen metabolism markers in synovial fluid were relatively unaffected by Si supplementation. Use of these markers in synovial fluid, rather than serum, allowed for analysis

at individual joints as collagen biomarker concentrations can vary by joint (Nicholson et al., 2010), and serum values may be inflated and poorly correlated to synovial fluid concentrations (Frisbie et al., 2008; Catterall et al., 2010; Nicholson et al., 2010). While C1,2C concentrations in LFF tended to higher in SIL, changes from d 0 were not different between the groups. Rather than as a result of supplementation, the higher concentrations likely resulted from SIL horses having higher C1,2C concentrations by chance. Additionally, C1,2C concentrations were similar among metacarpophalangeal and carpal joints with values similar to previous reports in both joints without OA (Frisbie et al., 2008; Nicholson et al., 2010). Values for CPII are similar to previous studies ranging from around 1,000 ng/ml (Donabedian et al., 2008; Nicholson et al., 2010) to over 3,000 ng/ml (Frisbie et al., 2008) in normal and OA-affected joints. Concentrations increase in OA-affected joints and in response to exercise (Frisbie et al., 2008; Nicholson et al., 2010). As horses can have lower concentrations of CPII in metacarpophalangeal joints as compared to carpal joints (Nicholson et al., 2010), LFF concentrations remained relatively unchanged and similar to concentrations in non-OA joints (Donabedian et al., 2008; Frisbie et al., 2008; Nicholson et al., 2010). With consistent exercise, CPII concentrations can increase (Frisbie et al., 2008), but a bout of high-intensity exercise changes degradation markers in synovial fluid very little in the 24-h post-exercise (Macnicol et al., 2020). As noted above with OC, activity may have increased during certain weeks, but this activity may not have been consistent enough to influence CPII concentrations. Overall, activity was likely low, leading to the decrease in CPII seen in the current study. As concentrations in both collagen markers were similar to previous studies and no differences between treatments were observed after d 0, Si supplementation did not increase collagen degradation or synthesis within joints.

Although supplemented horses received nearly 54 mg Si/d based on the manufacturer's recommendations, this amount falls short of proposed beneficial threshold in humans on a kg BW basis. In humans, research suggests 0.3 mg Si/(kg BW·d) as the minimum level to have positive influences on bone (Nielsen, 2014). In rats, favorable effects on bone are seen at a much higher supplementation rate of 54 to 94 mg and 37 to 60 mg Si/(kg BW·d) for females and males, respectively (Jugdaohsingh et al., 2015b). In broilers, differences in bone strength and ash among controls and varying Si concentrations are achieved beginning at 14 mg Si/(kg BW·d) (Scholey et al., 2018). Mean BW in the current study was around 540 kg, meaning SIL received 0.1 mg Si/(kg BW·d), so the amount of Si in the current study falls below the minimum level recommended for humans and well below the experimental amounts given to rats. To achieve the proposed human minimum level, horses in the current study would need to receive 162 mg Si/d or around 5 g of supplement, and to match minimum experimental amounts, nearly 7.5 g Si/d or 231 g of supplement would need to be fed. The supplement would then equal nearly 1 to 46% of the textured feed given. These amounts, especially the latter, may present palatability issues, and many of the experimental amounts are well above the threshold for humans previously suggested by Nielsen (2014). Another study in broilers from an early age using between 0.005 and 0.015 mg Si/(kg BW·d) (Sgavioli et al., 2016) found changes in the mineral profile of bones with supplementation but no difference in bone density or strength. However, these birds started receiving supplementation at an earlier stage of development compared to the horses in the current study, indicating that silicon supplementation may be more influential during growth. Implementing high experimental amounts that generate results in adult small animals may be difficult to achieve and unfeasible in large animals, and age at start of supplementation may play a role in bone response.

CONCLUSIONS

These results indicate that Si supplementation in older horses may not be as beneficial as in younger animals and that it may be difficult to achieve beneficial experimental amounts in large animals due to volume of Si supplement needed to be fed. In previous studies, Si supplementation showed potential to improve bone and cartilage health in many species, including horses and humans, but positive effects from Si were only achieved at high concentrations in the diet, making them difficult to replicate as animals get larger. Silicon can positively influence bone density, structure, and strength, as well as collagen metabolism in connective tissues, indicating the possibility to affect degraded cartilage and arthritis in older animals. Since arthritis is a prominent cause of lameness in the equine industry, supplementing Si would be an easy way to address this issue. However, Si supplementation did not improve lameness as compared to controls. Additionally, supplemented horses did not show increases in collagen degradation and synthesis markers in synovial fluid than controls, and concentrations of these markers did not change from baseline, indicating that cartilage turnover remained unaffected. While promising in bone and cartilage development in young animals, it may be too late for Si supplementation to affect skeletal health in mature animals and require too much supplementation for large animals to benefit.

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CHAPTER 4: Silicon supplementation affects mineral metabolism, footpad dermatitis, and hock burn but not bone density or strength in male broilers

INTRODUCTION

Nearly 20 million metric tons of broiler meat per year are produced by the United States alone (Food and Agriculture Organization of the United Nations, 2018). Due to genetic selection, commercial broiler chickens have rapid growth rates and high feed efficiency. As producers seek to increase production while reducing costs, birds will grow from about 40 g a day after hatching to around 3 kg in 42 days. This fast growth generates problems, ranging from pulmonary hypertension and sudden death syndrome (Julian, 1998) to leg deformities and fractures (Knowles et al., 2008). Because these birds have been selected for muscle gain and size, this selection creates a high muscle to bone ratio which puts additional strain on their legs. The fast growth also negatively impacts bone quality by increasing bone porosity (Williams et al., 2004) and decreasing ash and breaking strength per kg bodyweight (Williams et al., 2004; Shim et al., 2012). In addition to injuries and deformities, hock burns, keel blisters, and footpad dermatitis can cause additional pain, thereby reducing weight gain and efficiency, and may affect nearly 50% of birds in commercial operations (Opengart et al., 2018; Hartcher and Lum, 2019).

Because leg injuries produce welfare concerns and impact production (Bessei, 2006; Averos and Estevez, 2018), numerous interventions have been suggested as potential solutions, many of which have aimed at improving bone mineral density (BMD). Because bone typically responds to loading (Rubin and Lanyon, 1984), attempts at encouraging movement have been employed such as: adding perches (Tablante et al., 2003), reducing stocking density (Buijs et al., 2012), and increasing the photoperiod (Lewis et al., 2009). These methods have not convincingly improved leg health (Riber et al., 2018; Pedersen et al., 2020) and have not been implemented in commercial systems. Frequently, changes to nutrition have been suggested, as feed is easier to

manipulate than changes to the environment. Reducing metabolizable energy (Venäläinen et al., 2006) or limiting overall feed intake (Williams et al., 2004) can improve bone quality by slowing growth rate, but producers do not want slowed growth. Varying concentrations and sources of calcium and other minerals (Venäläinen et al., 2006; Bradbury et al., 2017; Güz et al., 2019) have positively influenced bone mineral content (BMC), BMD, and breaking strength. By manipulating these minerals, growth rate remains the same, but potential leg problems may be averted.

One mineral which may affect bone quality is silicon. Older studies showed that Si deficiency in chicks and rats produces more porous, less mineralized bone (Schwarz and Milne, 1972; Carlisle, 1980b; Elliot and Edwards, 1991). Silicon intake in humans has been linked to greater BMD (Kim et al., 2009; Jugdaohsingh et al., 2015b; Arora and Arora, 2017), and in other species, Si can increase bone formation markers such as osteocalcin (Jugdaohsingh et al., 2015b) and reduce bone resorption markers (Kim et al., 2009), including pyridinoline (Lang et al., 2001). In broilers, the inclusion of high levels of supplementary Si has improved tibial ash content and breaking strength (Sgavioli et al., 2016; Scholey et al., 2018). Since Si stimulates collagen synthesis, it may also impact meat quality, but feeding rice hulls, high in Si, at levels up to 7.5 mg/kg feed did not increase thawing losses or shear force on thigh or breast meat in broilers (Nakhon et al., 2019). Silicon also may also reduce the instance of footpad, hock, and keel lesions as it has been associated with increased hydroxyproline content as a measure of collagen in calves' skin (Calomme and Vanden Berghe, 1997). To build upon these previous studies, the objective of this study was to determine if supplementing bioavailable Si at two different concentrations could affect bone morphology, mineralization, and strength in a dose-dependent manner without negatively influencing welfare and meat quality. This study tested the

hypothesis that increasing Si concentrations would improve bone health measures as compared to controls and that these effects would be most prominent at the highest supplement concentration.

MATERIALS AND METHODS

Birds and Management

All procedures were approved by the Michigan State University Animal Care and Use Committee (IACUC #: PROTO201800040). Male broilers (n=375; Ross 708, Aviagen, Huntsville, AL) were raised from d 1 after hatching. Initially, broilers were divided into six 1.8 x 1.2 m pens of either 62 or 63 chicks per pen for brooding, but after the first week, chicks were divided randomly into 15 pens at 25 birds per pen. The photoperiod was stepped down from 24 h to 20 h over the course of the first 7 d on study and maintained at 20 h for the remainder of the study in accordance with the Ross Broiler Management Handbook (Ross, 2018). Chicks had ad libitum access to water and a commercially-available starter-grower feed (Kent Nutrition Inc., Muscatine, IA) at all ages. Water was supplemented with a bioavailable Si source based on randomly assigned treatment groups: Control (C; n=125), Normal (N; n=125) and High (H; n=125). The dosage for the N treatment group represented the therapeutic dosage recommended by the manufacturer (Siliforce, Agro-Solutions, Nuth, Netherlands) for horses (0.011 ml supplement/kg bodyweight) while the H treatment group (0.063 ml supplement/kg bodyweight). received the dosage tested in a previous equine mineral balance study (O'Connor et al., 2008). Water consumption with 15% wastage was calculated based upon age and projected weight according to estimates from previous broiler flocks (Williams et al., 2013), and Si was added to the waters of the N and H treatment groups using an appropriately-sized syringe to ensure proper dosage.

Pen Weights and Mortality

Birds were weighed twice weekly for dose adjustments. Five birds from each pen were randomly selected and weighed once weekly, and this weight was averaged per bird for pen dosing. Full pen weights were taken on a separate day once weekly to ensure accuracy. Mortality data included date, pen, and weight.

Feed and Water Consumption

Feed was weighed in bag every time before being added to the storage containers, (the empty bag weight was then subtracted). Containers were also weighed when empty and on a weekly basis to monitor consumption. At the start of the study, empty carboys with water lines and nipple drinkers were weighed and assigned to each pen. Water was measured in a graduated cylinder before being poured into the carboy. To measure consumption throughout, all water was drained out of the nipple drinkers and water lines back into the carboys, and carboys, lines, and drinkers were placed on scales and weights recorded. Water was then added while the carboys were still on the scales, and amount added was recorded every three days or as needed.

Welfare Scores

Toe damage, footpad dermatitis, hock burn, and keel blisters were assessed on d 42. Footpad dermatitis and hock burn were scored according to Bilgili et al. (2006) where 0 indicated no lesions, 1 indicated mild irritation, and 2 indicated severe irritation. Toe damage included broken toes or nails was scored similarly to keel blisters as present (1) or not present (0).

Blood Samples

Blood samples were collected from wing veins into vacutainer serum separation tubes (BD Vacutainer™: SST™, Becton, Dickinson and Company, Franklin Lakes, NJ) on d 42 for

serum osteocalcin (OC) and pyridinoline cross-links (PYD) analysis. Serum samples were centrifuged and placed into microcentrifuge tubes before freezing at -4° C. Serum samples were analyzed using OC (Microvue™ Osteocalcin EIA, Quidel, San Diego, CA) and PYD (Microvue™ Serum PYD EIA, Quidel, San Diego, CA) enzyme-linked immunosorbent assay. Silicon, boron (B), and calcium (Ca) in plasma as well as Si concentrations in the supplement were determined by inductively coupled plasma mass spectrometry (Agilent 7900 Inductively Coupled Plasma Mass Spectrometer, Agilent, Santa Clara, CA) by a certified laboratory (Michigan State University Veterinary Diagnostic Laboratory, Lansing, MI).

Computed Tomography Scans

Clinical QCT scans and analysis were conducted according to Robison and Karcher (Robison and Karcher, 2019) with the settings of 120 kV, 320 mAmp, and 0.625 mm slices. Briefly, right leg quarters were thawed in a chiller for 24 h prior to scanning. Legs were scanned with muscle and feathers intact and arranged in rows on plexiglass, and each scan included a solid Ca hydroxyapatite phantom (Image Analysis; Columbia, KY) of 0, 75, and 150 mg/cm^3 Ca. A DICOM of each row of leg quarters with an 11-cm field of view and bone algorithm was generated using Imageworks (General Electric Healthcare; Princeton, NJ) and imported for analysis into Mimics (Materialise; Plymouth, MI). The threshold for Hounsfield units (HU) was determined by applying a range from 200 to 600 HU with differences among thresholds of 25 HU based on thresholds used in the Robison and Karcher study (2019). Appropriate thresholds were set to 275 HU and 225 HU for the tibia and femur, respectively. After applying thresholds, whole bone volume and average HU were recorded for QCT BMC calculations. To determine bone density, average HU for each step of the Ca hydroxyapatite phantom was plotted against the known densities of the phantom to generate a standard curve. The following regression

equation generated was used to calculate density in mg Ca hydroxyapatite/cm^3: $y = 0.7589x - 4.646$, $R^2 = 0.99$ where y is density in mg Ca hydroxyapatite/cm^3 and x is HU. To calculate QCT BMC from CT scans, bone volume was multiplied by the density generated from the regression equation. Diameter, cross sectional area, and cortical area were measured.

Bone Breaking

After CT, four-point bending tests of tibias were conducted on a universal testing machine (Model 4202, Instron Corp., Canton, MA) according to ASABE Standards (ANSI/ASAE S459, 2017). Tibias were placed with the palmar cortex under tension with a span between outer posts of 7 cm (outer span) and a span from inner post to outer post of 2 cm (inner span). A 10 kN load cell and a speed of 20 mm/min was used. One tibia was excluded from testing as it had been broken at CT. Fracture force, Young's modulus, moment of inertia, and flexural rigidity of the tibias were determined. After breaking, caliper measurements were taken for bone length, fracture length, and distance of fracture from the proximal and distal end of the tibia. The proximal and distal locations of the fracture was defined as fracture start and fracture end, respectively. These points were used to calculate densities and other morphological data. Due to morphological changes within an individual tibia, moment of inertia and flexural rigidity were calculated at fracture start and fracture end.

Moment of inertia (I, mm^4) was calculated using the following equation (ANSI/ASAE S459, 2017):

$$I = 0.049[(B \cdot D^3) - (b \cdot d^3)]$$

Where B is the outer lateromedial diameter, D is the outer dorsopalmar diameter, b is the lateromedial medullary diameter, and d is the dorsopalmar meduallary diameter. Young's modulus of elasticity (E, N/mm^2) was calculated as:

$$E = \left(\frac{F}{V}\right)\left(\frac{a^2}{12I}\right)(3L - 4a)$$

Where $\left(\frac{F}{V}\right)$ is the slope of the force deformation curve from 1 to 2.5 mm extension, *a* is the inner span, and *L* is the outer span (Turner and Burr, 1993). Flexural rigidity (EI, N·mm^2) was calculated as the product of the moment of inertia and Young's modulus.

Bone Ash

Left leg quarters were autoclaved (733HCMC; Gentige, Wayne, NJ) at 121º C for 25 min in a method described by Cloft et al. (2018). After autoclaving, tissue and skin were removed, and tibias and femurs were separated (n=50/bone/treatment). Each bone was cut into thirds, wrapped in cheesecloth, and placed into a modified soxhlet for ether-extraction for 12 to 24 h, after which they were dried at ambient temperature in a hood for 24 h and weighed. After ether extraction, bones were placed into crucibles and further dried in a DN-81 constant-temperature oven (American Scientific, Portland, OR) at 105° C for 24 h. Dry bone weights were obtained after this period, and crucibles containing bones were placed in an ash oven (Thermolyne 30400, Barnstead International, Dubuque, IA) overnight at 600° C. Ash was allowed to cool and weighed.

Mineral Analysis

Bone ash was ground and microwave digested (MARS5, CEM Corp., Matthews, NC) in duplicate with nitric acid (70% trace-metal grade; Fisher Scientific, Pittsburgh, PA) as described by Shaw et al. (2002). Twenty-four digests were randomly selected for analytical Ca, and Ca concentrations were determined by inductively coupled plasma mass spectrometry (7900 Inductively Coupled Plasma Mass Spectrometer, Agilent, Santa Clara, CA) by a certified laboratory (Michigan State University Veterinary Diagnostic Laboratory, Lansing, MI). Due to the large amount of Ca in bone, digests were diluted 100x to ensure readings were within range

of the standard curve. The same inorganic standard was used for all samples, and digested peach leaves (Standard Reference Material® 1547 Peach Leaves, National Institute of Standards and Technology, Gaithersburg, MD) served as controls. Total mineral amount within ash was determined by multiplying the concentration in mg/g by total amount of ash digested.

Texture Analysis

On d 42, whole keels were collected, tagged, and frozen at -20° C until analysis. For each day of analysis, keels were thawed for at least 24 h, and the right breast fillet (*Pectoralis major*) was cut from each keel and repacked in a plastic cooking bag. Fillets were cooked in batches, and a temperature probe was placed with the heaviest fillet to ensure that all fillets were cooked to an internal temperature of 71° C. Bags were weighed before and after cooking to determine cooking losses. After cooling, four 1-cm diameter cores were drilled from each fillet for shear force measurements. Each core was sheared perpendicular to muscle fiber direction by a Warner-Bratzler shear blade attachment at a test speed of 4 mm/sec on a TA-XT Plus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY). Firmness and toughness were calculated from shear force using a macro within the texture analyzer software (Exponent Connect, Stable Micro Systems, Surrey, United Kingdom).

Statistics

Residuals from continuous data were tested for normality and log-transformed as necessary for analysis. Pen weights, as well as water and feed consumption, were analyzed using a mixed model procedure (PROC MIXED) in SAS 9.4 (SAS Institute, Cary, NC) with fixed effects of treatment and date, repeated effect of date, and random effect of pen. Welfare scores were analyzed using the generalized linear mixed model procedure (PROC GLIMMIX) with the fixed effect of treatment and distribution set as either binary for toe damage and keel blisters or

multinomial for footpad dermatitis and hock burn. The remainder of the measurements were analyzed using PROC MIXED with the fixed effect of treatment and random effect of pen. Significance was set at $P < 0.05$, and trends were considered at $0.05 \leq P < 0.10$. For significant effects, Tukey's *post hoc* analysis was used for multiple comparisons.

RESULTS

Pen Weights, Mortality, and Feed and Water Consumption

At placement, all groups weighed similarly, and growth rates were similar among groups (Figure 4.1). On d 42, all groups weighed similarly. Mortality was unaffected by group, and mean mortality was 12.5 ± 0.02%. Silicon supplementation tended to increase daily water consumption in N and H as compared to C (Figure 4.2; $P = 0.07$). Overall feed consumption was similar among groups (Figure 4.3; $P > 0.10$), and both feed and water consumption increased over time ($P < 0.001$ for both). Silicon intake from feed was 2.5 mg Si/kg, and the supplement provided 8,988 mg Si/l. Treatment did not affect feed conversion (C: 0.75 ± 0.03, N: 0.71 ± 0.03, H: 0.77 ± 0.03).

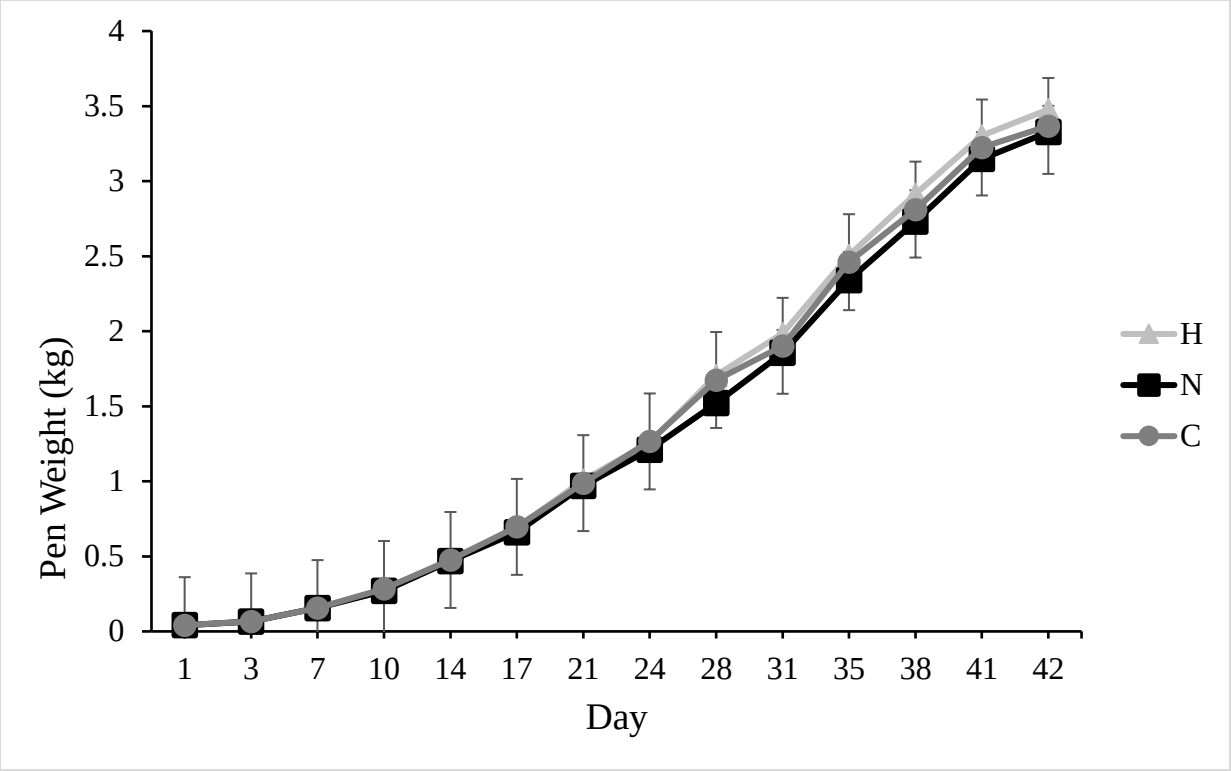

Figure 4.1. Mean bodyweight (± SE) of male broilers from d 1 after hatching to 42-d of age receiving from pens either no silicon supplementation (Control, C, n=5), normal supplementation (0.011 ml supplement/kg BW, Normal, N, n=5), or high supplementation (0.063 ml supplement/kg BW, High, H, n=5). Weights were taken as pen weights and averaged over number of birds weighed.

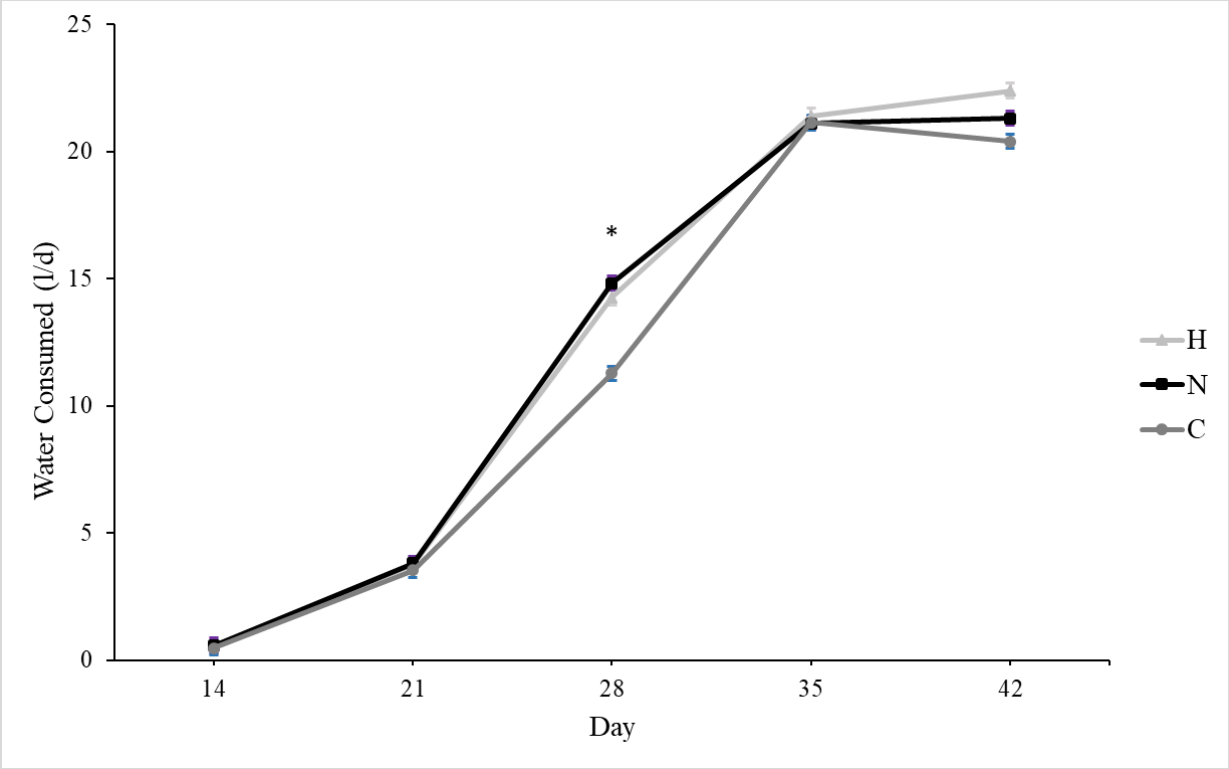

Figure 4.2. Mean water consumption (± SE) of broiler pens receiving either no silicon supplementation (Control, C, n=5), normal supplementation (0.011 ml supplement/kg BW, Normal, N, n=5), or high supplementation (0.063 ml supplement/kg BW, High, H, n=5). Supplementation tended to increase water consumption ($P = 0.07$). *Indicates difference between treatment groups (N and H) and control (C, $P < 0.005$).

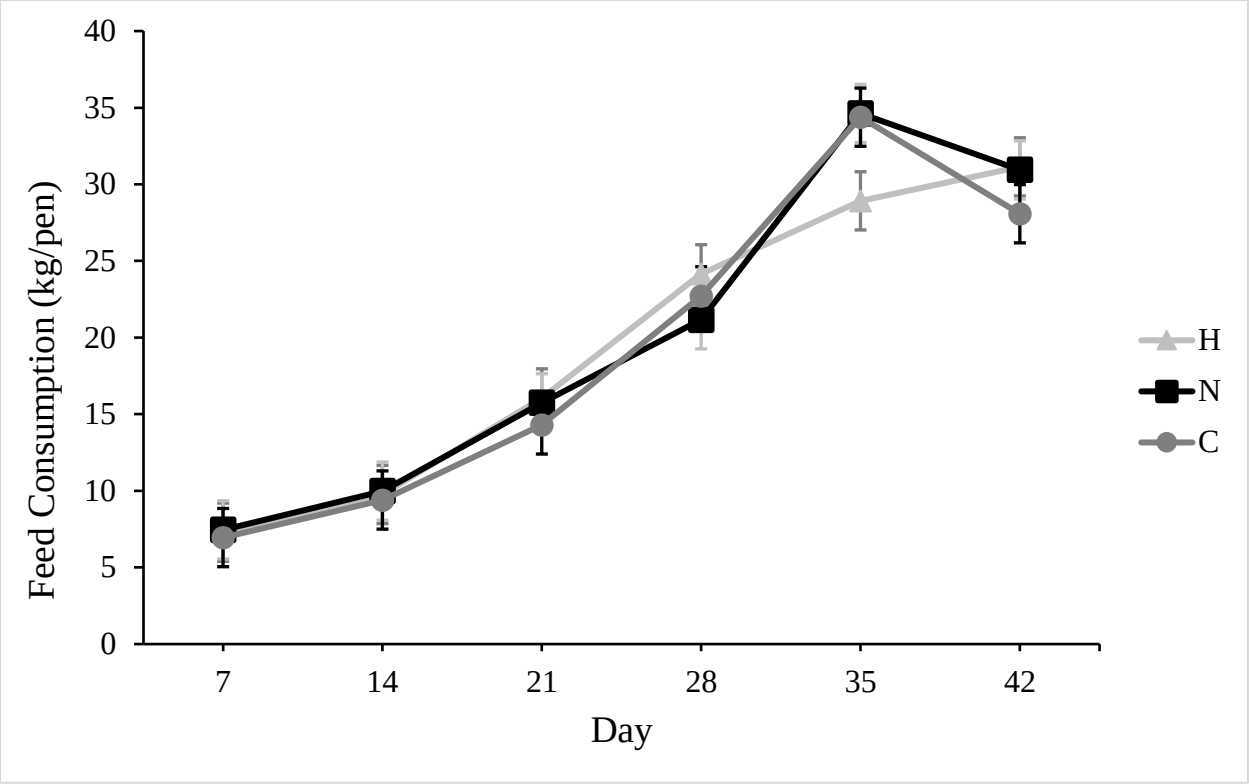

Figure 4.3. Mean weekly feed consumption (± SE) over six weeks of broiler pens receiving either no silicon supplementation (Control, C, n=5), normal supplementation (0.011 ml supplement/kg BW, Normal, N, n=5), or high supplementation (0.063 ml supplement /kg BW, High, H, n=5).

Welfare Scores

Groups scored similarly for toe damage and keel blisters. Footpad dermatitis and hock burn varied by treatment ($P = 0.002$ and $P = 0.007$, respectively; Figure 4.4). High birds scored lower in both measures than in N ($P < 0.05$ for both) or C ($P < 0.05$ for both).

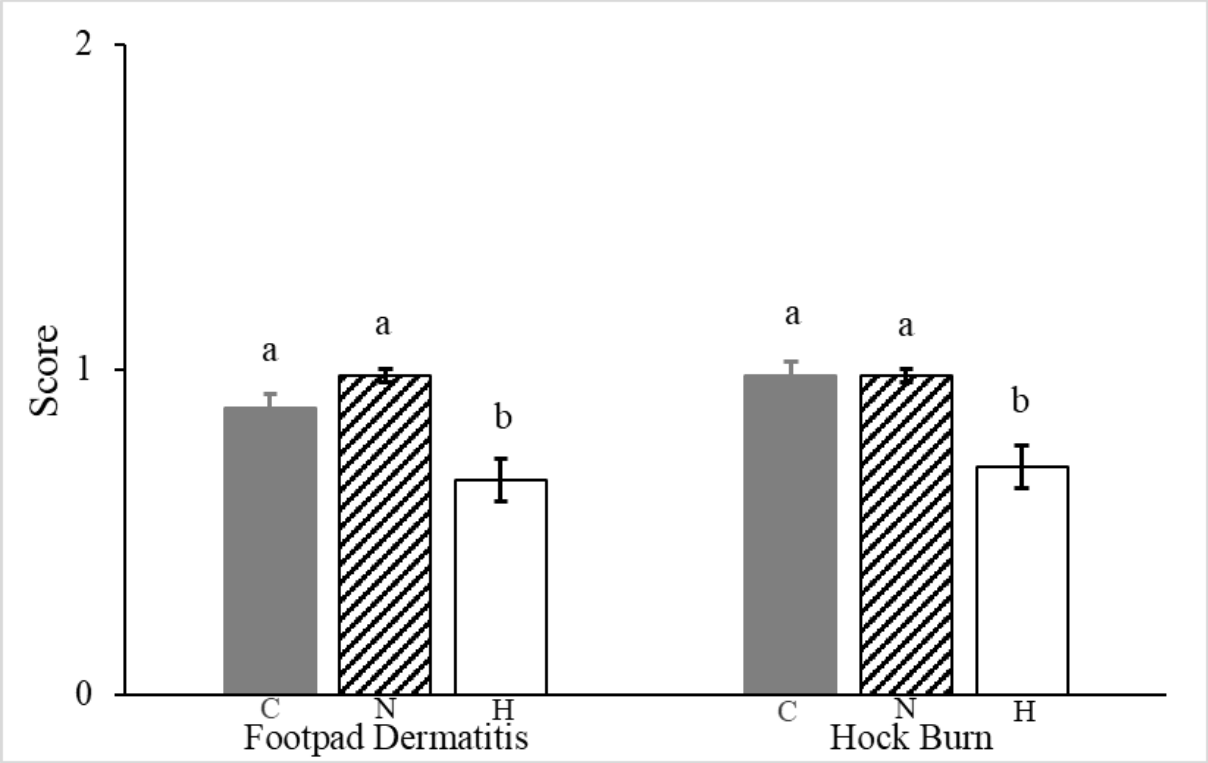

Figure 4.4. Mean welfare scores (±SE) for foot condition, footpad dermatitis, hock burn, and keel blisters on male broilers (10 birds/pen) from pens receiving no supplementation (Control, C, n=5), normal silicon supplementation (0.011 ml supplement/kg BW, Normal, N, n=5), or high silicon supplementation (0.063 ml supplement/kg BW, High, H, n=5) using typical industry scoring systems taken at 42 d. [ab]Bars lacking a common superscript differ ($P < 0.05$).

Bone metabolism markers and serum minerals

Treatment did not affect OC (C: 203 ± 8 µg/ml; N: 196 ± 9 µg/ml; H: 192 ± 8 µg/ml) nor PYD (C: 12 ± 1 nmol/l; N: 11 ± 1 nmol/l; H: 12 ± 1 nmol/l). Serum Si did not differ among C (1.25 ± 0.02 µg/ml), N (1.20 ± 0.02 µg/ml), or H (1.21 ± 0.02 µg/ml), but supplementation reduced serum B ($P < 0.001$) and increased serum Ca ($P < 0.001$). For serum B, N (0.24 ± 0.01 µg/ml) was lower than H (0.28 ± 0.01 µg/ml; $P < 0.001$), and C was higher than either supplemented group (0.32 ± 0.01 µg/ml; $P < 0.001$ for both comparisons). For serum Ca, C and N were similar (98 ± 2 µg/ml and 100 ± 2 µg/ml, respectively), but both were lower than H (106 ± 2 µg/ml; $P < 0.01$ for both comparisons).

Bone measures

Neither total ash nor ash percentage in the femur and tibia were affected by treatment. Calcium concentrations in bone ash did not differ by treatment, but total Ca was lower in N (1,255 ± 43 mg) than in C (1,409 ± 34 mg, $P = 0.02$) and tended to be lower than in H (1,380 ± 34 mg, $P = 0.07$). Fracture start and end inner and outer diameters, cortical area, cross sectional area were similar among treatments (Table 4.1). Fracture start slice density was greater in C and H than in N ($P = 0.03$, Table 4.2), but fracture end slice density was similar across groups. At fracture start, C had greater dorsal density than N ($P = 0.04$), but H was similar to both groups, and there were no differences among groups in any other cortex. Fracture end lateral cortical density tended to be highest in C than in N and H ($P = 0.07$), but treatment did not affect any other cortex at fracture end. Flexural rigidity tended to be greater in H ($P = 0.07$) when compared to C and tended to be similar to N ($P = 0.09$); no other breaking variable was different among groups (Table 4.3).

	Location of Measurement	Group Control	Normal	High
Bone Length (mm)		110.3 ± 0.7	108.3 ± 0.7	109.9 ± 0.7
Dorsopalmar Outer Diameter (mm)	Fracture Start	9.6 ± 0.2	10.3 ± 0.2	9.8 ± 0.2
	Fracture End	9.0 ± 0.1	9.0 ± 0.1	8.9 ± 0.1
Dorsopalmar Medullary Diameter (mm)	Fracture Start	6.0 ± 0.2	6.1 ± 0.2	6.5 ± 0.2
	Fracture End	5.0 ± 0.1	5.0 ± 0.1	5.1 ± 0.1
Lateromedial Outer Diameter (mm)	Fracture Start	11.4 ± 0.2	11.8 ± 0.2	11.5 ± 0.2
	Fracture End	10.4 ± 0.1	10.6 ± 0.1	10.5 ± 0.1
Lateromedial Medullary Diameter (mm)	Fracture Start	7.3 ± 0.3	7.5 ± 0.3	7.4 ± 0.3
	Fracture End	6.1 ± 0.2	6.1 ± 0.2	6.3 ± 0.2
Cortical Area (mm^2)	Fracture Start	52.7 ± 1.5	54.3 ± 1.4	56.1 ± 1.4
	Fracture End	51.0 ± 1.1	52.2 ± 1.1	50.5 ± 1.1
Cross Sectional Area (mm^2)	Fracture Start	89.1 ± 4.0	98.6 ± 4.0	91.1 ± 4.0
	Fracture End	73.9 ± 1.8	74.7 ± 1.8	74.6 ± 1.8

Table 4.1. Right tibiae morphological measures of male broilers (10 birds/pen) from pens receiving either no supplementation (Control, n=5), normal silicon supplementation (Normal, 0.011 ml supplement/kg BW, n=5), or high silicon supplementation (High, 0.063 ml supplement/kg BW, n=5) taken proximally (Fracture Start) and distally (Fracture End) according to fracture location after breaking. Scans were obtained just prior to four-point bending.

	Location of Measurement	Group Control	Normal	High
Slice Density	Fracture Start	549 ± 13[a]	503 ± 13[b]	540 ± 13[ab]
	Fracture End	617 ± 9	601 ± 9	609 ± 9
Lateral Cortical Density	Fracture Start	530 ± 16	499 ± 16	513 ± 16
	Fracture End	635 ± 14	601 ± 14	591 ± 14
Medial Cortical Density	Fracture Start	540 ± 15	494 ± 15	519 ± 15
	Fracture End	611 ± 14	600 ± 14	605 ± 14
Dorsal Cortical Density	Fracture Start	547 ± 15	494 ± 15	529 ± 15
	Fracture End	578 ± 15	553 ± 15	546 ± 15
Palmar Cortical Density	Fracture Start	528 ± 16	483 ± 16	510 ± 16
	Fracture End	603 ± 16	565 ± 15	574 ± 16

Table 4.2. Right tibiae density measures (mg Ca hydroxyapatite/cm^3) of male broilers (10 birds/pen) from pens receiving either no supplementation (Control, n=5), normal silicon supplementation (Normal, 0.011 ml supplement/kg BW, n=5), or high silicon supplementation (High, 0.063 ml supplement/kg BW, n=5) taken proximally (Fracture Start) and distally (Fracture End) according to fracture location after breaking. Scans were obtained just prior to four-point bending.[ab]Values within a row lacking a common superscript differ ($P < 0.05$).

	Location of Measurement	Group Control	Normal	High
Fracture Force, N		448 ± 8	462 ± 8	464 ± 8
Young's Modulus, x 10^7 N/mm^2	Fracture Start	1.4 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
	Fracture End	1.7 ± 0.1	1.8 ± 0.1	1.9 ± 0.1
Moment of inertia, mm^4	Fracture Start	43.4 ± 2.5	45.3 ± 2.5	43.8 ± 2.5
	Fracture End	33.4 ± 1.5	34.8 ± 1.5	33.4 ± 1.5
Flexural Rigidity, x 10^8 $N{\cdot}mm^2$	Fracture Start	5.6 ± 0.2	5.6 ± 0.2	6.3 ± 0.2
	Fracture End	5.6 ± 0.2	5.6 ± 0.2	6.3 ± 0.2

Table 4.3. Right tibiae biomechanical measures of male broilers (10 birds/pen) from pens receiving either no supplementation (Control, n=5), normal silicon supplementation (Normal, 0.011 ml supplement/kg BW, n=5), or high silicon supplementation (High, 0.063 ml supplement/kg BW, n=5) taken proximally (Fracture Start) and distally (Fracture End) according to fracture location after breaking. One Control tibia was excluded from analysis due to breaking during scanning.

Texture and Cooking Losses

Overall, treatment did not affect firmness as C (2,200 ± 163 g force) was similar to N (2,223± 168 g Force) and H (2,327 ± 194 g Force). Toughness was also similar among C, N, and H (8,791 ± 752 g force/sec, 9,097 ± 795 g force/sec, and 9,862 ± 971 g force/sec, respectively).

Cooking losses were similar among groups, and there were no differences for pre- or post-cooking weights.

DISCUSSION

Although Si supplementation seemed promising from previous research, the current study did not show improved bone measures with bioavailable Si supplementation. Texture remained unaffected by silicon supplementation as well. However, high Si supplementation did improve welfare scores that dealt with skin irritation, indicating its importance in predominantly collagen-based tissues.

Water consumption tended to be higher in supplemented groups as compared to controls, despite similar feed consumption among groups. The supplement provided in their water was stabilized using propylene glycol, which has a faintly sweet taste. In an early experiment, the addition of sucrose to water increased consumption (Jacobs and Scott, 1957), but a later experiment found that sucrose concentrations ≥ 1 M produced an aversion response and decreased water consumption (Cheled-Shoval et al., 2017). It is likely that the supplement added a slightly sweet taste to the water, enticing the birds to drink more or more often than their unsupplemented counterparts.

Supplementing Si at a high concentration appeared to influence the formation of footpad dermatitis and hock burn. In commercial operations, over 45% of birds may score a 1 or above (Opengart et al., 2018) on the same scale used in the current study (Bilgili et al., 2006). Since footpad dermatitis can affect weight gain, welfare, and paw quality (Shepherd and Fairchild, 2010), bioavailable Si supplementation may reduce the incidence or severity of these lesions. Silicon can stimulate type 1 collagen synthesis (Reffitt et al., 2003; Dong et al., 2016), affecting collagen quality in the skin and other soft tissues (Calomme and Vanden Berghe, 1997;

Jugdaohsingh et al., 2015c). Calomme and Vanden Berghe (1997) found higher hydroxyproline content in the dermis of calves supplemented orthosilicic acid, indicating a greater amount of collagen and stability. Since the broilers were supplemented during growth, the high Si concentration likely improved or increased the collagen in the footpads and legs of these birds, decreasing the incidence or severity of potential skin irritation.

While orthosilicic acid supplementation can increase osteoblast differentiation and osteocalcin concentration *in vitro* (Reffitt et al., 2003), the current study found no difference in osteocalcin concentrations in supplemented broilers as compared to controls, either due to the supplemental concentrations or differences in responses to Si between sexes. High levels of Si may also induce premature apoptosis in osteoblast-like cells *in vitro* (Shie et al., 2011), ultimately reducing activity and indicating a beneficial range that the current study exceeded. However, this effect shown in Shie et al. (2011) may have more to do with hyperosmolality of culture medium than direct effects of Si. There is some evidence that Si supplementation may be more beneficial for females than males. Most studies examining ovariectomized animals have found positive effects of Si (Nielsen and Poellot, 2004; Kim et al., 2009), while studies using exclusively male subjects have reported no differences in bone with Si supplementation (Kayongo-Male and Julson, 2008; Kim et al., 2014; Sgavioli et al., 2016). In male rats, a strong negative correlation between serum osteocalcin and increasing doses of Si can occur without similar effects in females despite similar osteocalcin concentrations (Jugdaohsingh et al., 2015b). Likely, this sex effect contributed to the similar concentrations among groups in the current study. Using only male broilers may have limited the results of the current study, but male broilers are more common in industry due to their larger size.

Although serum Si was unchanged by treatment, supplementation lowered B concentrations in both treated groups and raised Ca in H. In rats and calves, Si intake has been associated with increased serum Ca (Najda et al., 1993; Calomme and Vanden Berghe, 1997) but not in horses (O'Connor et al., 2008) or turkeys (Kayongo-Male and Julson, 2008). Higher concentrations of serum Ca may indicate greater availability for deposition into bone, but Ca concentrations within bone did not differ among groups in the current study. These results support previous findings on the effects of Si supplementation in Ca and Mg metabolism as greater serum Ca concentrations in the treated group did not lead to higher tissue concentrations (Najda et al., 1993). Though one study showed Si supplementation and higher serum Si affected femoral and vertebral mineral composition in male rats (Seaborn and Nielsen, 2002), other studies, including the current one, have not shown similar increases in bone minerals in males (Kayongo-Male and Julson, 2008; Jugdaohsingh et al., 2015b; Sgavioli et al., 2016). Since Si supplementation can decrease Mg retention without affecting Ca metabolism (Kim et al., 2014) and reduce Al absorption (Pérez-Granados et al., 2002), Si may interfere with the absorption of other trace elements, such as B, causing lower serum concentrations. The exact route of gastrointestinal Si uptake is unknown (Jugdaohsingh, 2007; Arora and Arora, 2017), though Si source matters for absorption and retention (O'Connor et al., 2008; Sripanyakorn et al., 2009). As indicated by the results of the current study and previous research, gastrointestinal Si uptake and source may interfere with the absorption and metabolism of other minerals which in turn may influence Si effects on other tissues.

Fracture force at breaking was similar across treatments, mirroring previous findings in turkeys and rats (Kayongo-Male and Julson, 2008), as well as water Si supplementation to broilers (Sgavioli et al., 2016). However, these findings contradicted other studies showing

greater breaking strength in Si-supplemented animals (Maehira et al., 2009; Scholey et al., 2018; Nakhon et al., 2019). This disparity was likely due to differences between four-point and three-point bending in bone mechanical testing (Sharir et al., 2008) as three-point bending generates high shear stress and four-point bending ensures almost no shear stress where loading is applied (Turner and Burr, 1993). Because four-point bending generates no shear stress, it is preferential for measuring mechanical properties.

In all the studies supplementing Si to broilers, the amounts used in the current study, 0.35 mg Si/kg BW for N and 1.98 mg Si/kg BW for H, most closely match those in Sgavioli et al. (2016). The previous study found differences in phosphorus, zinc, and manganese concentrations of tibiae above non-supplemented birds beginning at 0.35 mg Si/kg BW but no differences in strength or density even at the highest level of supplementation, 0.53 mg Si/kg BW. Other studies used much higher rates of supplementation. In Scholey et al. (2018), bone strength was improved in birds supplemented approximately 45 mg Si/kg BW, and tibia ash was increased in birds receiving a minimum 7.7 mg Si/kg BW. Nakhon et al. (2019) showed increased tibia breaking strength in birds supplemented 12.7 mg Si/kg BW but not in birds supplemented 17.8 mg Si/kg BW. Each of these studies used different rates and methods of supplementation and reported Si either as percent of supplement or mg/kg included in the diet, making standardization difficult. However, these amounts demonstrate that very high levels of supplementation are needed for bone measures to improve above adequate control diets and that better standardization would help interpret results of studies on the benefit of Si to bone.

CONCLUSIONS

Though deficiency will negatively affect bone, the results of this study suggest that the Si supplementation provided does not improve bone quality in male broilers. Even when

supplemented at high concentrations in a bioavailable form, Si did not improve bone density, morphology, or breaking strength above controls. Factors such as growth rate and mechanical loading likely play a greater role in developing bone quality than trying to supplement on top of good basic nutrition alone. However, the lower footpad dermatitis and hock burn scores in the high supplementation group support previous findings that Si stimulates type I collagen synthesis and helps to stabilize this molecule. This particular role may be more important for connective tissue rather than bone, and future research should focus on this aspect of Si supplementation to improve lameness in broilers.

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CHAPTER 5: Conclusions

The skeleton provides a complex system that is uniquely important for a wide range of functions. Its proper development and continued quality are influenced by multiple factors, including mineral nutrition. As Ca and Si deficiencies prove detrimental to growing bone and cartilage, altering the balance of these minerals may impact both the mature and immature skeletal system.

By doubling urinary Ca and urine output, one dose of furosemide creates a temporary negative Ca balance, but values return to baseline quickly. Evidence for increased intestinal Ca absorption following this dose comes from a decrease in fecal Ca concentrations. This potential mechanism to restore Ca balance in three days indicates that there likely is not a long-term Ca deficit. To determine mechanisms to restore Ca homeostasis, future studies should consider examining changes to parathyroid hormone and vitamin D as these two hormones are likely the cause for this change in fecal Ca concentrations. Additionally, studies should focus on changes to Ca balance, urine output, and plasma volume after frequent, repeated bouts of administration to determine if furosemide maintains its efficacy. Reductions in plasma volume to reduce pressure within the lungs is how furosemide supposedly works to reduce the incidence or severity of EIPH; if plasma volume is not reduced after several administrations, whether weekly, biweekly, or monthly, then it likely is not an effective long-term prophylactic for EIPH.

Silicon supplementation likely does not benefit older animals or cartilage quality, and current beneficial recommendations may be too low or difficult to scale up for large animals. Supplemented mature horses did not show a change in collagen metabolism in synovial fluid, indicating that cartilage turnover remained unaffected, nor did lameness improve. Previous experiments that have achieved improvements in bone or cartilage quality in adult animals fed Si

in large amounts which, when expressed on a per kg BW basis, are difficult to translate into large animals. Studies should determine a minimum beneficial threshold for Si supplementation as most current studies vary widely in amount given. Additionally, these studies should express these amounts on a mg Si per kg BW basis rather than raw intakes or amount in diet so that these amounts are standardized and easier to determine if these amounts can be realistically achieved in larger animals.

Providing Si on top of an already adequate diet likely does not influence better bone development. In growing male broilers, Si supplementation did not improve overall bone quality. However, supplementation may benefit tissues wither greater collagen turnover (such as skin), supporting previous findings of greater collagen content and synthesis in the skin and connective tissues of supplemented animals. Moving forward, sex should be considered in experimental design as results from the current study and others indicate supplementation may benefit females more than males. Additionally, analyzing Si's effects and concentrations in different connective tissues may provide more benefit than supplementing for bone alone. Future studies should focus on the types of collagen Si most strongly associates with and stabilizes, as this finding may provide some insight into Si's exact function in this molecule.

Both Ca and Si play important roles in developing bone health. However, once grown, small changes to Ca balance are quickly rectified so bone health is not compromised. Additionally, supplementing Si on top of already adequate diets does not have as profound of an effect as it did in early studies with deficiency. Likely, bone is relatively hard to manipulate through nutrition alone. Growth rate and mechanical loading likely play a larger role in developing bone and cartilage quality than trying to supplement on top of good basic nutrition.