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# COMPARISON OF BONE GROWTH IN STALL- VERSUS PASTURE-REARED HORSES

By

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ABSTRACT

COMPARISON OF BONE GROWTH IN STALL- VERSUS PASTURE-REARED HORSES

By

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Sixteen Arabian yearlings were randomly assigned to two experimental groups, stalled and pastured, to investigate the effects of stalling versus pasture-rearing on bone growth over a 140-day period. Following an 84-d pre-training period, six horses from each group were randomly chosen to complete a 56-d training period. Serum osteocalcin, Ca, P, 25-hydroxyvitamin D, and parathyroid hormone concentrations were determined from blood samples taken every 14 d. Urinary deoxypyridinoline, pyridinoline, Ca, and P concentrations and mineral content of the third metacarpal, as determined by radiographic bone aluminum equivalencies (RBAE), were determined every 28 d from 24-hr urine collections and radiographs of each horse's left front leg, respectively. Lateral RBAE was lower in the stalled horses at d 28 and remained lower throughout most of the project, while pastured horses had increasing lateral RBAE. Horses kept in stalls had lower RBAE of the medial cortex at d 28. Medial RBAE tended to remain lower in stalled horses than in pastured horses throughout most of the project. Serum osteocalcin concentrations were lower and urinary deoxypyridinoline concentrations were higher in the stalled horses at d 14 and d 28, respectively, compared with the pastured horses, and subsequently returned to baseline. Results suggest that housing yearling/two-yr-old horses in stalls may negatively affect normal bone growth experienced by horses maintained on pasture.

Dedicated to my mother, father, and sister.

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# INTRODUCTION

A major concern with young performance horses is the high incidence of skeletal injury. The practice of transferring young, growing horses from pasture to stalls prior to yearling sales or commencement of training may predispose them to injury. Previous research has demonstrated a decrease in bone mineral content of the third metacarpal in young horses soon after the onset of race training, as well as a change in housing from pasture to stalls (Nielsen et al., 1997). The decrease in bone mineral content could result from increased or decreased strain rates on the bone associated with training or the change in housing. Transferring young horses from pasture to stalls results in a slowdown in the rate of bone formation due to a decrease in physical activity (Maenpaa et al., 1988). Studies of other species demonstrated similar decreases in bone strength in response to confinement rearing (Knowles and Broom, 1990; Marchant and Broom, 1996). Thus, yearling horses maintained on pasture with free access to exercise may have a skeletal structure that is better prepared for the increased biomechanical forces that occur during training and competition than horses housed in stalls with limited access to exercise.

The objectives of this study were to determine if bone development is negatively affected when yearlings are taken from pasture to be housed in stalls and allowed limited exercise, and to determine the consequential effects of the change in housing on bone modeling/remodeling at the onset of training.

# CHAPTER 1

# REVIEW OF LITERATURE

## Bone Architecture

All bones are composed of both cortical and cancellous bone (Marks and Popoff, 1988). Cortical bone makes up the majority of the total skeletal mass (Jee, 1988). Characterized by its compactness, cortical bone forms the outer shell of all bones. In contrast, cancellous bone, characterized by a framework of rods, plates, and arches, individually called trabeculae, and a high bone marrow content, is found primarily in the interior of bones. For example, the diaphysis, or central cylindrical shaft, of the third metacarpal is composed primarily of cortical bone surrounding a marrow cavity. The epiphyses, located at either end in long bones, and metaphyses, the regions connecting the diaphysis and epiphyses, are mainly made up of trabecular bone surrounded by an outer layer of cortical bone. The cavities of cancellous bone and the bone marrow in the diaphysis are lined with the endosteum, a membranous layer of differentiated bone cells (osteoblasts and osteoclasts). The periosteum on the outer surfaces of most bones are lined with a layer of undifferentiated cells (mesenchymal stem cells). When stimulated, these cells can be recruited to increase bone growth and facilitate fracture repair. The periosteum itself is covered by a thin layer of connective tissue.

Bone tissue exists in either a primary or a secondary form (Jee, 1988). Primary, or immature, bone, also referred to as woven bone, is characterized by collagen fibers that lack an ordered arrangement, osteocytes that are fewer in number and less uniformly placed within the bone extracellular matrix, and a lower mineral content, when compared

with secondary bone. Primary bone is present in the skeleton when developing bone tissue initially forms, an injury occurs, or a skeletal abnormality is present. Secondary, or lamellar, bone, with its patterned orientation of collagen fibers, evenly distributed osteocytes, and high mineral content, replaces most primary bone. Both primary and secondary bone can be either cortical or cancellous.

The osteon, or Haversian system, is the main structural unit of cortical bone (Jee, 1988). Each osteon is comprised of a large, central vascular channel, or Haversian canal, surrounded by circular sheets of collagen fibers, or concentric lamellae. Haversian canals themselves contain blood vessels, nerves, and connective tissue. Volkmann's canals, set transversely to the longitudinal Haversian canals, form a network for osteocytes that provides nutrients and allows intercellular signaling. Osteocytes are the main cells of mature bone, located within the central canals, formed from osteoblasts that have surrounded themselves with mineral (Currie, 1988). Osteocytes can regulate mineral homeostasis. Volkmann's canals support the osteocytes through connections to bone surface cells, bone marrow, and other Haversian canals (Jee, 1988). Cancellous bone does not contain Haversian systems. Instead, it contains trabecular packets within its framework, or trabeculae, that are functionally analogous to the osteon. Throughout the lamellae of both cortical and cancellous bone are small cavities, or lacunae, occupied by osteocytes and connected by thin tubular channels, or canaliculi (Marks and Popoff, 1988). The canaliculi contain the cytoplasmic processes of the osteocytes, which allow communication with other osteocytes through gap junctions and provide nutrients through connections to the bone surface. In addition to osteocytes, other cells present in bone are osteoblasts, which are found along active bone surfaces, and osteoclasts, which usually

reside in or near Howship's lacunae, the resorption pits located on the bone surface (Jee, 1988). Osteoblasts are bone-forming cells that synthesize and secrete unmineralized bone matrix (osteoid), participate in calcification of bone, and regulate mineral homeostasis through the flux of calcium and phosphate in and out of bone. Osteoclasts are giant multinucleated cells, originating from bone marrow, whose primary function is the resorption of bone, either internally or on the surface of bone (Jaworski, 1984; Weryha and Leclere, 1995). Also present in bone are bone-lining cells. These thin, elongated cells are found directly apposed to inactive bone surfaces (Marks and Popoff, 1988). Bone-lining cells function primarily as an ion barrier separating interstitial fluid from the bone fluids of the lacunar-canalicular system. Bone-lining cells are postulated to be involved in sensing the magnitude, distribution, and rate of mechanical strain placed on bone during loading and transmitting this information to the active bone cells, stimulating the appropriate bone formation or resorption response (Jee, 1988). Osteocytes have also been hypothesized to be responsible for this transmittance (Lanyon, 1987), due to their wide distribution throughout the organic matrix and their ability to communicate with each other and other bone surface cells through canalicular processes (Jee, 1988).

The process of bone degradation by osteoclastic cells involves the release of calcium and phosphate ions into the extracellular fluid, followed by proteolytic degradation of the organic matrix of bone (Marks and Popoff, 1988). The surface of an active osteoclast, characterized by its membrane infoldings, is called the ruffled border (Jee, 1988). A clear zone surrounds the ruffled border and provides a tight seal for maintenance of an appropriate microenvironment for bone resorption through its adhesion to the bone surface. Acid hydrolases stored in Golgi complexes, located in the cytoplasm

of the osteoclast cell, are transported to the ruffled border region, where the membrane of the primary lysosome fuses with the ruffled border and releases the acid hydrolases into the extracellular space created between the bone and the cell by the clear zone (Marks and Popoff, 1988). This creates an acid microenvironment where the bone can be degraded by acidic proteases. The breakdown products of the bone are taken up in digestive vacuoles and secondary lysosomes, further degraded, and released into vascular spaces nearby. Once minerals are released, the organic matrix is resorbed (Jee, 1988). Cells of the osteoblast lineage are believed to play a role in the initiation of osteoclastic resorption by their destruction of the osteoid tissue lining the bone matrix (Weryha and Leclere, 1995). Bone formation, on the other hand, involves matrix formation, followed by mineralization. The synthesis of organic matrix by active osteoblasts occurs at the common boundary between osteoblastic cells and osteoid that is already present (Jee, 1988). Mineralization subsequently occurs at the interface between osteoid and the most recently mineralized bone. The lag time between matrix formation and mineralization in the adult is approximately 10 days. During this time the matrix is undergoing a series of steps in preparation for mineralization. The process of mineralization lasts several months and is divided into two distinct phases (Jee, 1988). The primary phase occurs over several days and results in approximately 70 percent of total mineralization. This phase is thought to be regulated by osteoblasts on the osteoid surface and osteocytes within the lacunae of osteoid. The secondary phase lasts several months and provides most of the remaining 30 percent of mineralization. The availability of mineral to the matrix and the chemical composition of the fluid surrounding the matrix may be the governing elements of the secondary phase of mineralization. The mechanism for the activation of mineralization can

be bone resorption or can occur independently of any local resorption (Jaworski, 1984). Various interleukins, prostaglandins, growth factors, and hormones have been implicated in the regulation of osteoclast and osteoblast cell activity (Weryha and Leclere, 1995).

In the young, growing animal, cartilaginous growth plates, also referred to as epiphyseal-metaphyseal complexes, separate the epiphyses and metaphyses of the long bones. Proliferation, and hence elongation, of the long bone occurs at this site (Currie, 1988). The growth plate itself is made up of five zones, referred to as the resting, proliferative, maturation, hypertrophic, and calcification zones (Jee, 1988). Each zone provides a distinct and important function in the elongation of long bones. The resting zone, closest to the epiphysis, functions as a source of progenitor cells, which have the capacity to undergo mitosis and differentiation into chondrocytes (Currie, 1988). These differentiated cells, assembled into longitudinal columns, comprise the proliferative zone. Within this zone, differentiated cells actively undergo mitosis and synthesis and secretion of cartilage matrix. The synthesis of matrix and the preparation of the matrix for calcification occurs in the zone of cell maturation (Jee, 1988). In the hypertrophic zone, cells are characterized by their enlargement and intracellular glycogen and calcium stores, the latter of which is actively removed from the cells and used in the mineralization of the cartilage matrix (Currie, 1988). This initial step in the conversion of cartilage to bone occurs in the calcification zone, located closest to the metaphysis. The next step in the conversion involves blood vessels and osteoblasts entering the mineralized cartilage. Osteoblasts secrete an organic matrix (osteoid) containing type I collagen, noncollagenous proteins, phosphoproteins, and lipids, which is subsequently mineralized by the deposition of inorganic salts, primarily calcium phosphate, to form bone (Currie, 1988; Marks and

Popoff, 1988). In essence, bone elongation occurs by the growth plate growing away from the diaphysis (Currie, 1988). Chondrocytes eventually die due to a lack of available nutrients resulting from matrix calcification, and any remaining cartilage is removed by osteoclastic cells. Eventually this primary, or immature, bone, also referred to as the spongiosa, is removed and replaced with secondary, or mature, bone at the region of the metaphysis of the long bone. Growth at the epiphyseal-metaphyseal complex continues until cells of the resting zone can no longer undergo mitosis and chondrocytes are no longer available to make up the proliferative zone. Upon cessation of growth, the band of cartilage at the growth plate is completely replaced by cancellous bone and the adjacent epiphysis and metaphysis fuse together. This process is referred to as closure of the epiphyses. In the horse, epiphysial closure of the long bones will occur between 9 and 30 mo of age, depending on the specific bone (Evans et al., 1990).

While elongation occurs at the growth plate, the diaphysis of the long bone develops circumferentially by a combination of bone deposition and degradation (Currie, 1988). Bone formation occurs on the bone surface, immediately internal to the periosteum, while resorption takes place on the inner surface of the shaft wall. Internal resorption will result in the creation of a cavity for bone marrow. Initially, while the diameter of the shaft expands, the shaft wall thins as a result of regulated rates of formation and degradation during growth. Ultimately, the wall thickens as bone formation on the surface of the bone accelerates, thus increasing the mechanical strength of the bone. The flaring of the ends of a long bone during growth occurs by formation of bone on the periosteal surface and osteoclastic resorption on the endosteal surface of the diaphyseal shaft, while, at the same time, bone formation on the endosteal surface and periosteal

resorption are occurring in the metaphyseal region (Jee, 1988). This results in a long bone with a narrow shaft at the junction of the metaphysis and flaring epiphysis, greater diaphyseal diameter, and an enlarged marrow cavity.

## Bone Modifications

The skeletal architecture functions primarily to protect the body's soft tissues, such as the skull and the ribs, and is determined genetically (Lanyon, 1987). The general shape and composition of load-bearing bones, responsible primarily for the locomotion of the skeleton, is also determined genetically. In the absence of functional loading, these bones will develop into their recognizable form. However, only through an adaptive response to physical loading will the structural features that enable these bones to withstand repetitive loading without damage develop. These structural features include girth, cross-sectional shape, cortical thickness, longitudinal curvature, and bone mineral content.

During bone growth, bone modeling and remodeling are occurring simultaneously. The modeling mechanism, most obvious during long bone growth in a young animal, causes modifications in the shape and size of the bone (Jee, 1988). In essence, modeling determines the amount and form of bone in the adult animal's body. Modeling occurs by the apposition or resorption of bone on the bone surface. Examples of modeling include drift, or shifting, of the midshaft, flaring of the ends of long bones, and increasing bone volume during growth (Jaworksi, 1984). In contrast, the replacement of primary, or immature, bone by secondary, or mature, bone, as well as the replacement of old or damaged bone, is referred to as remodeling (Jee, 1988). In horses, approximately 50% of the replacement of immature bone with mature bone occurs by 3 yr of age, and the

remodeling mechanism then continues throughout life, as secondary bone is continuously being destroyed and replaced (Riggs and Evans, 1990). In remodeling, bone degradation and formation occur at the same skeletal location, whereas either formation or resorption occur in the modeling process, but at different surfaces and rates (Jee, 1988; Burr et al., 1989). In essence, no change in the shape or gross amount of bone present occurs with the remodeling process because of the coupling of bone resorption and formation, whereas an increase or decrease in the total amount of bone or a change in shape or location of bone will occur with modeling, based on the independent activation of resorption or formation (Jaworski, 1984).

In addition to sites of immature and old bone replacement, the areas of bone that endure the most stress during loading, especially tendon attachment sites, and sustain maximal loads, are at points of remodeling (Norwood, 1978). Three major phases make up the remodeling cycle. Osteoclastic resorption of primary, damaged, or old bone occurs first, followed by a short reversal phase, during which the bone surface is prepared for formation, and finally osteoblastic deposition of secondary or new bone. Layers of bone may be removed and then replaced from the surface of the bone, or osteoclasts may tunnel their way through the inner cortex of bone, leaving behind a canal that is subsequently filled by osteoblastic cells, producing a secondary Haversian system (Riggs and Evans, 1990). Burr et al. (1989) includes an activation phase, prior to resorption, at which time the bone is stimulated to change in response to mechanical stress or strain by the activation of preosteoclastic cells. The adaptive response of remodeling occurs by engagement of new osteoblasts and/or osteoclasts, rather than increasing the activity of already established cells. The specific mechanism that links the mechanical loading

stimulus to the activation of the bone cells responsible for the remodeling process is unknown (Lanyon, 1989). The functional unit of the remodeling mechanism is referred to as the "bone remodeling unit" or "bone multicellular unit" (Jee, 1988). This unit includes the group of cells responsible for a quantum of bone resorbed and replaced at a particular site, as well as the quantum of bone involved.

When the bone is loaded, it deforms due to the strain. If, when the bone is unloaded, the shape of the bone returns to its original form, the bone is said to have been loaded within its elastic region (Lanyon, 1989). Little modeling or remodeling occurs within this region. When the bone can no longer resist the change in shape and continues to deform, in response to an increase in loading magnitude, it has reached a point beyond the elastic region. This is referred to as entering the yield region and occurs when the level of physical activity is increased to a point beyond what the skeletal structure is accustomed. Continual loading within this region without allowing the skeleton to adjust to the new level of activity through remodeling will eventually result in damage to the bone. Norwood (1978) has proposed that one remodeling cycle takes approximately 4 mo to complete in the horse. The resorption phase is said to last approximately 1 mo, the reversal phase about 1 wk, and the bone deposition phase approximately 3 mo. It is during the period of time between the start of resorption and the completion of bone deposition in the reparative remodeling mechanism that the bone is susceptible to injury due to its weakness and porosity. The increase in porosity that occurs is related to the number of Haversian systems that are being actively remodelled at the same time. The more Haversian systems simultaneously undergoing remodelling, the greater the porosity of the bone, resulting in the strains that are applied to the bone at this time to be increased

(Nunamaker, 1986). If adequate time is not given for the bone to heal and modify itself before additional stress is placed upon it, the risk of injury increases (Lanyon, 1984). Damage can also result from a single massive load applied to the skeletal system or from fatigue failure due to repetitive loading (Lanyon, 1987).

## Skeletal Response to Exercise

Wolff's Law states that as the biomechanical load on a bone changes, the internal structure of the bone will modify to accommodate the new stress placed upon it (Norwood, 1978). While the general shape of load-bearing bones is genetically determined, modeling and remodeling ensure that the structure of the load-bearing bones is sufficient in strength and rigidity to withstand repetitive loading without damage (Lanyon, 1987). In young, growing racehorses or other performance horses, the adaptation of the skeletal structure is a response to the mechanical loads experienced as a result of training. This adaptive response serves to develop optimal functional characteristics of bone, as well as tendon and muscle, for the physical activity most commonly encountered, which is the primary objective of training (Lanyon, 1989). Bone cells responsible for the adaptive response directly or indirectly recognize and appropriately match the skeletal structure to the functional load applied during training (Lanyon, 1987). As loading increases beyond the appropriate strain environment previously utilized, as when the next stage in training is introduced, bone formation predominantly occurs, decreasing the strain placed on the load-bearing bones by increasing the amount of bone tissue present. Unless the magnitude, rate, or distribution of the strain applied during training changes thereafter, further modifications to the skeletal

architecture are unnecessary and subsequent loading of the same type will not elicit a greater adaptive response (Lanyon, 1989). The skeletal structure is said to have reached an optimal strain environment for that physical activity (Lanyon, 1984).

Woo et al. (1981) conducted a study to determine the effects of a prolonged, moderate intensity exercise program on the composition, mechanical properties, and structural properties of the femur of immature swine. The study included both a non-exercised control group and an exercised group that underwent 12 mo of conditioning on a treadmill at an exercise level consistent with 65 to 80% of maximum heart rate. The mechanical properties, bone density, and biochemical contents of the femurs in the non-exercised and exercised groups were not different after completion of the study. However, cortical thickness, cross-sectional area, total volume, ash content, and calcium content were significantly higher in the exercised group. Thus, the skeletal differences between the groups were attained by an increase in bone quantity, with no affect on bone quality. In response to mechanical loading, an adaptive mechanism was stimulated to remodel the cortical bone, increasing the amount of bone tissue present and thereby decreasing the strain encountered during exercise.

A study by McCarthy and Jeffcott (1992) examining the effects of treadmill exercise on the equine third metacarpal bone showed similar results to that of Woo and colleagues (1981). Investigators in this study compared the structural properties and cellular activity of cortical bone in the third metacarpal of two groups of young horses. One group was kept relatively inactive through restricted housing and the other group was subjected to an intense treadmill exercise program at near maximal speeds. Results indicated that the cortical bone of the exercised horses experienced little bone remodeling,

but extensive bone formation occurred on the dorsal cortex, resulting in an increase in thickness of this cortex. This suggests that the strains endured by the third metacarpal during treadmill exercise were below that which would cause microdamage to the bone and initiate bone remodeling, but were high enough to stimulate bone formation in an attempt to adapt to the new functional load placed on the bone. Increasing the bone mineral content of the dorsal cortex of the third metacarpal improved the bone's ability to withstand loading on that particular cortex.

Buckingham and Jeffcott (1991) compared the effects of a long term submaximal exercise program on the bone mass of a group of yearling Standardbreds to that of a non-exercised control group. Bone mineral density increased in the exercised group, but decreased in the non-exercised group, although the changes were small and lacked significance. Even so, results indicated a possible trend toward increasing bone strength and density as a result of exercise. What may be more apparent from the results of this study is that while low intensity exercise did not result in a high degree of bone hypertrophy, it was at least not detrimental.

## Skeletal Response to Limited Physical Activity

An appropriate skeletal structure can only be maintained if mechanical loading is performed on a regular basis (Lanyon, 1989). If loading falls beneath that normally sustained by the skeletal environment, bone formation will slow or cease and bone resorption will predominantly occur until the skeletal structure and functional load applied match (Rubin, 1984). Transferring young horses from pasture to stalls during the winter months has been shown to result in decreased serum osteocalcin concentrations (indicative

of osteoblastic activity), indicating a slowdown in the rate of bone formation associated with decreased physical activity (Maenpaa et al., 1988). Immobilization of young, growing rats by sciatic denervation was shown to increase bone resorption and dramatically decrease bone formation over 6 wk, resulting in a lower bone mineral content (Yeh et al., 1993). Kannus et al. (1996) demonstrated a marked decrease in osteocalcin immunoreactivity in the rat patella after 3 wk of immobilization, indicating reduced bone formation. These examples support the existence of an adaptive response between mechanical loading and the skeletal structure. If the functional strain on the load-bearing limbs is suddenly reduced, the skeletal architecture responds by lowering bone mass to a level appropriate for the new strain (Lanyon, 1984).

In the extreme case of complete skeletal disuse, Rubin and Lanyon (1984) determined that the predictable response is primarily bone resorption and decreased bone mineral content. Removal of the natural strain loading of an intact rooster ulna by functional isolation resulted in a 12% decrease in bone mineral content over 6 wk. An insignificant amount of new bone formation did occur, due to the coupling of resorption and formation in the remodeling mechanism. A similar study in humans showed a 10.4% decrease in bone mineral of the calcaneus and a total body bone mineral loss of 1.4% in healthy adult men voluntarily subjected to 17 wk of bed rest (LeBlanc et al., 1990). In the study by Rubin and Lanyon (1984), only four strain cycles per day, applied externally, were necessary to prevent bone resorption and maintain a functional level of bone mass. This indicates that a functional strain environment for the load-bearing bones can only be maintained if loading occurs on a continuous basis.

Placing yearling horses in stalls in preparation for yearling sales or the

commencement of training, without any exercise more rigorous than hand-walking, is common in the horse industry. Few, if any, studies have examined the skeletal effects of stall-rearing yearlings. In addition to the study by Maenpaa et al. (1988) looking at the transfer of foals from pasture to stalls and its effects on bone formation, concern about the effects of stalling on bone growth stems from various research studies discussing the effects of confinement rearing on other livestock species. Laying hens housed in battery cages were found to have only 54% of the humeri strength of birds that were kept in a perchery (Knowles and Broom, 1990). Fleming et al. (1994) showed that the breaking strength and radiographic density of the humeri from battery caged laying hens were 40 to 50% lower than values obtained for birds housed in two different perchery systems. A similar decrease in humerus breaking strength of 45% was demonstrated in caged birds in a study by Norgaard-Nielsen (1990). Similarly, sows housed in stalls were found to have only two-thirds of the humeri and femur breaking strength of group-housed sows (Marchant and Broom, 1996).

Research suggests that limiting a young, growing horse's access to physical activity of such a magnitude as to signal the bone to remodel and become stronger may have negative consequences. These consequences may include the development of a skeletal system that is of inferior strength and thus unprepared to handle training and competition. Young horses should enter training with a skeletal system that is optimally prepared for the strain that mechanical loading will place upon it. Frost (1987) states that cortical bone deposits will be increased by the modeling mechanism if functional loading of bone is vigorously increased in the juvenile skeleton. Additionally, future deposits will be retarded if disuse of bone occurs. If the modeling mechanism primarily occurs in the young,

growing animal, changing the architecture of the bone while the animal is young and still capable of altering bone structure would be advantageous. This may contribute to the prevention of career-threatening, or even life-threatening, injuries. However, if the skeletal systems of young performance horses are being ill-prepared for training and competition due to stalling, they may be predisposed to injury, which could result in a shortened, or even terminated, competitive career. Determining if bone development is negatively affected when young, growing horses are taken from pasture to be housed in stalls and allowed limited exercise would be financially and emotionally advantageous for the horse industry.

## Measuring Skeletal Changes

Current research is being conducted to assess the capabilities of biochemical markers of bone turnover in horses as a supplement to other noninvasive methods of assessing bone mineral content, including radiographic photometry, photon absorptiometry, and ultrasonography. Development of such biochemical markers would provide a complimentary, and perhaps more sensitive, means of identifying equine athletes at risk of injury. Biochemical markers investigated in this study are bone matrix components released into the blood or urine during the formation of new bone by osteoblasts or the resorption of old or damaged bone by osteoclasts, respectively (Price et al., 1995; Delmas, 1993). In young, healthy individuals, bone formation exceeds bone resorption. In physically active adults, bone formation and degradation are tightly regulated to maintain bone mass. Development of bone marker assays may aid in detecting situations when the skeletal system is more actively engaged in bone resorption

than production and thus the risk of skeletal injury is higher. Assays for biochemical indicators of bone turnover in serum and urine already exist in human medicine for the clinical investigation of osteoporosis and other metabolic bone diseases (Delmas, 1993). These assays are now being utilized for equine research. In this study, the biochemical indicators of bone formation and bone resorption analyzed were serum osteocalcin and urinary pyridinoline and deoxypyridinoline, respectively.

Osteocalcin, or bone gla protein (BGP), is one of the most abundant noncollagenous proteins of mammalian bone (Gomez et al., 1994). Osteocalcin contains three amino acid residues of the vitamin K-dependent gamma-carboxyglutamic acid and an associated disulfide bond (Price, 1982). The 49 amino acid protein has weak calcium ($Ca^{2+}$) binding properties, but a strong affinity for hydroxyapatite (Gomez et al., 1994; Price, 1982). The role of gamma-carboxyglutamic acid residues in osteocalcin is to fully enable osteocalcin to bind to hydroxyapatite. Decarboxylation of gamma-carboxyglutamic acid to glutamate greatly weakens osteocalcin's $Ca^{2+}$ binding properties and lowers its affinity for hydroxyapatite, although the non-gamma carboxylated protein will continue to bind hydroxyapatite, albeit weakly (Price, 1982). Osteocalcin does not appear in mineralized tissues until the initial mineral phase has matured into hydroxyapatite, indicating the importance of hydroxyapatite binding to the accumulation of the protein in bone (Price, 1982). The kidney is the main route of excretion for circulating osteocalcin, following proteolysis (Gomez et al., 1994).

Osteocalcin has been found in osteoblasts, osteocytes, and dentin (Gomez et al., 1994). However, certain characteristics of osteocalcin suggest that osteoblasts are the cells within bone that are responsible for osteocalcin production. First, osteocalcin

contains a 4-hydroxyproline residue in its structure, indicating the presence of prolyl hydroxylase (Price et al., 1976). The prolyl hydroxylase enzyme has been used as a marker for distinguishing osteoblasts from osteoclasts in culture. Second, osteocalcin has been shown to be synthesized exclusively by clonal osteosarcoma cells that exhibit features of the osteoblast phenotype, such as high alkaline phosphatase activity and a high degree of responsiveness to PTH (Nishimoto and Price, 1980). Thus, measurements of serum osteocalcin appear to directly reflect the activity of osteoblastic cells (Price, 1982).

Osteocalcin is synthesized by bone cells at new bone mineralization sites. A fraction of newly synthesized osteocalcin that fails to bind to the organic matrix of bone during bone formation is released intact from these mineralization sites into blood, where it becomes a measurable marker of osteoblastic activity (Gomez et al., 1994; Price, 1982). Osteocalcin is not released from the bone matrix at any other time except new bone formation (Price, 1982). The specific function of osteocalcin in bone metabolism is unknown, although it is likely that osteocalcin regulates mineral formation. Its synthesis by osteoblastic cells supports a role in bone formation, however the hormonal regulation of osteocalcin synthesis complicates this idea. Osteocalcin synthesis is stimulated by 1,25 dihydroxyvitamin $D_3$ (1,25-$(OH)_2D_3$), the active form of vitamin D, although osteocalcin synthesis will occur in the absence of vitamin D. However, 1,25-$(OH)_2D_3$ also inhibits collagen synthesis, the basic unit of bone formation (Price, 1982). The function of 1,25-$(OH)_2D_3$ is to adjust bone metabolism during periods of stress involving inadequate mineral levels from dietary intake through its stimulation of osteocalcin synthesis, implying that it helps regulate mineral homeostasis during bone resorption.

Brown et al. (1984) determined that serum osteocalcin correlated positively with

markers of osteoblastic activity, including relative osteoid volume, relative osteoid surfaces, tetracycline labeled surfaces, and bone formation rate. Interestingly, serum osteocalcin and bone resorption surfaces were not positively correlated. As an increase in resorption surfaces is indicative of an increase in osteoclast cell activity, the lack of a positive correlation with osteocalcin supports the idea that osteoblasts, and not osteoclasts, synthesize osteocalcin. Carter et al. (1996) reported an inverse correlation between osteocalcin and growth rate ($P < 0.01$), bone strength ($P < 0.01$), metacarpal ash ($P < 0.10$), femur ash ($P < 0.01$), and femur ash weight ($P < 0.01$). Interpretation of these results by the investigators indicated the high specificity of osteocalcin as a marker of bone mineralization and/or turnover.

Osteocalcin's direct correlation with bone metabolism and the relative ease of radioimmunoassay measurement have led to its attractiveness as a means of evaluating bone disorders and the effects of physical activity on bone formation (Price et al., 1980). When compared to two other commonly used biochemical markers, plasma alkaline phosphatase activity and urinary hydroxyproline, osteocalcin has an advantage in that it is a specific bone protein, whereas alkaline phosphatase and hydroxyproline both have several tissues of origin. In human medicine, serum osteocalcin can monitor changes in the rate of bone formation common in patients with metabolic bone disease (Brown et al., 1984; Delmas, 1993). Osteocalcin is increased in patients with bone diseases characterized by increased bone resorption and increased bone formation, including Paget's disease, bone metastases, primary hyperparathyroidism, renal osteodystrophy, and osteopenia (Price et al., 1980). Brown et al. (1984) determined that serum osteocalcin reflects bone formation, but not bone resorption, in patients with postmenopausal

osteoporosis.

Lepage et al. (1990) measured serum osteocalcin concentrations in 50 clinically normal female Standardbred horses of varying ages to determine differences in serum levels with age. Mean osteocalcin concentrations for animals less than 1 yr of age, between 1.5 and 2.5 yr, and older than 3.5 yr of age were 47.3, 35.7, and 6.7 ng/mL, respectively. These results indicated that serum osteocalcin concentrations decrease with age in female horses, suggesting a significant slowdown in the rate of bone formation in adult horses compared to young horses. A higher degree of variation occurred in the 1.5 to 2.5 yr age group, which was attributed to differences in adaptation of bone turnover to exercise, since 18 mo of age corresponded to the commencement of training.

Another study examined the effects of transferring foals from pasture to stalls for the winter months on biochemical markers of bone formation (Maenpaa et al., 1988). A 23.4% decrease in serum osteocalcin concentrations occurred within 1 mo of when the foals were stalled. Investigators interpreted this decrease to indicate a slowdown in the rate of bone formation in response to decreased physical activity associated with stalling.

Urinary pyridinoline and deoxypyridinoline, pyridinium cross-links of collagen, are biochemical indicators of bone resorption or collagen degradation (Robins et al., 1994). Pyridinoline is present in bone and cartilage matrix, as well as in other connective tissues, while deoxypyridinoline is present in bone collagen of the organic matrix (Delmas, 1993; Robins et al., 1994). The function of pyridinoline and deoxypyridinoline is to provide structural rigidity and strength for bone collagen. These crosslinks provide stability due to their location between adjacent collagen fibers within the extracellular matrix (Gomez et al., 1996). When collagen is degraded by various proteases, crosslinks are released into

circulation and ultimately are excreted in urine. Studies have shown that the concentrations of pyridinoline and deoxypyridinoline found in urine are essentially derived from bone, based on a similar molar ratio of pyridinoline to deoxypyridinoline in bone and urine, therefore explaining their use as indicators of bone resorption (Gomez et al., 1996; Robins et al., 1994).

Urinary excretion of pyridinoline and deoxypyridinoline has been shown to be unaffected by renal dysfunction (Robins et al., 1994). However, arthritic conditions in humans have been shown to significantly increase levels of pyridinoline from sources other than bone, suggesting that deoxypyridinoline may be a more specific and reliable marker for bone resorption. Once released into the circulation, pyridinoline and deoxypyridinoline cannot be reutilized in collagen production because both are products of a posttranslational modification of collagen molecules that have already been secreted and become part of the extracellular matrix (Delmas, 1993). Pyridinoline and deoxypyridinoline are not metabolized prior to excretion by the kidneys and are unaffected by diet, adding further to their reliability as indicators of bone degradation. Approximately 40% of the pyridinium crosslinks are excreted in urine in a free form and 60% in a peptide-bound form (Delmas, 1993).

High-performance liquid chromatography (HPLC) has been used to measure total amounts of crosslinks, while direct enzyme immunoassay (ELISA) methods have been developed to measure free pyridinoline and deoxypyridinoline, with no significant interaction with the peptide-bound forms of the crosslinks (Robins et al., 1994). Studies have shown that results from immunoassay methods are highly correlated with results from HPLC assays measuring total crosslinks (Gomez et al., 1996; Robins et al., 1994).

Measures of pyridinium crosslinks have been found to be useful in human medicine for assessing metabolic bone diseases and risk of disease, as well as monitoring therapy (Robins et al., 1994). Concentrations of free pyridinoline and deoxypyridinoline in the urine are higher in children than adults, due to greater activity of the bone modeling and remodeling mechanisms during growth (Gomez et al., 1996; Robins et al., 1994). These findings suggest that measurements of the pyridinium crosslinks may be useful monitors of growth and the presence of growth abnormalities (Robins et al., 1994).

A study comparing the effects of estrogen alone and estrogen and androgen together on biochemical markers of bone formation and resorption in postmenopausal women included pyridinoline and deoxypyridinoline among the markers measured (Raisz et al., 1996). Owing to the fact that estrogen deficiency is known to play a role in the changes that occur in bone metabolism after menopause, and androgens are suspected to be involved as well, both replacement therapies used in this study were expected to affect both bone formation and bone resorption. Results indicated that both therapies showed a similar decrease in urinary excretion of deoxypyridinoline and pyridinoline, interpreted as an indication of a slowdown in the rate of bone resorption.

The applicability of these biochemical markers of bone formation and resorption to the horse have exciting possibilities, in terms of determining normal bone growth patterns and identifying abnormal bone breakdown. The impact that these markers may have on reducing the potential for career- and life-threatening injuries in horse racing and other equine performance activities is encouraging.

Supplemental to the use of biochemical markers of bone metabolism are various noninvasive methods of evaluating bone. Radiographs can be used for both qualitative

and quantitative evaluation of bone. Meakim et al. (1981) has developed a method to determine radiographic bone aluminum equivalence (RBAE), a measurement of bone mineral content, using dorsal-palmar radiographs. A study by Williams et al. (1991) evaluating the capabilities of noninvasive techniques to estimate bone mineral content (BMC) and bone strength of the third metacarpal in cattle demonstrated a correlation coefficient ( r ) between BMC and radiographic photometry of .967 ($P < .0001$). This value was shown to be higher than r values between BMC and two other noninvasive methods evaluated, photon absorptiometry (.908, $P < .0001$) and ultrasonography (.406, $P < .0001$). Studies have shown that RBAE of the third metacarpal is an adequate means of demonstrating the changes in bone mineral content that occur during normal bone growth (El Shorafa et al., 1979; Meakim et al., 1981; Frey et al., 1992) and exercise (Nielsen et al., 1997). However, RBAEs determined by the above method can only indicate changes in the section of bone with the greatest optical density. A method that can measure total changes occurring in the third metacarpal may be more valuable.

Single photon absorptiometry has been shown to be effective in determining bone mineral content of the third metacarpal (Jeffcott et al., 1987). However, disadvantages of this method include the necessity of sedating horses in order to get the most accurate bone scans, which take 60 s each to complete, and preparing the horse and performing the procedure requires approximately 30 min per horse. Therefore, this method is impractical for utilization in a research setting when large numbers of horses are involved.

A method for determining cross-sectional area of the third metacarpal using ultrasonography has been reported (McCartney and Jeffcott, 1987). In contrast to single photon absorptiometry, ultrasonography has been reported to be a relatively simple and

reliable technique for measuring changes in bone. However, ultrasonography has a limitation in that the changes that it measures are volumetric in nature.

These studies suggest that the use of radiographic photometry to estimate bone mineral content of the third metacarpal in horses may be the most efficient and effective method in a research setting.

# CHAPTER 2

# MATERIALS AND METHODS

## Management of Animals

Sixteen Arabian yearlings, 11 geldings and five fillies, with a mean age of 18.6 mo (range 16.6 to 19.9), were pair-matched by sex and age and randomly placed into two experimental groups. One group was housed in 3.0 m by 3.4 m box stalls while the second group was maintained on a 28,327 $m^2$ pasture. The project was conducted during the winter months to minimize dietary differences that may have occurred if grazing had been available to the pastured horses. Each horse was fed 1.8 kg of a commercial concentrate (Strategy for Performance or Breeding Horses, donated by Purina Mills, Lansing, MI) per d, divided into two equal feedings (0600 and 1800), and had ad libitum access to mixed alfalfa-grass hay, satisfying NRC (1989) recommendations for long yearlings and two-year-olds (Table 1). The horses were maintained on this diet through the duration of the study. Based on 24-hr hay intake trials conducted every 28 d, each stalled horse consumed an average of 10.9 kg and each pastured horse an average of 9.8 kg of roughage per d. The horses were vaccinated against rhinopneumonitis, tetanus, influenza, and equine encephalomyelitis. In addition, horses had their hooves trimmed and were dewormed routinely, and had their wolf teeth removed prior to the start of training.

Table 1. Calculated analysis of total ration on an as-fed basis

| Nutrient | Units | Ration |
|---|---|---|
| DE | Mcal/kg | 2.169 |
| CP | % | 14.7 |
| Ca | % | .75 |
| P | % | .29 |

## Training of Animals

The project was divided into two phases, consisting of an 84-d pre-training period and a 56-d training period. During the pre-training period, pastured horses were allowed free access to exercise, while stalled horses were walked 1 hr per d on a mechanical walker. Six horses from each group were randomly selected to enter the training phase of the project. One horse from the pastured group was removed from the project after d 84 due to an accidental injury, hence only five horses were represented in the pastured group during the 56-day training period. Each horse was ridden 5 d per wk, following the morning feeding, and the stalled horses were walked 1 hr on a mechanical walker on all non-riding days.

During the first wk of the 56-d training period, the horses were introduced to a saddle and rider, gentled to ride, and taught to guide in a round pen of 14.0 m in diameter. During this stage, the horses were asked to walk, trot, and canter in both directions for 20 to 30 min per d, the length of time depending on the progress of the horse. Riders were randomly assigned to horses (within a rider's capability). During the second through the fourth wk of the training period, the horses were ridden in either a 30.5 m by 61.0 m outdoor arena or 22.9 m by 54.9 m indoor arena. Some of the horses were occassionally ridden for a brief period of time in the round pen and subsequently ridden in the outdoor or indoor arena. As during the first wk of training, the horses were asked to walk, trot, and canter in both directions for 20 to 30 min per d. Horses were ridden on a 768 m exercise track 5 d per wk for the remaining 4 wk of the 56-d training period. Each horse was trotted one lap to warm up, galloped three laps, and trotted a second lap in order to cool down. Average training distances per horse per d during this 4-wk stage were 1378

m at the trot and 2146 m at the gallop.

On d 122 and 131, the horses were ridden in the indoor arena due to inclement weather and poor track conditions. Horses were exercised similar distances in the indoor arena on d 122 and 131 as on the exercise track during the last 4 wk of the 56-d training period. On d 87, 94, 108, and 115, the horses were ridden in the round pen to accomodate a behavior research project involving the same horses (Rivera et al., 1997).

## Sample Collection

Dorsal-palmar radiographs of each horse's left front leg were taken to determine RBAE of the lateral and medial cortices of the third metacarpal. Blood samples were taken via jugular puncture at 11 hr post-feeding (Lepage et al., 1991) to determine serum osteocalcin, 25-hydroxyvitamin D (Vit D), parathyroid hormone (PTH), and serum Ca and P concentrations. Twenty-four-hr urine collections were taken from four randomly chosen geldings from each treatment group to measure urinary deoxypyridinoline, pyridinoline, and urinary Ca and P concentrations. Body weight, height at withers, third metacarpal circumference, and body condition score (Henneke et al., 1984) were recorded for each horse every 28 d. Horses were weighed on a livestock scale. Additionally, 24-hr intake trials were conducted every 28 d to determine the average kg of hay consumed per d per stalled or pastured horse. On d 14, 42, 70, 98, and 126, horses from each experimental group were randomly selected to undergo visual observation to determine the number of elective strides taken, and in what gait, during a 24-hr period. Blood samples were collected every 14 d, while radiographs were taken and urine collections were conducted every 28 d.

## Urine Collections

Twenty-four-hr urine collections were conducted on d 0, 28, 56, 84, 112, and 140. Eight geldings randomly chosen for collection were allowed to become accustomed to wearing a saddle pad, collection harness, and surcingle prior to the first 24-hr collection on d 0. Horses were tied in individual stalls, within reach of a hay feeder and feed tub, through the duration of each collection. Urine was collected in a rubber bus tire inner tube hung beneath the sheath of each horse by a collection harness and surcingle. Urine was emptied from the inner tube at regular intervals to decrease the likelihood of losing a sample or contamination. The horses were monitored during the entire 24 hr and were allowed to drink water periodically throughout each collection. After 12 hr of each 24-hr period, a 10% sample of urine was measured and retained for each horse. After completion of each collection, another 10% sample of urine was measured and retained from the amount of urine collected during the latter 12 hr. The two 10% urine samples for each horse were mixed thoroughly and from that a final urine sample for each horse was taken at each collection, stored in a 250 ml bottle, and frozen for analysis following the completion of the project. The total volume of urine excreted during each 24-hr period was recorded for each horse.

## Elective Exercise Observations

Observations of daily elective strides were conducted on d 14, 42, 70, 98, and 126. Two horses from each experimental group were randomly selected to be visually observed for three 1-hr periods on observation days. The three periods were scattered throughout the day, the first period in the morning (between 0600 and 0900), second period in the

afternoon (between 1500 and 1800), and third period in the evening (between 2100 and 2400). The number of elective strides taken per horse, and in what gait, were visually observed and recorded by hand. Stride numbers from the three time periods were pooled by gait for each treatment group, divided by the number of horses observed (2), and divided again by the number of observation periods (3). This number was then multiplied by 24 hr, or 23 hr in the stalled group, to determine the average number of elective strides taken per horse at the walk, trot, and canter on that observation day. The stalled horses were also visually observed for 1 hr on the mechanical walker on each observation day. Stride numbers from that time period were pooled by gait and divided by the number of horses observed (2) to calculate the average number of strides taken per stalled horse at the walk, trot, and canter for 1 hr on the mechanical walker on that observation day. The data collected for all observations days were then pooled by gait and treatment to determine the average number of daily elective strides per horse, based on a 24-hr period for pastured horses and a 23-hr period plus a 1-hr period on the mechanical walker for stalled horses.

## Sample Analysis

Dilutions were made of serum and urine for determination of serum osteocalcin, urinary deoxypyridinoline and pyridinoline, serum and urinary Ca, and serum P. Serum samples for determination of Vit D and PTH and urine samples for measurement of urinary P did not require dilution. Serum osteocalcin and urinary deoxypyridinoline and pyridinoline concentrations were determined by competitive enzyme immunoassay procedures (Metra Biosystems, Inc., Mountain View, CA). The results obtained from

deoxypyridinoline and pyridinoline assays were corrected for variations in urine concentration by dividing the deoxypyridinoline or pyridinoline value (nM) by the creatinine value (mM) of each sample. Final values were expressed as nM deoxypyridinoline or pyridinoline per mM creatinine. Concentrations of creatinine in the urine were determined by colorimetric assay (Sigma-Aldrich, St. Louis, MO). Urinary deoxypyridinoline concentrations were also determined on a per d basis by multiplying the concentrations obtained from the deoxypyridinoline assay by the total volume of urine excreted per horse during the 24-hr period of each urine collection, and then multiplying by the molecular weight. Radioimmunoassays were used to determine Vit D and PTH concentrations in the serum (INCSTAR Corporation, Stillwater, MN). Due to limited funding, enough assay kits were purchased only to determine whether differences appeared to exist between treatment groups. Hence, not all samples were analyzed. Coefficient of variation (CV) for assays used were determined using unknown samples provided by the company. Serum and urinary Ca were analyzed by atomic absorption using a Smith-Hieftje 4000 (Thermo Jarrell Ash, Franklin, MA). Concentrations of P in the serum and urine were determined using a DU 7400 spectrophotometer (Beckman, Fullerton, CA).

## Radiographs

Dorsal-palmar radiographs of the left third metacarpal were taken to determine RBAE values using the technique described by Meakim et al. (1981). An aluminum stepwedge penetrometer was exposed simultaneously with each radiograph for use as a standard of reference, necessary for the comparison of radiographs. Dorsal-palmar views

were taken with the cassette positioned parallel to the cranial surface of the leg and centered midway between the proximal and distal ends of the third metacarpal. Medial-lateral views of the third metacarpal were not included in the sample collection due to insufficient financial support.

A Bio-Rad Model GS-700 imaging densitometer (Hercules, CA) was used to scan the radiographs transversely at the nutrient foramen of the third metacarpal. A logarithmic regression, developed using the thickness of the steps on the stepwedge, provided a means to estimate bone mineral content in RBAE at the maximum optical density reading of the lateral and medial cortices.

The scans of the dorsal-palmar radiograph of the third metacarpal and the aluminum stepwedge penetrometer were used to measure total RBAE in $mm^2$ Al. The area under the stepwedge curve corresponding to the steps with thicknesses of 14, 17, 20, 23, and 26 mm Al and the total area under the curve of the third metacarpal were determined. The total area under the five steps from the stepwedge scan was 1270 $mm^2$. The total RBAE was then determined by multiplying the total area under the curve of the third metacarpal by 1270 $mm^2$ Al and then dividing by the area under the stepwedge curve.

## Statistical Analysis

Physical characteristics, biochemical bone markers, and bone mineral content data were analyzed as repeated measures using PROC MIXED analysis (SAS, 1997). Treatment means, pairwise differences between the means, standard errors of the means and differences, and t-tests indicating statistical significance of the means differences were

obtained by inclusion of the LSMEANS statement in the analysis (SAS, 1997). Treatment, day, and day*treatment were included in the model to determine the effects of stalling on normal bone growth. Differences were considered significant at $P < .05$. When differences existed between the stalled and pastured groups at the start of the project, d 0 values (baseline) for each horse were subtracted from all other values for that animal and analysis was performed on the change from baseline. Results were graphed with the SEM indicated for each mean.

# CHAPTER 3

# RESULTS

## Weight, Height, Third Metacarpal Circumference, and Body Condition Score

No treatment differences occurred in body weight, height at withers, third metacarpal circumference, or body condition score. However, horses kept in stalls had higher body weight at d 140 than horses maintained on pasture ($P < .05$). Stalled horses began the project at an average weight of 349 $\pm$ 10.2 kg and increased to 405 $\pm$ 11.1 kg by d 140 for an average gain of 56 kg. Pastured horses had an initial weight of 342 $\pm$ 10.1 kg and increased to 367 $\pm$ 11.3 kg by d 140 for an increase of 25 kg. Body weight for both treatment groups increased steadily from d 0 to d 84 and remained relatively steady through the duration of the training period to d 140. Mean height for the stalled horses at d 0 was 143 $\pm$ 1.0 cm, increasing 3 cm to 146 $\pm$ 1.5 cm by d 140. Pastured horses also began the project at an average height of 143 $\pm$ 1.0 cm and increased 3 cm to 146 $\pm$ 1.6 cm by d 140. Third metacarpal circumference was 18.4 $\pm$ 0.4 cm at the start of the project and 18.6 $\pm$ 0.3 cm by d 140 in the horses kept in stalls. Pastured horses had an initial third metacarpal circumference of 18.6 $\pm$ 0.4 cm compared to 19.0 $\pm$ 0.4 cm by d 140. Body condition score (BCS) remained relatively unchanged for both treatment groups. Stalled horses began the project at an initial BCS of 6.2 $\pm$ 0.1 and increased 0.5 to a BCS of 6.7 $\pm$ 0.2 at d 140. Body condition score for the pastured horses was 6.1 $\pm$ 0.1 at d 0 and 6.4 $\pm$ 0.2 at d 140 for an increase of 0.3. Body condition score for both treatment groups decreased from d 84 to d 112, as horses entered training, and subsequently increased through d 140.

## Elective Exercise

The number of strides per day of each gait, calculated from visual observation, indicated large variations between treatment groups in elective physical activity (Table 2).

Table 2. Calculated daily elective strides per horse from observation.

| | Walk | Trot | Canter | Total |
|---|---|---|---|---|
| Pastured | | | | |
| 24 hr on pasture | 4076 | 389 | 674 | 5139 |
| Stalled | | | | |
| 23 hr in stalls | 804 | 0 | 0 | 804 |
| 1 hr on mechanical walker | 3171 | 55 | 10 | 3236 |
| Total for 24 hr | 3975 | 55 | 10 | 4040 |

## Radiographic Bone Aluminum Equivalence

Analysis of the radiographs showed a trend toward a difference between the two treatment groups at d 0 in RBAE of both the lateral ($P < .15$) (Table 3) and medial cortices ($P < .1$) (Table 4) of the third metacarpal. Therefore, baseline values for lateral and medial RBAE for each horse were subtracted from RBAE values for the appropriate cortex on all other days for that animal. Analysis of the change in lateral RBAE from d 0 (Figure 1) showed that stalled horses had lower lateral RBAE than pastured horses at d 28 ($P < .05$), which remained lower through most of the duration of the project, while horses maintained on pasture had increasing lateral RBAE ($P < .05$). Pasture-reared horses had greater lateral RBAE at d 28, 56, and 140 ($P < .05$), and had a tendency to be greater at d 112 ($P = .07$). The change in RBAE of the medial cortex (Figure 2) tended to be different between treatments ($P = .1$). Horses kept in stalls had lower medial RBAE at d 28 ($P < .05$), which tended to remain lower than horses maintained on pasture through most of the project ($P = .1$). Medial RBAEs were greater at d 28 ($P < .05$) and tended to be greater at

Table 3. Means table for lateral RBAE (mm Al).

| Day | 0 | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|---|
| Stall | 20.423† | 19.495 | 19.499 | 20.062 | 19.843 | 19.841 |
| SEM | 0.313 | 0.315 | 0.304 | 0.425 | 0.415 | 0.269 |
| Pasture | 19.770 | 19.646 | 19.908 | 20.208 | 20.339 | 20.189 |
| SEM | 0.313 | 0.315 | 0.315 | 0.442 | 0.446 | 0.293 |

† in table indicates pasture different than stalled at given day ($P < .15$)

Table 4. Means table for medial RBAE (mm Al).

| Day | $0^{b}$ | $28^{c}$ | $56^{bc}$ | $84^{ab}$ | $112^{ab}$ | $140^{a}$ |
|---|---|---|---|---|---|---|
| Stall | 21.363† | 20.335 | 20.495 | 21.608 | 20.957 | 21.501 |
| SEM | 0.215 | 0.280 | 0.295 | 0.601 | 0.416 | 0.185 |
| Pasture | 20.788 | 20.566 | 20.938 | 21.214 | 21.762 | 21.533 |
| SEM | 0.215 | 0.280 | 0.308 | 0.629 | 0.450 | 0.202 |

† in table indicates pasture different than stalled at given day ($P < .1$)

$^{abc}$Days lacking a common superscript differ ($P < .05$)

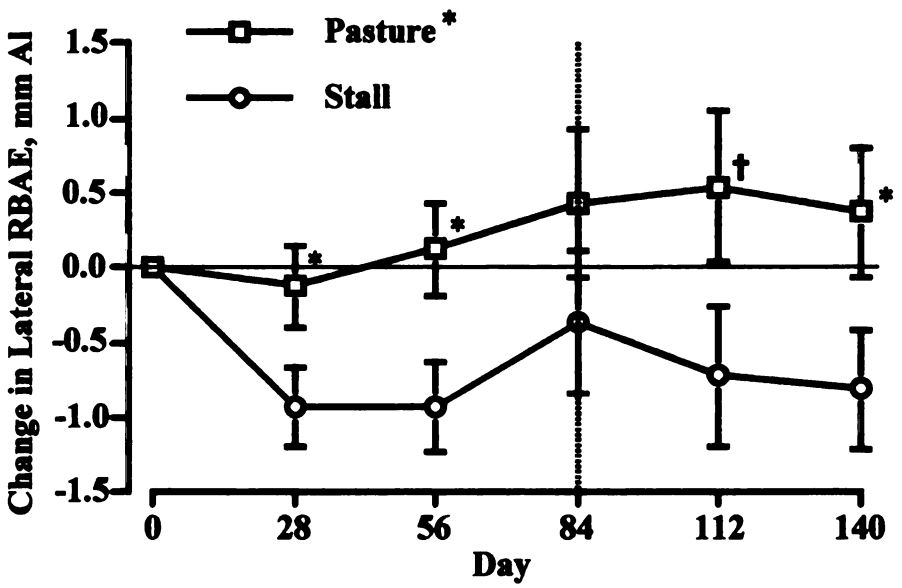


Figure 1. Change in Lateral RBAE (mm Al) versus day of project.

* in legend indicates pasture different than stalled ($P < .05$)

* in graph indicates pasture different than stalled at given day ($P < .05$)

† in graph indicates pasture different than stalled at given day ($P = .07$)

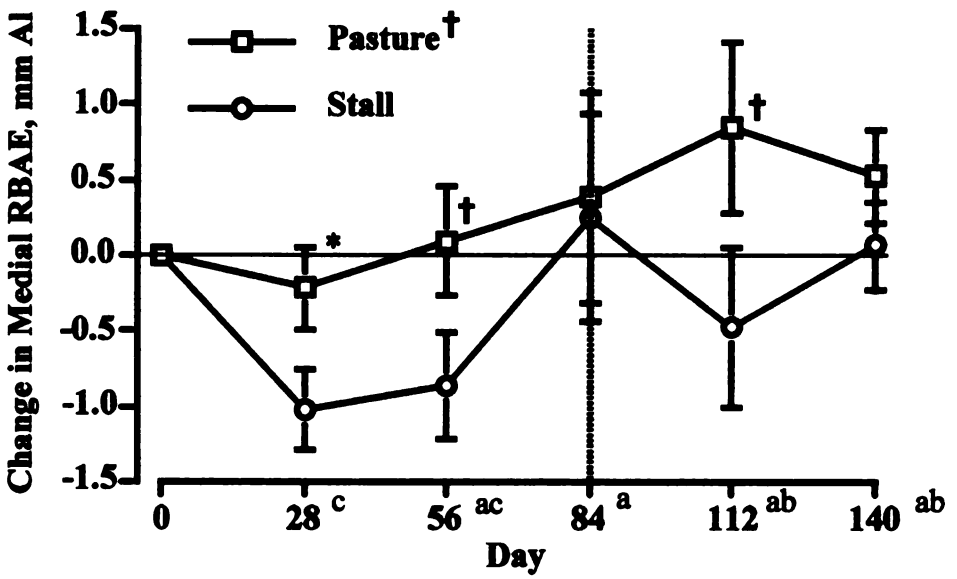


Figure 2. Change in Medial RBAE (mm Al) versus day of project.
† in legend indicates pasture different than stalled (P = .1)
* in graph indicates pasture different than stalled at given day (P < .05)
† in graph indicates pasture different than stalled at given day (P < .1)
[abc]Days lacking a common superscript differ (P < .05)

d 56 and d 112 (P < .1) in horses maintained on pasture, compared with horses housed in stalls. Radiographic bone aluminum equivalence of the medial cortex increased from d 28 to d 112. No treatment differences occurred in total RBAE of the third metacarpal (Table 5). For both treatment groups, total RBAE remained relatively unchanged throughout the project, except for a slight decrease from d 112 to d 140.

## Serum Osteocalcin

No treatment differences occurred in serum osteocalcin concentrations (Figure 3), however serum osteocalcin was lower in the stalled horses at d 14 when compared to horses maintained on pasture (P < .05). Following d 14, osteocalcin concentrations in the stalled horses returned to baseline. A high degree of variation in osteocalcin

Table 5. Means table for total RBAE ($mm^2$ AI)

| Day | 0[ab] | 28[ab] | 56[ab] | 84[ab] | 112[a] | 140[b] |
|---|---|---|---|---|---|---|
| Stall | 526.808 | 486.477 | 523.633 | 546.850 | 539.985 | 500.840 |
| SEM | 27.159 | 35.500 | 30.032 | 83.375 | 52.816 | 35.497 |
| Pasture | 491.257 | 505.700 | 574.647 | 528.663 | 577.435 | 482.279 |
| SEM | 27.159 | 35.500 | 30.912 | 85.882 | 56.353 | 38.496 |

[ab]Days lacking a common superscript differ ($P < .05$)

concentrations present in the serum of pastured and stalled horses occurred during the latter half of the project. Stalled horses tended to have greater osteocalcin than pastured horses at d 98 ($P < .1$), while pastured horses indicated a trend toward greater osteocalcin concentrations than horses housed in stalls at d 140 ($P < .1$). Serum osteocalcin decreased in both treatment groups from d 112 to 126, followed by an increase through d 140.

## Urinary Deoxypyridinoline and Pyridinoline

Urinary deoxypyridinoline concentrations based on creatinine (Figure 4) were greater at d 28 in horses housed in stalls than in horses maintained on pasture ($P < .01$). Similar results occurred in urinary deoxypyridinoline concentrations based on total daily urinary output ($P < .1$) (Figure 5). Following d 28, deoxypyridinoline in the stalled horses returned to baseline. No other treatment or time differences occurred in urinary deoxypyridinoline. Urinary pyridinoline concentrations (Table 6) appeared to decrease in both treatment groups through d 84, and subsequently increase slightly through d 140. No treatment differences occurred in pyridinoline concentrations.

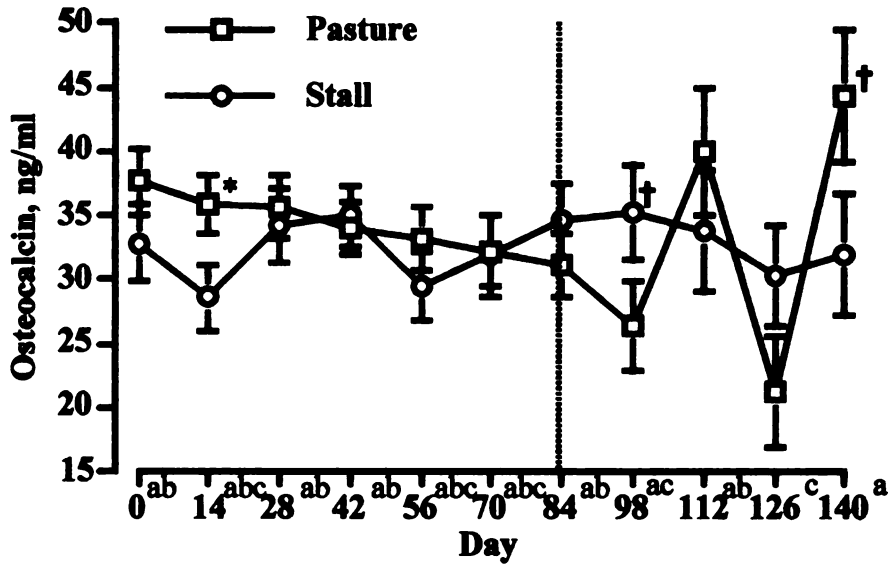


Figure 3. Serum osteocalcin (ng/ml) versus day of project.
* in graph indicates pasture different than stalled at given day ($P < .05$)
† in graph indicates pasture different than stalled at given day ($P < .1$)
[abcd]Days lacking a common superscript differ ($P < .05$)

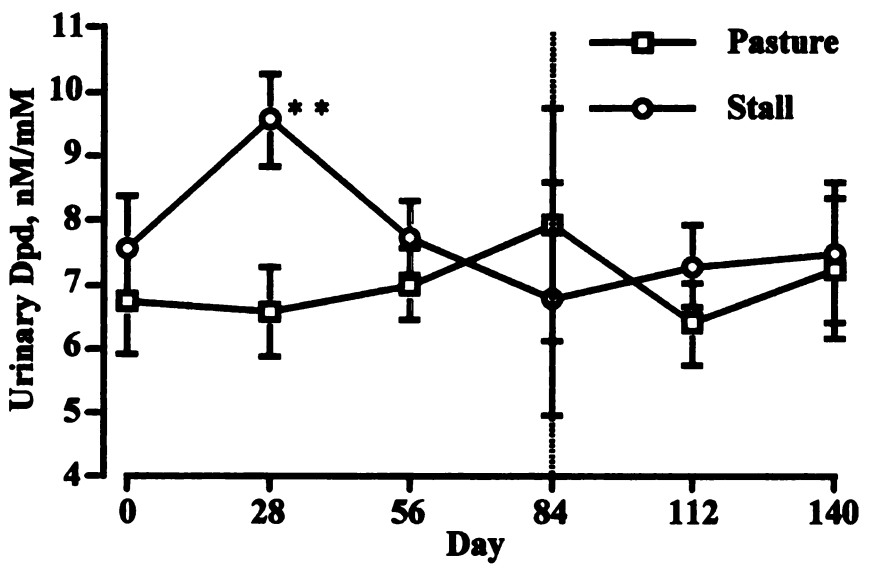


Figure 4. Urinary deoxypyridinoline (nM/mM) versus day of project.
** in graph indicates pasture different than stalled at given day ($P < .01$)
n = 4 for each treatment group

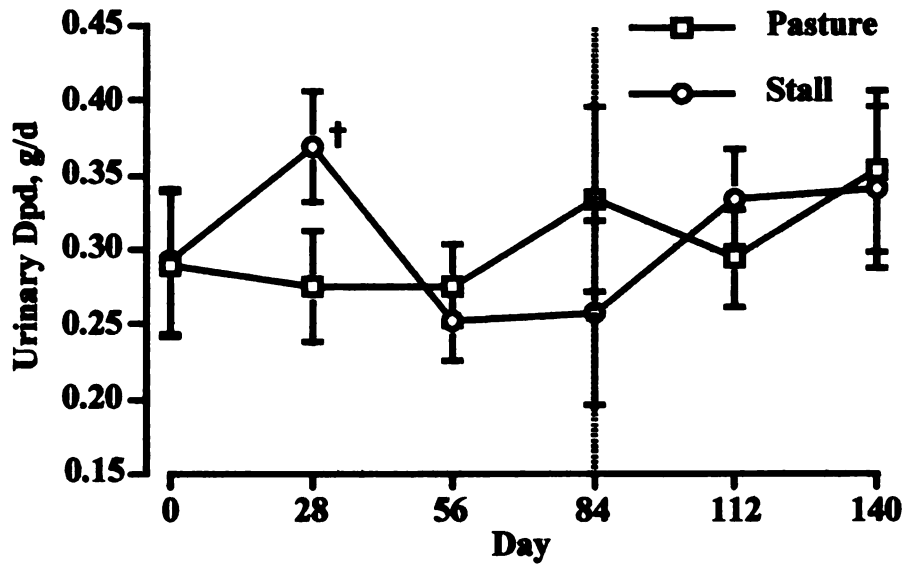


Figure 5. Urinary deoxypyridinoline (g/d) versus day of project.
† in graph indicates pasture different than stalled at given day (P < .1)
n = 4 for each treatment group

Table 6. Means table for urinary pyridinoline (nM/mM).

| Day | 0[a] | 28[a] | 56[a] | 84[b] | 112[ab] | 140[a] |
|---|---|---|---|---|---|---|
| Stall | 263.266 | 219.822 | 180.592 | 145.179 | 144.456 | 184.594 |
| SEM | 39.386 | 36.616 | 23.109 | 15.507 | 21.898 | 24.571 |
| Pasture | 155.831 | 156.248 | 153.397 | 123.926 | 158.813 | 174.615 |
| SEM | 39.386 | 36.616 | 23.109 | 14.202 | 21.898 | 24.571 |

[ab]Days lacking a common superscript differ (P < .05)
n = 4 for each treatment group

## Serum Minerals, Vitamins, and Hormones

No overall treatment differences occurred in serum Ca concentrations (Table 7). In both the stalled and pastured horses, serum Ca appeared to increase slightly from d 0 to d 14, followed by a decrease through d 84, remained relatively stable up to d 112 and then increased through d 140. A high degree of variation occurred between treatments in serum Ca concentrations during the latter half of the project. At d 84, horses housed in stalls tended to have greater serum Ca ($P < .1$), however pastured horses had greater serum Ca at d 98 ($P < .01$). Following d 98, concentrations of serum Ca did not differ between treatment groups. Serum P concentrations (Table 8) were lower in the stalled horses at d 0 when compared to horses maintained on pasture ($P < .01$), and remained lower through most of the project ($P < .05$). Pasture-reared horses had greater serum P concentrations at d 0, 28, 112, and 140 ($P < .01$), and at d 70 ($P < .05$), however stalled horses had greater serum P at d 98 ($P < .05$). Serum P increased in both treatment groups from d 0 to d 14 and then decreased below baseline values by d 28, increasing slightly through d 84. From d 84 to d 98, serum P decreased markedly, changed little through d 126, and then increased through d 140. No treatment differences occurred in serum Vit D or PTH concentrations (data not shown).

## Urinary Minerals

No treatment differences between stalled and pastured horses occurred in urinary Ca (Table 9) or P (Table 10) concentrations. In both treatment groups, urinary Ca decreased from d 0 to d 84 and then increased through d 140 to concentrations similar to those found in the first half of the project. Urinary P concentrations in stalled and

Table 7. Means table for serum calcium (mg/dl).

| Day | 0[a] | 14[a] | 28[a] | 42[a] | 56[b] | 70[cd] |
|---|---|---|---|---|---|---|
| Stall | 12.778 | 13.275 | 13.067 | 13.244 | 11.926 | 11.317 |
| SEM | 0.273 | 0.241 | 0.440 | 0.268 | 0.425 | 0.254 |
| Pasture | 13.188 | 13.484 | 13.350 | 12.793 | 12.111 | 11.265 |
| SEM | 0.273 | 0.241 | 0.440 | 0.268 | 0.425 | 0.254 |

| Day | 84[e] | 98[cde] | 112[de] | 126[bc] | 140[bc] |
|---|---|---|---|---|---|
| Stall | 11.127† | 10.446** | 11.051 | 11.575 | 11.902 |
| SEM | 0.306 | 0.295 | 0.267 | 0.247 | 0.403 |
| Pasture | 10.396 | 11.588 | 10.601 | 11.230 | 11.230 |
| SEM | 0.306 | 0.295 | 0.292 | 0.271 | 0.441 |

**in table indicates pasture different than stalled at given day ($P < .01$)

† in table indicates pasture different than stalled at given day ($P < .1$)

[abcde]Days lacking a common superscript differ ($P < .05$)

Table 8. Means table for serum phosphorus (mg/dl).

| Day | 0[c] | 14[a] | 28[d] | 42[bc] | 56[c] | 70[ab] |
|---|---|---|---|---|---|---|
| Stall* | 4.829** | 6.216 | 4.471** | 5.499 | 5.371 | 5.371* |
| SEM | 0.192 | 0.251 | 0.142 | 0.184 | 0.202 | 0.265 |
| Pasture | 5.635 | 5.964 | 4.996 | 5.370 | 5.264 | 6.138 |
| SEM | 0.192 | 0.251 | 0.142 | 0.184 | 0.202 | 0.265 |

| Day | 84[ab] | 98[d] | 112[d] | 126[d] | 140[ab] |
|---|---|---|---|---|---|
| Stall | 5.635 | 5.072* | 4.096** | 4.605 | 4.951** |
| SEM | 0.267 | 0.293 | 0.135 | 0.238 | 0.344 |
| Pasture | 6.020 | 4.123 | 4.956 | 4.678 | 6.959 |
| SEM | 0.267 | 0.293 | 0.146 | 0.261 | 0.377 |

* in heading indicates pasture different than stalled ($P < .05$)

** in table indicates pasture different than stalled at given day ($P < .01$)

* in table indicates pasture different than stalled at given day ($P < .05$)

[abcd]Days lacking a common superscript differ ($P < .05$)

pastured horses appeared to follow each other in a slight increase throughout the project, although urinary P in the pastured horses increased to a greater degree than in the stalled horses following d 112, creating a tendency for pastured horses to have greater urinary P at d 140 ($P < .1$).

Table 9. Means table for urinary calcium (g/d).

| Day | $0^{a}$ | $28^{a}$ | $56^{b}$ | $84^{c}$ | $112^{ab}$ | $140^{ab}$ |
|---|---|---|---|---|---|---|
| Stall | 0.446 | 0.582 | 0.303 | 0.154 | 0.308 | 0.372 |
| SEM | 0.122 | 0.210 | 0.062 | 0.032 | 0.086 | 0.120 |
| Pasture | 0.677 | 0.704 | 0.334 | 0.262 | 0.456 | 0.589 |
| SEM | 0.122 | 0.210 | 0.062 | 0.032 | 0.086 | 0.120 |

$^{abc}$Days lacking a common superscript differ ($P < .05$)
n = 4 for each treatment group

Table 10. Means table for urinary phosphorus (g/d).

| Day | $0^{d}$ | $28^{cd}$ | $56^{ac}$ | $84^{ab}$ | $112^{cd}$ | $140^{a}$ |
|---|---|---|---|---|---|---|
| Stall | 62.222 | 143.638 | 122.266 | 159.413 | 124.478 | 111.841† |
| SEM | 21.053 | 61.161 | 46.499 | 76.238 | 44.237 | 113.857 |
| Pasture | 56.928 | 41.520 | 129.003 | 313.308 | 119.228 | 420.558 |
| SEM | 21.053 | 61.161 | 46.499 | 76.238 | 44.237 | 113.857 |

† in table indicates pasture different than stalled at given day ($P < .1$)
$^{abcd}$Days lacking a common superscript differ ($P < .05$)
n = 4 for each treatment group

# CHAPTER 4

# DISCUSSION

A horse's skeletal system will reach full maturity at approximately 4 yr of age (El Shorafa et al., 1979). Lawrence et al. (1994) reported that maximum bone mineral content and breaking strength are not reached in the equine third metacarpal until approximately 6 yr of age, and maximum breaking load is not reached until around 5 yr of age. The most significant changes in the cross-sectional area and inertial properties of the third metacarpal bone, indicative of the bone's resistance to compressive stress and of the bending strength of the bone by alterations in mass around its central axis, respectively, have been shown to occur between a Thoroughbred's yearling and 2-yr-old yr (Nunamaker et al., 1989). El Shorafa et al. (1979) demonstrated that in the horse, bone mineral content increases sharply through the first year of life, and subsequently increases further up until approximately 7 yr of age, but at a much slower rate. These studies indicate that the skeletal system of the young, growing horse is very dynamic and has the capability of developing to its full genetic and functional potential. During growth, the modeling and remodeling mechanisms are important in the development and maintenance of an optimal skeletal structure for a young horse in training and competition.

If the functional strain on the limbs is suddenly reduced, as in bed rest (LeBlanc et al., 1990) or immobilization (Kannus et al., 1996), the skeletal architecture responds by lowering bone mass to a level appropriate for the new strain (Lanyon, 1984). Results of this study suggest that housing yearling horses in stalls with limited access to exercise may negatively affect bone growth compared to that experienced by yearlings allowed to

remain on pasture, thus predisposing the former group to injury when higher strain rates are introduced during training or competition. These negative effects, as indicated by analysis of RBAEs, osteocalcin concentrations, and deoxypyridinoline concentrations, were most apparent between 14 and 28 d of placement of yearlings in stalls.

Nielsen et al. (1997) determined that fewer skeletal-related injuries occurred to young horses in initial training when RBAEs were greater in the medial and lateral cortices of the third metacarpal, compared with the palmar cortex, at the commencement of training. The majority of changes to the bone mineral content of the third metacarpal in response to training occurred in the medial and lateral cortices. Based on the findings of this study, estimated bone mineral content in the present study was determined for only the medial and lateral cortices of the third metacarpal.

Radiographic bone aluminum equivalencies have been highly correlated with bone mass (Meakim et al., 1981), which, in turn, has been described as the best measurable determinant of bone strength (Kimmel, 1993). The lower RBAEs of both the medial and lateral cortices of the third metacarpal in the stalled horses, compared with the pastured horses, at d 28 of the project indicated not only loss of bone, but also loss of bone strength. This is supported by the results of a study conducted by Nielsen et al. (1997) examining the changes in skeletal strength of 53 Quarter Horses put into race training at 18 mo of age. Using a technique correlating RBAE and total bone mineral content (Nielsen and Potter, 1997), Nielsen et al. (1997) demonstrated a decrease in RBAE of the total cross-sectional area of the third metacarpal soon after the onset of race training. The lowest RBAEs were attained 60 to 100 days after the start of training, at which time speed was introduced into the training program and the highest number of skeletal-related

injuries occurred. Prior to the start of training, the horses were kept on pasture with free access to exercise. Upon commencement of training, all horses were maintained in stalls at all times except when they were being exercised. It was not determined whether the decrease in bone mineral content was the result of bone remodeling caused by increased strain rates on the bone associated with training or the result of bone modeling resulting from decreased strain rates associated with the change in housing from pasture to stalls at the start of training.

Although RBAE measurements in the present study did not indicate differences in total bone mineral content between the stalled and pastured horses, if the results obtained by Nielsen et al. (1997) were in response to the change in housing more so than to the commencement of training, these studies support the hypothesis that limiting a young horse's access to free exercise will result in negative effects on bone development. Horses were transferred from pasture to stalls and placed into training at d 0 of the project conducted by Nielsen et al. (1997). The lowest total RBAEs of the third metacarpal did not appear to occur until 60 to 100 d after the commencement of training and change in housing. However, after d 0, a second set of radiographs was not taken until d 64 of the project. Therefore, we do not know if the lowest RBAEs were actually attained prior to d 60, as in the present study, in which the lateral and medial cortices of the third metacarpal attained the lowest RBAEs within 28 d of stalling and subsequently returned to baseline after d 28. In the present study, lateral RBAEs remained lower in the stalled horses than in the horses maintained on pasture through the duration of the project. Similarly, medial RBAEs tended to remain lower in stall-reared horses when compared with pastured horses though most of the project. Both lateral and medial RBAEs were unaffected by the

commencement of training at d 84. A lower intensity conditioning program was used in the first two 28-d periods of training in the study by Nielsen et al. (1997), when compared with the training regimen used in the 56-d training period in the present study. Therefore, the decrease in bone mineral content of the third metacarpal demonstrated soon after the onset of race training in the study by Nielsen et al. (1997) was most likely the result of bone modeling due to decreased strains on the bone associated with the change in housing from pasture to stalls at the start of training. The apparent longer period of bone loss, compared to that in the present study, may have been the result of the horses receiving some mechanical strain on the load-bearing bones associated with training, causing a slowdown in the rate of bone loss. The immediate decrease in bone mineral content in the stalled horses in the present study is not surprising, considering that housing conditions were the only treatment differences at d 0 of the project.

A study was conducted at the University of Florida looking at the effects of race training on bone mineral content of the third metacarpal in young Thoroughbred horses under field conditions (Porr et al., 1997). Two treatment groups were designated by differences in maturity. Group A consisted of horses visually determined to be more mature than horses in Group B. As colts are normally larger in body size than fillies at around 2 yr of age, Group A was made up of 11 colts and only 5 fillies. Group B consisted of horses that appeared to be less developed than horses in Group A, and therefore contained 8 fillies and only 5 colts. The mean age of the horses at the commencement of training was younger in Group A than in Group B. In addition to the uneven numbers of male and female horses in each group, primary housing conditions differed between groups. Fillies were placed, as a group, into a large pasture, while colts

were paired by temperament and placed into small paddocks. Investigators in that study concluded that the decrease in bone mineral content observed in Group A shortly after commencement of training was the result of horses in Group A beginning training at an earlier age than Group B, which demonstrated an increase in bone mineral content upon commencement of training. However, results of the present study suggest that the differences observed between groups in the response of bone mineral content of the third metacarpal to the onset of training was the result of the differences in the primary housing conditions between groups. Group A consisted of more male horses housed in small paddocks, while Group B consisted of more female horses maintained in a large pasture as a group.

In comparison to the stalled horses, medial RBAEs in the pastured horses tended to increase in a pattern of growth, and lateral RBAEs appeared to follow a similar pattern, although day differences were statistically insignificant for lateral RBAE. During normal bone growth, which, according to El Shorafa et al. (1979), is not completed until approximately 4 yr of age in the horse, bone formation is occurring faster than bone resorption (Maenpaa et al., 1988), resulting in a gradual increase in bone mineral content. The horses kept in stalls showed an increase in medial RBAE from d 56 through d 84 ($P < .05$), thus returning to baseline. This may indicate that the skeletal systems of the stalled horses had adapted to a new, lower functional load and subsequently returned to a pattern of bone growth appropriate for their optimal strain environment. After commencement of training at d 84, medial RBAEs in stalled horses ceased increasing and even appeared to decrease. If training had not been introduced at d 84, it is speculated whether the stalled horses would have eventually returned to a similar bone mineral content and growth

pattern as the pastured horses.

No treatment differences occurred in total RBAE of the third metacarpal and, except for a slight decrease from d 112 to d 140 for both treatment groups combined, remained relatively unchanged throughout the project. The slight decrease at d 112 may indicate an adaptive response to training, although lateral and medial RBAEs did not follow this pattern of demineralization and therefore its relevance is skeptical.

Osteocalcin is a biochemical marker of bone turnover synthesized predominantly by osteoblasts, or bone-forming cells (Price et al., 1980), and is thus considered a measure of osteoblastic activity (Price, 1982). In the present study, lower serum osteocalcin concentrations in the stalled horses at d 14 support the apparent bone loss demonstrated by RBAEs as an indication of a slower rate of osteoblastic activity resulting from a decrease in physical activity. Maenpaa et al. (1988) reported a similar immediate decrease in osteocalcin in foals transferred from pasture to stalls for the winter months. A marked decrease in serum osteocalcin concentrations occurred within 1 mo of stalling in response to the diminished mechanical strain on the limbs of the foals associated with stalling.

Except for the low serum osteocalcin in the stalled horses at d 14 and the unexplainable variation in osteocalcin concentrations in both the pastured and stalled horses during the 56-d training period, osteocalcin concentrations in the serum of both treatment groups remained relatively stable. This stability suggests a steady rate of osteoblastic cell activity considered normal in young horses experiencing growth and development. Studies have shown that serum osteocalcin concentrations are high in young horses and decrease with age, indicating a slowdown in the rate of bone formation in mature horses compared to foals (Lepage et al., 1990). Regardless of whether bone

formation was due to bone growth by the modeling mechanism or the replacement of immature or damaged bone by the remodeling mechanism, osteocalcin concentrations measured in the present study were relatively normal for long yearling/two-yr-old horses. Lepage et al. (1990) determined that mean osteocalcin concentrations for female horses less than 1 yr of age and between 1.5 and 2.5 yr were 47.3 and 35.7 ng/mL, respectively. The mean osteocalcin concentration for both treatment groups combined was 33.1 ng/mL in the present study.

In contrast to osteocalcin, urinary deoxypyridinoline and pyridinoline are released by the activity of osteoclastic cells, or bone-degrading cells, and are thus considered indicators of bone resorption (Gomez et al., 1994). However, differences in the origin and activity of these pyridinium cross-links may influence their comparative usefulness as markers of bone resorption. Pyridinoline is primarily present in bone and cartilage matrix and deoxypyridinoline is present in bone collagen of the organic matrix (Delmas, 1993; Robins et al., 1994). These crosslinks are released into circulation by the action of osteoclastic cells as bone collagen is digested during the degradation process (Gomez et al., 1996). Pyridinoline has also been reported to be released from sources other than bone in response to other degenerative conditions (Robins et al., 1994). Therefore, except for a slight decrease in urinary pyridinoline concentrations in both treatment groups through d 84 of the present study, followed by a slight increase through d 140, it is not surprising that no treatment differences occurred in pyridinoline concentrations. Additionally, the small number of horses represented in each treatment group (n = 4) during urine collections may have contributed to the lack of specificity of pyridinoline as a marker of bone resorption. A difference between treatments may have been more

apparent with larger sample numbers.

Urinary deoxypyridinoline concentrations were greater in the stalled horses than in the horses maintained on pasture at d 28. Allowing that deoxypyridinoline is a reliable marker of bone degradation, results indicate that stalled horses were experiencing a period of increased bone resorption 28 d after being placed into stalls. The apparent decrease in bone formation activity at d 14, as indicated by low serum osteocalcin, leads to the speculation of whether urinary deoxypyridinoline could have been at its highest concentration when osteocalcin was at its lowest. Without urine samples at d 14 for determination of deoxypyridinoline concentrations, this remains unanswerable. Vico et al. (1987) demonstrated an inverse relationship between bone resorption and bone formation in healthy male subjects voluntarily subjected to 120 d of bed rest. Bone biopsy data showed that a decrease in mineralization rate and a simultaneous increase in bone cell resorption activity occurred in completely immobilized subjects.

In comparison to the stalled horses in the present study, pastured horses maintained a relatively stable concentration of urinary deoxypyridinoline through the duration of the project. Black et al. (1997) demonstrated a strong correlation between both osteocalcin and deoxypyridinoline concentrations and skeletal growth patterns in foals. Osteocalcin and deoxypyridinoline concentrations reportedly decreased as specific growth rates, including body weight, girth, and wither height, gradually decreased from birth to 1 yr of age. Although deoxypyridinoline concentrations did not follow a similar pattern in the present study, this may be related to differences in the age of the horses participating in each study and a faster growth rate occurring in the younger horses.

Stalled horses quickly equilibrated to the decrease in strain on the load-bearing

bones, as demonstrated by low osteocalcin concentrations in the stalled horses, as well as high deoxypyridinoline concentrations, returning to baseline following d 14 and d 28, respectively. This equilibration suggests that an adaptive mechanism was triggered to appropriately match the skeletal architecture to the new functional load (Lanyon, 1987). The lower loads placed on the bone directly or indirectly indicated to the bone cells responsible for the adaptive response that unnecessary energy was being used to maintain and transport the initial skeletal mass (Rubin and Lanyon, 1985; Lanyon, 1987). The process of bone modeling lowers bone mass until a new optimal strain environment is reached (Lanyon, 1984).

No overall treatment differences occurred in serum Ca concentrations in the present study, although a slight increase in serum Ca appeared to occur in the stalled horses from d 0 to d 14. The immediate increase in serum Ca appears to reflect the release of Ca from bone into the circulation during bone resorption. This is supported by high urinary deoxypyridinoline concentrations in the stall-reared horses at d 28, indicative of a high rate of bone resorption, and the low lateral and medial RBAEs at d 28, indicating a loss of bone mineral content. Krook (1968) has demonstrated an increase in serum Ca associated with the release of Ca into the circulation during bone resorption. Nielsen et al. (1998) also reported an inverse relationship between serum Ca and RBAEs during a period of bone resorption in the early stages of a training program.

Following d 14, serum Ca concentrations in both the stalled and pastured horses decreased until the commencement of training at d 84, remained relatively stable through d 112, and then increased through d 140. The decrease in serum Ca through d 84 may be attributable to the deposition of Ca into bone from the circulating Ca pool in support of a

period of bone formation (Bronner, 1993). This makes sense in the horses maintained on pasture because they appeared to be experiencing a pattern of skeletal growth, based on medial and lateral RBAEs. Medial RBAE increased in the stalled horses from d 56 through d 84 ($P < .05$), possibly indicating that they too were experiencing bone growth at their new optimal strain environment, albeit at lower RBAEs than those found in the pastured horses. Both lateral and medial RBAEs were unaffected by the commencement of training, which may explain the lack of activity in serum Ca concentrations in both treatment groups through d 112 of the project.

Serum Ca concentrations are maintained within a narrow range through tight regulation by the body (Krook and Lowe, 1964). A secondary function of bone remodeling, in addition to an adaptive response to mechanical strain on the load-bearing bones and replacement of old or damaged bone, is to maintain serum Ca levels (Riggs and Evans, 1990). In addition to bone, Ca can be taken from the diet and muscle tissue in order to maintain conditions of mineral homeostasis in the blood (Bronner, 1993). Due to the body's tight regulation of the Ca pool, large deficiencies in dietary Ca intake are necessary to cause marked variations in serum Ca concentrations. In the present study, serum Ca concentrations were within the normal range for horses (Duncan and Prasse, 1986) and diets were fed to satisfy NRC (1989) recommendations for the age and activity of the horses. Therefore, the variation in Ca concentrations in the serum of the stalled and pastured horses were not likely the result of Ca intake.

Lepage et al. (1991) determined that serum phosphate concentrations follow an inverse pattern to serum Ca concentrations, serum phosphate being low when serum Ca is high, based on analysis of both minerals over a 24 hr period. This pattern was not

observed in the present study. In fact, P concentrations in the serum of stalled horses increased dramatically from d 0 to d 14 ($P < .01$), similar to the apparent increase in serum Ca concentrations during the same 14-d period in the stalled group. This increase in serum P may also be the result of the release of bone mineral into circulation during a period of bone resorption.

Serum P concentrations in the stalled and pastured horses were different at d 0, the stalled horses having lower P concentrations when compared with horses maintained on pasture. Serum P concentrations are not as tightly regulated as Ca concentrations in the serum, therefore variations in dietary intake will quickly affect serum P. Cymbaluk and Christison (1989) demonstrated that feeding varying amounts of P in the diet directly influences serum P concentrations in horses. For example, feeding a diet higher in P results in higher P concentrations in the serum. In the present study, the difference in serum P concentrations between the two treatment groups at d 0 may be reflective of a reduction in hay intake, and thus a decrease in dietary P intake, by the stalled horses due to the stress caused by the change in housing from pasture to stalls. The high degree of variation in serum P concentrations between the two treatment groups during most of the project may also be attributable to variations in dietary P intakes. Serum P concentrations were within the normal range for horses (Duncan and Prasse, 1986).

Urinary Ca output can accumulate from a number of sources, including the diet, muscle tissue, and bone (Bronner, 1993). In human subjects, fasting urinary Ca measured from a morning sample and corrected by creatinine excretion is useful in detecting a notable increase of bone resorption activity (Delmas, 1993). Using urine samples from 24-hr urine collections, urinary Ca concentrations were easily determined in the present

study by atomic absorptiometry. Urinary Ca concentrations are highly variable, depending on, for example, the age of the animal, growth factors, and dietary intake. In the present study, daily concentrations of Ca excreted in the urine appeared low, compared to other research data (Schryver et al., 1974; Nielsen et al., 1997). Failure to solubilize calcium carbonate prior to analysis of urinary Ca may have caused concentrations to be low. Interestingly, visual appraisal of the graphs for total RBAE of the third metacarpal and urinary Ca in the stalled horses indicated an inverse relationship. As total RBAEs appeared to decrease from d 0 to d 28, subsequently increase through d 84, and then decrease from d 84 to d 140, urinary Ca in the stalled horses appeared to increase through d 28, decrease through d 84, and then increase through d 140. Decreasing RBAEs indicated periods of bone resorption, during which Ca was released from bone into the circulation and ultimately excreted in the urine or feces. In contrast, increasing RBAEs indicated a period of bone formation, hence the reduction in Ca excretion through the urine. Arnaud et al. (1986) demonstrated an increase in renal and fecal excretion of Ca in healthy subjects immobilized by bed rest for 20 wk.

No treatment differences occurred between pastured and stalled horses in urinary P concentrations, except for a tendency for pastured horses to have greater urinary P at d 140. The amount of P provided by the diet, which was calculated to meet NRC (1989) recommendations, was probably adequate to meet the needs of the horses in this study.

Both vitamin D and PTH play a major role in maintaining serum Ca homeostasis by enhancing Ca absorption efficiency and mobilizing Ca from stores in the bone (Holick, 1996). Parathyroid hormone synthesis and secretion is stimulated by low serum Ca concentrations, which, in turn, increases tubular reabsorption of Ca in the kidney and

stimulates the production of active 1,25-dihydroxyvitamin $D_3$ (1,25-$(OH)_2D_3$) from 25-hydroxyvitamin D (Vit D) in the kidney. Concentrations of Vit D have been shown to accurately reflect concentrations of 1,25-$(OH)_2D_3$ in the serum (Kumar, 1990). Normal serum Ca concentrations are restored by the direct stimulation of osteoclastic resorption by 1, 25-$(OH)_2D_3$ and PTH as they increase differentiation of preosteoclasts into new osteoclasts and increase the activity of existing osteoclasts (Jee, 1988). Additionally, the efficiency of intestinal Ca absorption is increased by 1, 25-$(OH)_2D_3$ (Holick, 1996). Serum Ca, once within a normal range, regulates synthesis and secretion of PTH from the parathyroid gland (Pocotte et al., 1991). The role of Vit D in bone metabolism appears to be the maintenance of adequate mineral concentrations for the deposition of hydroxyapatite in the bone matrix, accomplished by maintaining intestinal Ca absorption efficiency (Holick, 1996).

No treatment differences occurred in serum Vit D and serum PTH concentrations in the present study. Holick (1994) reported that 80 to 90% of the human body's requirement for vitamin D can be obtained from exposure to sunlight alone. Pastured horses had free access to sunlight, except for short 20 to 30 min training periods that were conducted in a round pen or indoor arena. Stalled horses had access to direct sunlight while being walked daily on a mechanical walker during the 84-d pre-training period and on non-riding days during the 56-d training period, as well as during training periods conducted in an outdoor arena or on the exercise track. Additionally, each horse had access to sunlight through a window in each individual stall. Adequate Vit D would allow Ca absorption efficiency, and, in turn, mineral homeostasis to be maintained (Holick, 1996). Adequate serum Ca, demonstrated in the present study, would subsequently

suppress PTH synthesis and secretion (Pocotte et al., 1991). Roussel et al. (1987) demonstrated that manipulation of serum Ca concentrations in healthy horses produced an expected fluctuation in PTH concentrations. Results of this study support Dalin and Jeffcott (1994) in their report that PTH and Vit D have limited usefulness as markers of bone metabolism, under conditions of adequate sunlight exposure.

Stalling did not appear to influence body weight, height at withers, third metacarpal circumference, or body condition score, as no treatment differences occurred in any of these body measurements. Body weight in both treatment groups increased through the start of training at d 84, which is attributable to normal growth. Body condition score dropped for both stalled and pastured horses between d 84 and d 112 and subsequently increased through d 140, most likely due to the increased energy needs associated with training. Horses housed in stalls appeared to be heavier in weight compared to pastured horses, although body condition scores were not different between treatments. The visual appearance of weight gain in the stalled horses was most likely due to a greater hay intake, compared to the horses maintained on pasture, causing enlargement of the gastrointestinal tracts of the stalled horses.

Initial conditioning did not appear to alleviate the negative effects of stalling on bone formation, as demonstrated by lower lateral RBAEs in the stalled horses than in the pastured horses during the training period. Perhaps the magnitude of strain applied to the metacarpal bone during training was insufficient to elicit an adaptive response (Lanyon, 1987). Microstrain applied to bone will increase linearly with an increase in velocity, thus galloping will place more strain on the load-bearing bones than walking or trotting (Nunamaker, 1986; Pratt, 1982). In the present study, based on observations of elective

exercise, each pastured horse cantered an average of 674 strides per day and each stalled horse cantered only 10 strides per day on the mechanical walker, therefore the pastured horses had free choice as to the speed used, while speed was restricted in the stalled horses. A study of competitive-age horses suggests that introducing speed work, or short, fast sprints, into the early stages of a training program, or even prior to the start of training, may place sufficient strain on the bone to prevent bone atrophy and facilitate normal bone growth (Nunamaker et al., 1990). Research conducted by Bruin (1993) involved repeatedly sprinting three-mo-old weanlings at short distances over a cement surface covered with approximately 2 to 3 cm of sand three times per wk for 21 mo. Although no biochemical or bone mineral content measurements were taken, at 2 1/2 yr of age, these horses were competing in 3-day eventing without much lameness. In essence, the most efficient and effective means of skeletal development appears to be short, fast sprinting, not the endurance training typically enforced in the early stages of a race conditioning program. This modification in training may result in the development of a skeletal structure appropriately prepared for both training and competition.

A study by Rubin and Lanyon (1985) reported a dose-response relationship between the magnitude of strain externally applied to the ulna of skeletally mature turkeys and the bone mass present. Strains below a certain peak magnitude resulted in bone loss, but above that magnitude bone formation exceeded any remodeling activity, resulting in bone mineral deposition and thus a stronger skeletal structure. Strain rate and distribution have also been shown to influence the remodeling process (Rubin and Lanyon, 1985). Caution should be used, however, in implying that the more diverse the training program, and therefore the more strain magnitudes, rates, and distributions that the bone has been

allowed to adapt to, the smaller the potential for injury due to the greater strength of the bone. The equine skeletal system must be gradually conditioned for the particular activity in which the horse is being prepared to compete, in order to maximize the strength and resistance to failure of bone with the least amount of bone tissue necessary, reducing the energy necessary to maintain and transport the bone tissue (Rubin, 1984; Lanyon, 1987). Simply, racehorses should be conditioned with at least some speed work, not just hand-walking. The more intense the activity, assuming the duration is not extensive and the force not too great so as to cause irreparable damage, the greater the adaptive response and ultimately the skeletal strength and durability (Lanyon, 1989). While Standardbred horses are conditioned at speeds at which they compete, Thoroughbred horses are often conditioned at speeds much lower than what they are expected to compete at during a race. The incidence of dorsal metacarpal disease is much lower in Standardbred horses. Hence, young Thoroughbred racehorses that are still growing are most likely at a higher risk of injury due to their inappropriately prepared skeletal structure (Nunamaker et al., 1990). Rubin and Lanyon (1984) also determined that the daily duration of training need not be excessive in order to stimulate bone. Using a functionally isolated, intact rooster ulna, artificially loading the bone with 36 strain cycles per day for 6 wk resulted in a 33% increase in bone mineral content. Beyond 36 cycles per day, no additional new bone formation occurred, indicating that a greater response from the cells responsible for the adaptive mechanism of bone remodeling did not occur. Only 4 peak strain cycles, similar to fast strides in the horse (Rubin and Lanyon, 1982), per day were determined necessary to prevent disuse osteoporosis in the rooster ulna.

Although it was not tested in this study, free access to exercise may have provided

sufficient loading on the legs of pastured horses to promote normal bone growth. The study by Nielsen et al. (1997) suggests that the horses may have experienced greater mechanical loading on their legs while on pasture, prior to d 0 of the project, than during the initial stages of conditioning, during which time they were maintained in stalls. Results of the present study indicate that housing yearling/two-yr-old horses in stalls without access to forced or free exercise may impair normal bone growth, compared with horses maintained on pasture.

# CHAPTER 5

# CONCLUSIONS AND RECOMMENDATIONS

A major concern with young performance horses is the high incidence of skeletal injury associated with a skeletal structure that is poorly prepared to handle the stress of training and competition. Young, growing horses are routinely housed in stalls in preparation for yearling sales or the commencement of training, without any exercise more intense than being hand-walked. Results of the present study suggest that housing yearling/two-yr-old horses in stalls with limited access to exercise may negatively affect bone growth compared to that experienced by horses allowed to remain on pasture and exercise freely.

Limiting a horse's access to free exercise can result in a reduction in strain applied to the bone, stimulating bone mineral resorption as part of the bone's adaptive response (Burr et al., 1989). Implications of this phenomena to the industry involve not only yearlings stalled in preparation for sales or training, but also young performance horses laid up following injury and subsequently returned to training. Results of the present study agree with Moyer and Fisher (1991) in suggesting that training should resume at a point retroactive to when injury occurred to account for the possible bone loss associated with a reduction in physical activity, thus preventing reoccurring or additional injuries. Bloomfield (1997) reported that after prolonged bed rest in healthy male subjects, the loss in bone mass was not fully regained after 6 mo of normal weightbearing activity. Even more significant, losses in muscle mass and strength resulting from bed rest were fully reversed weeks or even months before bone losses were recovered, thus contributing to

the risk of injury. Weinreb et al. (1997) conducted a study to investigate the recovery of bone loss during a reloading period following a short period of unloading in young, growing rats. After only 9 d of the complete unloading of one hind limb by external fixation, complete recovery of cancellous and cortical bone masses occurred after 2 and 3 wk, respectively, indicating that the rate of bone loss was much faster than the rate of bone mass recovery.

While incorporating speed work into a conditioning program is one option for facilitating normal bone growth, and thus possibly preventing injuries that may delay or even terminate a competitive career, other precautionary measures may be gleaned from this study. Allowing young horses primarily confined to stalls, and exercised under structured and supervised control, access to free exercise on pasture may supply the sufficient loading necessary to strengthen the skeletal system, as well as the opportunity for young horses to learn coordination and social skills. Further research is necessary to determine what length of time on pasture is necessary to establish a skeletal structure that is better equipped to handle the stress of training and competition. Providing young, growing horses with an environment that enhances bone development may result in a skeletal structure that is more functionally capable of withstanding the strains encountered during training. However, if free exercise is not a viable option or is out of the trainer's control, training must be modified accordingly to account for the possible bone loss experienced as a result of stalling. The younger the horse, the greater the potential for injury and benefit, therefore training must be monitored carefully (Ordidge, 1985).

Urinary deoxypyridinoline and serum osteocalcin appeared to be useful markers of bone metabolism in the present study. In contrast, the lack of treatment differences in

urinary pyridinoline concentrations suggest that pyridinoline's usefulness as a marker of bone resorption in limited. Deoxypyridinoline and osteocalcin clearly showed an increase in bone resorption and a decrease in bone formation, respectively, in the stalled treatment group shortly after placement in stalls. The pastured horses maintained relatively stable concentrations of both deoxypyridinoline and osteocalcin, indicating little alteration in osteoclastic and osteoblastic cell activity, which would be expected during a steady rate of bone growth. The change in bone resorption and formation activity in the stalled horses was supported by the decrease in bone mineral content in both the lateral and medial cortices of the third metacarpal in the stalled group. Thus, the noninvasive radiographic method of evaluating bone and the biochemical markers of bone resorption and formation appear to work in concert and suggest the usefulness of using both techniques in the determination of alterations in bone metabolism in a research setting.

# APPENDIX A

# APPENDIX A

## Means Tables

Appendix Table 1A. Means table for body weight (kg).

| Day | 0 | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|---|
| Stall | 348.769 | 367.500 | 389.432 | 399.663 | 392.831 | 405.060 |
| SEM | 10.165 | 10.580 | 11.030 | 11.419 | 11.296 | 11.090 |
| Pasture | 342.386 | 358.807 | 368.864 | 376.818 | 377.371 | 367.213 |
| SEM | 10.114 | 10.580 | 11.030 | 11.389 | 11.396 | 11.276 |

Appendix Table 2A. Means table for height at withers (cm).

| Day | 0 | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|---|
| Stall | 142.625 | 142.500 | 142.688 | 145.688 | 146.525 | 145.811 |
| SEM | 1.045 | 1.083 | 1.092 | 1.388 | 1.520 | 1.525 |
| Pasture | 143.031 | 142.813 | 143.313 | 144.625 | 145.433 | 146.494 |
| SEM | 1.045 | 1.083 | 1.092 | 1.388 | 1.548 | 1.575 |

Appendix Table 3A. Means table for third metacarpal circumference (cm).

| Day | 0 | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|---|
| Stall | 18.375 | 18.344 | 18.438 | 18.563 | 18.888 | 18.625 |
| SEM | 0.373 | 0.302 | 0.240 | 0.277 | 0.626 | 0.337 |
| Pasture | 18.563 | 18.813 | 18.625 | 18.781 | 19.790 | 19.040 |
| SEM | 0.373 | 0.302 | 0.240 | 0.277 | 0.651 | 0.358 |

Appendix Table 4A. Means table for body condition score.

| Day | 0 | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|---|
| Stall | 6.219 | 6.500 | 6.688 | 6.625 | 5.885 | 6.699 |
| SEM | 0.131 | 0.120 | 0.112 | 0.177 | 0.150 | 0.175 |
| Pasture | 6.125 | 6.25 | 6.375 | 6.500 | 5.900 | 6.400 |
| SEM | 0.131 | 0.120 | 0.112 | 0.177 | 0.161 | 0.190 |

Appendix Table 5A. Means table for change in lateral RBAE (mm Al).

| Day | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|
| Stall | -0.928 | -0.924 | -0.361 | -0.721 | -0.812 |
| SEM | 0.271 | 0.297 | 0.478 | 0.472 | 0.397 |
| Pasture | -0.124 | 0.123 | 0.427 | 0.542 | 0.370 |
| SEM | 0.271 | 0.307 | 0.493 | 0.505 | 0.431 |

Appendix Table 6A. Means table for change in medial RBAE (mm Al).

| Day | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|
| Stall | -1.028 | -0.868 | 0.245 | -0.478 | 0.061 |
| SEM | 0.269 | 0.358 | 0.685 | 0.530 | 0.286 |
| Pasture | -0.222 | 0.093 | 0.377 | 0.843 | 0.521 |
| SEM | 0.269 | 0.368 | 0.705 | 0.565 | 0.310 |

Appendix Table 7A. Means table for serum osteocalcin (ng/ml).

| Day | 0 | 14 | 28 | 42 | 56 | 70 |
|---|---|---|---|---|---|---|
| Stall | 32.818 | 28.580 | 34.231 | 34.947 | 29.463 | 31.842 |
| SEM | 2.966 | 2.569 | 2.888 | 2.311 | 2.761 | 3.172 |
| Pasture | 37.653 | 35.820 | 35.663 | 34.022 | 33.130 | 32.189 |
| SEM | 2.616 | 2.266 | 2.547 | 2.038 | 2.435 | 2.797 |

| Day | 84 | 98 | 112 | 126 | 140 |
|---|---|---|---|---|---|
| Stall | 34.566 | 35.205 | 33.781 | 30.312 | 31.961 |
| SEM | 2.848 | 3.740 | 4.765 | 3.968 | 4.727 |
| Pasture | 31.115 | 26.373 | 39.976 | 21.188 | 44.230 |
| SEM | 2.512 | 3.594 | 4.994 | 4.277 | 5.147 |

Appendix Table 8A. Means table for urinary deoxypyridinoline (nM/mM).

| Day | 0 | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|---|
| Stall | 7.541 | 9.558 | 7.727 | 6.791 | 7.278 | 7.488 |
| SEM | 0.830 | 0.713 | 0.561 | 1.811 | 0.644 | 1.082 |
| Pasture | 6.738 | 6.575 | 6.989 | 7.936 | 6.385 | 7.242 |
| SEM | 0.830 | 0.713 | 0.561 | 1.811 | 0.644 | 1.082 |

n = 4 for each treatment group

Appendix Table 9A. Means table for urinary deoxypyridinoline (g/d).

| Day | 0 | 28 | 56 | 84 | 112 | 140 |
|---|---|---|---|---|---|---|
| Stall | 0.292 | 0.369 | 0.253 | 0.258 | 0.333 | 0.341 |
| SEM | 0.048 | 0.037 | 0.028 | 0.062 | 0.033 | 0.054 |
| Pasture | 0.290 | 0.275 | 0.275 | 0.333 | 0.294 | 0.352 |
| SEM | 0.048 | 0.037 | 0.028 | 0.062 | 0.033 | 0.054 |

n = 4 for each treatment group

# APPENDIX B

# APPENDIX B

## Proc Mixed Tables

Appendix Table 1B. Proc mixed table for body weight (kg).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 1.57 | 0.2307 |
| Day | 5 | 58 | 30.70 | 0.0001 |
| Day*Treatment | 5 | 58 | 6.37 | 0.0001 |

Appendix Table 2B. Proc mixed table for height at withers (cm).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 0.00 | 0.9899 |
| Day | 5 | 60 | 7.07 | 0.0001 |
| Day*Treatment | 5 | 60 | 1.50 | 0.2045 |

Appendix Table 3B. Proc mixed table for third metacarpal circumference (cm).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 0.88 | 0.3634 |
| Day | 5 | 60 | 1.09 | 0.3779 |
| Day*Treatment | 5 | 60 | 0.64 | 0.6678 |

Appendix Table 4B. Proc mixed table for body condition score.

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 1.80 | 0.2014 |
| Day | 5 | 60 | 12.71 | 0.0001 |
| Day*Treatment | 5 | 60 | 0.79 | 0.5609 |

Appendix Table 5B. Proc mixed table for lateral RBAE (mm Al).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 0.22 | 0.6446 |
| Day | 5 | 58 | 1.74 | 0.1404 |
| Day*Treatment | 5 | 58 | 1.15 | 0.3465 |

Appendix Table 6B. Proc mixed table for medial RBAE (mm Al).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 0.09 | 0.7716 |
| Day | 5 | 58 | 4.90 | 0.0008 |
| Day*Treatment | 5 | 58 | 2.13 | 0.0741 |

Appendix Table 7B. Proc mixed table for change in lateral RBAE (mm Al).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 6.35 | 0.0245 |
| Day | 4 | 44 | 0.81 | 0.5260 |
| Day*Treatment | 4 | 44 | 0.29 | 0.8849 |

Appendix Table 8B. Proc mixed table for change in medial RBAE (mm Al).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 2.49 | 0.1371 |
| Day | 4 | 44 | 3.65 | 0.0118 |
| Day*Treatment | 4 | 44 | 1.28 | 0.2916 |

Appendix Table 9B. Proc mixed table for total RBAE ($mm^2$ Al).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 0.02 | 0.8996 |
| Day | 5 | 58 | 2.67 | 0.0308 |
| Day*Treatment | 5 | 58 | 1.26 | 0.2914 |

Appendix Table 10B. Proc mixed table for serum osteocalcin (ng/ml).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 0.23 | 0.6371 |
| Day | 10 | 121 | 4.50 | 0.0001 |
| Day*Treatment | 10 | 121 | 3.66 | 0.0003 |

Appendix Table 11B. Proc mixed table for urinary deoxypyridinoline (nM/mM).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 6 | 1.12 | 0.3301 |
| Day | 5 | 30 | 0.83 | 0.5373 |
| Day*Treatment | 5 | 30 | 1.18 | 0.3398 |

n = 4 for each treatment group

Appendix Table 12B. Proc mixed table for urinary deoxypyridinoline (g/d).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 6 | 0.01 | 0.9232 |
| Day | 5 | 30 | 2.42 | 0.0590 |
| Day*Treatment | 5 | 30 | 2.33 | 0.0667 |

n = 4 for each treatment group

Appendix Table 13B. Proc mixed table for urinary pyridinoline (nM/mM).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 6 | 2.22 | 0.1867 |
| Day | 5 | 29 | 2.92 | 0.0297 |
| Day*Treatment | 5 | 29 | 0.87 | 0.5159 |

n = 4 for each treatment group

Appendix Table 14B. Proc mixed table for serum calcium (mg/dl).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 0.08 | 0.7850 |
| Day | 10 | 121 | 21.00 | 0.0001 |
| Day*Treatment | 10 | 121 | 1.87 | 0.0552 |

Appendix Table 15B. Proc mixed table for serum phosphorus (mg/dl).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 14 | 6.97 | 0.0194 |
| Day | 10 | 121 | 16.20 | 0.0001 |
| Day*Treatment | 10 | 121 | 6.00 | 0.0001 |

Appendix Table 16B. Proc mixed table for urinary calcium (g/d).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 6 | 2.75 | 0.1486 |
| Day | 5 | 30 | 7.10 | 0.0002 |
| Day*Treatment | 5 | 30 | 0.40 | 0.8432 |

n = 4 for each treatment group

Appendix Table 17B. Proc mixed table for urinary phosphorus (mg/d).

| Source | NDF | DDF | F-Value | P-Value |
|---|---|---|---|---|
| Treatment | 1 | 6 | 0.81 | 0.4040 |
| Day | 5 | 30 | 3.03 | 0.0249 |
| Day*Treatment | 5 | 30 | 3.67 | 0.0104 |

n = 4 for each treatment group

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