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# THE EFFECTS OF SHORT TERM HIGH INTENSITY EXERCISE ON BONE PARAMETERS OF IMMATURE ANIMALS

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ABSTRACT

THE EFFECTS OF SHORT TERM HIGH INTENSITY EXERCISE ON BONE PARAMETERS OF IMMATURE ANIMALS

By

Kristina Marie Hiney

The ability of short duration, high intensity to increase bone formation in immature animals was investigated in two experiments using two species, the equine and bovine. In each study, three treatments compared the effects of confinement, voluntary activity and enforced exercise on bone parameters. Eighteen Holstein calves 8 wk of age were assigned to one of three treatment groups – group housed (GR), confined with no exercise (CF), or confined with exercise (EX). Exercise consisted of running 50 m/d, 5 d/wk. Confined calves remained stalled for the 42-d trial. Fused third and fourth metacarpal bones were scanned using computed tomography for determination of bone geometry and three point bending tests to failure were performed. Exercise resulted in increased dorsal cortex thickness, compared to the GR and CF calves ($P<0.05$). The exercised calves had a smaller medullary cavity than CF and GR and a larger percentage of cortical bone than CF ($P<0.05$). The metacarpi from EX tended to have a higher fracture force than CF ($P<0.1$). Analysis of the changes in concentration of the bone marker, osteocalcin, indicated greater bone formation in EX than CF ($P<0.05$).

In the equine study, eighteen Quarter Horses were divided randomly into three treatment groups similar to the bovine study: group housed (GR), confined with no exercise (CF), and confined with exercise (EX). The EX group was sprinted 82 m/d, 5 d/wk.

Radiographs of the left third metacarpal bone were taken to estimate changes in bone mineral content and cortical widths. Dorsal, medial and total RBAE, tended to differ by a treatment*day interaction, with values increasing over time only in the EX group. Normalized medial and total RBAE tended to differ ($P<0.1$) with treatment, with EX greater than CF. Dorsal-palmar cortical width in EX was greater than GR on d 56 ($P = 0.07$). The dorsal palmar medullary cavity decreased in EX compared to GR ($P<0.05$), while dorsal and medial cortical width increased only in the EX horses ($P<0.1$). Both studies indicate an adaptation in bone geometry indicating that short duration exercise may be effective in improving bone mass and therefore skeletal strength.

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Okay, they are dragging me off the stage now, later folks, thanks again.....

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

Lameness is a key concern among producers of many species in animal agriculture, including the swine, poultry, dairy and equine industries. Frequently, unsound animals must be removed from the herd or flock and euthanized. While this is a financial concern, these losses also raise issues of animal welfare and well-being. While the causes for lameness among species may vary, often they can be traced to the musculoskeletal system. In the equine industry, lameness is of particular concern, as it is the leading cause of wastage among racehorses (Jeffcott et al., 1982; Rossdale et al., 1985). The majority of injuries are musculoskeletal in nature, with potentially catastrophic results. In a survey conducted by Jones (1989), 58.1% of all two-year-old racehorses experienced an injury. As most young racehorses are placed into training as long yearlings, the rate of injuries may be due, in part, to the relatively immature skeletal structure of the animal.

Recently, attention has been focused on the impact of early management of young animals on skeletal strength. Unfortunately, some management practices of domestic livestock species may jeopardize skeletal integrity. Within the equine industry, weanlings and yearlings are often confined in small box stalls for several months to facilitate preparation for sales or futurities. Stall confinement decreases bone mass in young horses relative to age-matched horses on pasture (Hoeskstra et al., 1999; Bell et al., 2001; Firth et al., 1999). Following confinement, these horses are often placed into rigorous training, which may render them more susceptible to injury than horses with greater bone strength. Therefore, the goal of much research within the horse industry is

to identify optimal management practices that better prepare the young horse to begin training.

One approach to decreasing lameness is to strengthen the skeleton prior to the initiation of traditional "under-saddle" training. Young animals appear to adapt more readily to exercise (Loitz and Zernicke, 1992; Iwamoto et al., 2000), and increasing bone mass may be easier if training begins early. Training in young horses increases bone mass, provided the exercise is of sufficient intensity (McCarthy and Jeffcott, 1991). Therefore, training at high speeds before "under-saddle" training begins may better prepare the young horse than waiting until a later age when the skeleton is more mature.

One of the difficulties in performing studies of bone physiology in the equine is the lack of accurate, non-invasive determinants of bone structure. Computed tomography, while highly accurate, is difficult to perform due to the expense and risk involved with anesthetizing large animals. Terminal studies provide the most definitive information, but again, the expense and use of an animal species considered by some to be a companion animal limit the option of terminal research. However, the use of a bovine model is proposed as a suitable alternative. Holstein bull calves are commonly slaughtered in industry, and are similar in structure and size to young horses. In addition, bovine skeletal metabolism and adaptation has not been extensively researched. The hypothesis of the study was that confinement in young animals causes a reduction in bone mass or strength through a slowing of bone formation, and this reduction in bone growth can be alleviated or possibly enhanced by providing either a group housing system or short periods of forces exercise. Therefore the objectives of these studies were to investigate the usefulness of short periods of intense exercise in improving bone quality

in two different species, as well as to compare bone response to confinement and an environment which provides ad libidum exercise.

The specific goals of this project were to:

1. determine if short term, high intensity exercise enhances skeletal mass and architecture compared to confined or group housed animals.
2. determine if a reduction in bone mass or bone growth due to confinement could be alleviated by short term exercise
3. evaluate the use of the bovine model for studies in basic bone physiology and comparative studies in the equine.
4. Determine the influence of voluntary behavior on skeletal parameters

# CHAPTER II

# REVIEW OF LITERATURE

Bone is a rather unique, highly adaptive tissue that alters its shape and structure in response to changes in its external environment. The skeletal system responds to patterns of loading or strain in order to achieve a balance between skeletal strength and skeletal mass (Rubin, 1984). While a more massive skeleton would be stronger and able to resist higher forces, it would not be metabolically economical to the animal. Maintaining the minimal bone mass required to meet the animal's needs of skeletal strength is an evolutionary advantage as it reduces the energy expenditure involved with locomotion, synthesis, and maintenance of extraneous tissue (Loitz and Zernicke, 1992).

This phenomena of the adaptability of bone, first described in the late nineteenth century, is known as Wolff's Law (Woo et al., 1981). Numerous studies conducted in both humans and animals show the positive effect of exercise on bone mass (Heinonen et al., 1993; Snyder et al., 1992; Biewener and Betram, 1994) while bed rest (Donaldson et al., 1970; LeBlanc et al., 1990), confinement (Marchant and Broom, 1996) or weightlessness (Whedon et al., 1977) lead to a reduction of bone mass. The premise of Wolff's Law relies on the ability of cells within bone to sense variations in mechanical strain that fall outside the desired biological window. Cellular processes are then activated which can alter the extracellular architecture and return strains to a desired level. For example, in animals subjected to ulnar osteotomy, the radius altered its mass in response to the sudden increase in strain, and returned recorded strains to pre-operative levels (Lanyon et al., 1984; Goodship et al., 1979). Similarly, when the mechanical strain

is too low, "excess" bone is removed. Therefore the goal of much current research is to develop training and management practices which optimize skeletal strength through a clearer understanding of bone biology.

Basic Bone Biology

In order to prescribe programs to promote skeletal strength, an understanding of basic bone biology and the mechanisms governing this system is essential. The skeletal system is a highly specialized form of connective tissue providing numerous essential functions. While its primary role is that of support, protection, and locomotion, the skeleton is also the main reservoir of many minerals essential for proper cellular function. A complex relationship between bone cells and the extra cellular matrix allows the skeleton to respond to demands between both the need for effective support and the maintenance of mineral homeostasis.

Bone is comprised of specialized cells within an extensive extracellular organic matrix strengthened by the deposition of calcium salts, primarily in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Briefly, the four predominant cell types found in bone tissue include: osteoblasts, osteoclasts, osteocytes and bone-lining cells. Osteoblasts are the cell type responsible for the formation of bone. Osteoblasts not only produce bone matrix, but also regulate the mineralization of the organic matrix. As osteoblasts mature, they either undergo apoptosis, or become completely surrounded by mineralized tissue and are then referred to as osteocytes (Marks and Ogdren, 2002). Osteocytes exist within obloid spaces called lacunae, and are believed to be the actual mechano-sensors of the tissue. Thus, they must communicate with not only other

osteocytes, but other cell types as well. Cellular projections through the canaliculi and gap junctions allow transfer of electrical signals, as well as intracellular and extracellular transport of signal molecules between osteocytes and the bone surface. Similar communication and the passage of the mechanical signal has also been shown in osteoblasts (Martin, 2000) and osteocytes (Nomura and Takano-Yamamoto, 2000).

Bone surfaces not actively undergoing bone formation or resorption are covered by flat, inactive cells, or bone-lining cells. These cells may be the precursors of osteoblasts, capable of differentiating when needed (Marks and Odgren, 2002). Bone-lining cells covering the vertebrae of rat tails developed structural features of osteoblasts following a single loading bout (Chow et al., 1997). Bone-lining cells may help regulate osteoclastic activity as well. Osteoclasts in resorption pits were found to be in close contact with bone-lining cells and cytoplasmic extensions of bone-lining cells to osteoclasts suggested transfer of signals between these cell types (Everts et al., 2002).

Finally, osteoclasts, multi-nucleated cells of hemopoietic origin (versus the mesenchymal cell origin of those previously discussed) are responsible for the resorption of mineralized bone. As they orignate from hemopoietic stem cells, they use vascular channels to migrate to the site of absorption. The mononuclear cells then fuse with other cells to become the fully active osteoclast. Osteoclasts are distinguished by their ruffled border, a highly infolded section of the plasma membrane, which rests directly on the bone surface to be eroded. Osteoclasts also have a clear zone, which surrounds the ruffled border and serves as the point of attachment to the bone matrix. Osteoclasts are abundant with lysosomal vesicles containing the proteases and $H^+$ ion concentrations necessary to solubilize the inorganic matrix (Marks and Ogdren, 2002).

Mechanotransduction

The mechanical stresses placed on the bone must be translated into cellular signals, a process referred to as mechanotransduction. The appropriate response, either to increase or decrease bone formation and/or resorption, should be proportional to the degree of strain experienced. The force on the bone is suggested to be transmitted via a flow of interstitial fluid, sensed as alterations in streaming potential and shear stresses (Nomura and Takano-Yamamoto, 2000). Mechanical forces are transformed into biological impedances resulting in specific cellular events. The means by which bone senses and responds to its loading environment has not been completely defined, but several cellular pathways and signaling mechanisms are involved. Possibilities for the exact chemical mediator include changes in ion channel sensitivity altering the flux of $Ca^{+2}$ into the cell (Hung et al., 1995), activation of focal adhesion kinases, or modifications of the cytoskeleton, or integrins and CD44 receptors which anchor bone cells to the extracellular matrix (Nomura and Takano-Yamamoto, 2000), or perhaps combination of all of these.

Once the mechanical signal, which can occur after only a brief period of load application, has been received by the osteocytes, changes in cellular function occur quickly. Increased RNA production and glucose consumption have been observed in organ culture between 6 and 24 h post-loading (Markus, 2002) with other reports of increased RNA production within minutes after application of mechanical strain (Einhorn, 1992). Other factors linked with bone formation such as prostacyclin, prostaglandin E2 ($PGE_2$), and nitric oxide (NO) are released from bone surface-lining

cells and osteocytes 5 min following loading (Forwood and Turner, 1994) and transforming growth factor ß (TGF-ß), insulin-like growth factor – 1 (IGF-1), and c-fos RNA increase within 2 to 4 h (Raab-Cullen et al., 1994a). In fact, a number of genes have been discovered which have increased expression levels in response to mechanical stress including glutamate/aspartate transporter, nitric oxide synthase, c-fos, and cox-2 (Nomura and Takano-Yamamoto, 2000) which sustain the release of prostaglandins and NO. These changes are believed to be mediated by the rapid and transient release of $PGE_2$, especially as mechanical effects can be abolished by indomethacin, a $PGE_2$ antagonist (Markus, 2002).

Numerous other factors may be involved in the cellular response to loading. Signals are potentially transmitted through the cytosol through the action of various hormones as estrogen, prostaglandin, parathyroid hormone (PTH), di-hydroxy vitamin $D_3$ ($1,25\text{-}(OH)_2D_3$). Also, glucocorticoid receptors are found on osteocytes. Parathyroid hormone, previously known only for its role in Ca homeostasis, is now accepted as modulating the osteocytic response to mechanical strain. (Nijweide et al., 2002; Noble and Reeve, 2000). Mechanical stimulation via $PGE_2$ activates several classic $2^{nd}$ messenger systems, including protein kinase A and protein kinase C which increase concentrations of cAMP and activate arachidonic acid and $PGE_2$ release (Nijweide et al., 2002). Elevation of various transcription factors such as AP-1, the product of the c-fos gene, also result in a cascade of cellular events as it serves as a general activator for a large number of genes (Yamauchi et al., 1996). Mechanical stimulation increases levels of growth factors such as IGF-1 and -II, which stimulate collagen and DNA synthesis in osteogenic cells (Damien et al., 2000; Lean et al., 1995). As bone formation following

external loading was 5-fold greater in mice overexpressing IGF-1 than wild type mice (Gross et al., 2002), IGFs may play an important role in mediating bone formation.

While changes in concentrations of 2$^{nd}$ messengers may take only minutes, it does appear that the actual response of producing osteoid takes 3 to 4 days after activation (Forwood and Turner, 1994; Pead et al., 1988a). These common cellular events described above are followed by those more specific to bone tissue, such as the increased production of bone matrix proteins such as type I collagen, osteopontin, and osteocalcin. Also, load stimulus may not just increase bone formation, but can reduce bone resorption as well. Rubin et al. (1999) found a 40% reduction in osteoclast formation in marrow cultures subjected to strain.

In order for the bone tissue to respond in the appropriate fashion, the osteocytes that sense strain must coordinate the osteoblasts and osteoclasts responsible for forming or resorbing bone. Communication can potentially occur through the release of growth factors or other mediators, or through direct cell-to-cell contact. Bone resorption is regulated by osteoblasts or cells of the osteoblast lineage, rather than osteoclasts, the cells ultimately responsible for resorption, as osteoclasts cultured without osteoblasts fail to form resorption pits (Takashi et al., 2002). In fact, receptors for most osteolytic signals are found on osteoblasts rather than osteoclasts (Rodan and Martin, 1981) and their presence may be essential for resorption. While vitamin D, parathyroid hormone, interleukins, and prostaglandin $E_2$ all stimulate the formation of osteoclasts (Takashi et al., 2002), the differentiation of immature osteoclasts appears to be mediated through cell-to-cell contact with osteoblasts and their production of macrophage colony stimulating factor (M-CSF). Cultured osteoclasts were unable to resorb bone in the

absence of stimulatory factors released by osteoblasts (McSheehy and Chambers, 1986). Both osteocalcin and osteopontin, proteins synthesized by osteoblasts, may play a role in the recruitment and attachment of osteoclasts, the first step in the initiation of the bone remodeling cycle (Rodan and Rodan, 1995; Reinholt et al., 1990).

Bone resorption itself also releases growth factors from the bone matrix that, in turn, promote the recruitment and activation of osteoblasts, coupling these two processes tightly together. The release of inorganic phosphate from the demineralization of bone also may induce apoptosis of osteoblasts in the immediate area, removing the stimulus of resorption (Meletti et al., 2000). Therefore the function and activity of all bone cell types are tightly interwoven between the initial mechanical sensing by osteocytes, followed by biomechanical coupling, cellular communication, and the effector response.

## Adaptation of Bone

Regardless of the exact pathway by which strain is translated into cellular signals, the reaction of bone is to preserve its integrity. Bone failure occurs not only from sudden traumatic failure but also through progressive weakening of bone (Carter et al., 1981). Indeed, fatigue failure may be one of the most common causes of musculoskeletal injuries in horses (Estberg et al., 1996). Fatigue damage, due to the repetitive loading of bone, produces micro-cracks within cortical bone (Burr et al., 1985), decreasing its stiffness and increasing the strain magnitude (Nunamaker et al., 1990). This initiates the local repair mechanism of bone remodeling (Mori and Burr, 1993; Burr et al., 1985), a tightly coupled process where removal of old, weakened bone precedes replacement with new, undamaged bone. Bone is therefore able to withstand some damage that can be

repaired through normal processes before failure occurs. Continuous remodeling also prevents the accumulation of older, more densely mineralized bone which is more brittle (Ott, 2002).

Osteocyte apotosis in the region of damaged bone may initiate the activation of remodeling as well as the increase in strain (Verborgt et al., 2000) while formation alone is associated with a decrease in osteocyte apotosis (Noble et al., 1997). Bone-lining cells first remove osteoid covering the bone surface via matrix metalloproteinases which allows the subsequent attachment of osteoclasts (Everts et al., 2002). Activated osteoclasts remove a volume of bone during the resorption phase, lasting approximately 3 wk in humans (Parfitt and Chir, 1987). In cortical bone, this results in the removal of a tunnel or cone of bone. Bone resorption is followed by a reversal phase, normally 1 to 2 wk in duration, during which small mononuclear cells, potentially identified as bone-lining cells, are attracted to the resorption pit and clean it of any remaining undigested non-mineralized collagen (Everts et al., 2002). After bone-lining cells deposit a thin layer of collagen (the cement line), osteoblasts begin to lay down the new organic matrix, which later will be mineralized to form an osteon (Parfitt and Chir, 1987). The complete process of bone formation and mineralization for one bone remodeling unit may take as long as three months. Typically, bone remodeling results in no change in bone mass; however, with advancing age, the formative phase of remodeling may fail to keep pace with the resorptive phase and thus senile osteoporosis can occur.

In addition to local repair of damaged bone and an alteration of bone mass or density, the skeletal system evolved with the ability to alter the geometry of the bone, sometimes referred to as modeling. Discrepancies in the literature between the terms

bone modeling and remodeling can lead to some confusion. Some authors separate the two processes into separate categories in which bone modeling refers to only addition of bone mass and never a reduction while remodeling is any change in existing bone (Frost, 1990; Burr et al., 1985). Others refer to bone modeling as a non-localized process by which bone alters its shape, either by the addition of removal of bone on localized surfaces (Jee, 1988; Kimmel, 1993). The most commonly accepted definition is a change in shape of the bone or drift. Drift can occur by either slowing or increasing the relative rate of formation or resorption of bone. Therefore, a decrease in periosteal bone formation on a certain aspect of the bone does not always have to be interpreted as a negative outcome, but merely a corrective geometric adaptation in placing more bone where needed and away from areas with less strain (Mosely et al., 1997). Several studies have reported that geometric and biomechanical properties of long bones improve without an increase in bone mass (Barrengolt et al., 1993; Woo et al., 1981). A geometric change without a change in mass or density can therefore still be beneficial to the animal and should not be overlooked.

Extracellular Matrix

While most studies examining bone strength and integrity focus on bone mineral density, the non-mineral, or organic portion of the extracellular matrix also changes with exercise or disease. Type I collagen, common in many tissues, makes up 95% of the organic matrix of bone, with the remainder consisting of proteoglycans and noncollagenous proteins. Type I collagen has a triple helix motif composed of three polypeptide chains of repeating Gly-X-Y amino acid sequences. Most typically X is

proline and Y is hydroxyproline. Hydroxyproline serves to stabilize the triple helix and is found almost exclusively in collagen. Thus, the concentration of hydroxyproline within a sample of bone can provide an indication of collagen content which may change with age and exercise. McDonald et al. (1986) found an increase in hydroxyproline content of the femur and humerus of 7 mo old rats trained for 12 wk. However, exercise decreased the hydroxyproline content in the femoral neck (Salem et al., 1993) of immature rats but concentrations of hydroxyproline did not change with age from 8 to 18 wk of age.

Fibrils of type I collagen are spontaneously formed following the post-translational modification of N and C terminal propeptides of procollagen. The fibrils then undergo further modification through the action of lysyl oxidase by cross-linking lysine or hydroxylysine residues within fibrils. The final mature products are pyridinium crosslinks, whose metabolism is often monitored as an index of bone activity, which will be discussed in more detail at a later point. The process of cross-linking confers additional strength to the extracellular matrix and thus to the whole bone. However, Eyre et al. (1984) reported that collagen crosslinks, while improving the mechanical properties of soft tissue, were less important in bone than other tissues. The pattern of collagen cross-linking may play a role in regulating bone mineralization, as the ratio of hydroxylysylpyridinoline to lysylpyridinoline was twice as high in bone calluses as control bone 3 wk after fracturing (Wassen et al., 2000). This ratio returned to normal levels 21 wk after fracture when collagen was fully mineralized.

Concentrations of pyridinium crosslinks within the bone increase in the first two decades in humans and remain at a constant level thereafter (Eyre et al., 1988). Others

have also reported a maturation-related increase in collagen crosslinks (Eyre et al., 1984; Salem et al., 1989). The constant process of bone remodeling prevents the accumulation of more crosslinks after skeletal maturity, as the bone matrix is replaced. This is most likely due to remodeling rather than steric hinderance of mineral formation between fibrils as low bone turnover in osteopetrotic rats is associated with high concentrations of pyridinium crosslinks (Wojtowicz et al., 1997). Few studies have examined the effects of exercise and immobilization however. Crosslink concentrations increased with age and exercise in the femoral neck of rats (Salem et al., 1993) while Yamauchi et al. (1988) reported no change in crosslink concentration in primates immobilized for 7 mo. In roosters exercised at an intensity great enough to temporarily decrease the radial and longitudinal growth of the tibiotarsus and femur, runners and controls had similar crosslink concentrations (Maynard et al., 1995).

Bone and Activity

The degree of the bone modeling/remodeling response is dependent on the magnitude (Robling et al., 2001; Mosley et al., 1997; Rubin and Lanyon, 1985), number, rate (Mosley and Lanyon, 1998; Turner et al., 1995), distribution (Rubin and Lanyon, 1984) and frequency of cyclic strain (Turner and Pavalko, 1998). The magnitude and velocity of the fluid flow (and thus the streaming potentials and bioelectric current) depends on all of these factors which can act independently or additively. The number, as well as the magnitude, influence osteoregulation independently. High peak strain magnitudes are more osteogenic than low strain, regardless of cycle number (Rubin and Lanyon, 1985; Kerr et al., 2001). In addition, bone appears to be more responsive to

dynamic loading as opposed to static loading, which either has no effect (Lanyon and Rubin, 1984) or may depress bone growth (Robling et al., 2001). Presumably this is due to the absence of stimuli of fluid flow through the canniculi once the static load is in place. Osteogenic response is positively correlated to strain rate, which refers to the speed at which a strain is developed, independent of frequency, when all other factors are held equal (Mosely and Lanyon, 1998; Turner and Pavalko, 1998) and is again related to the velocity of the fluid flow (Burr et al., 2002). Mosley and Lanyon (1998) showed that higher strain rates are more osteogenic than increasing strain magnitude. Others have proposed that the diversity of strain, compared to absolute magnitude, initiated the adaptive response (Biewener and Bertram, 1993), with more uncommon strains creating a larger response. For example, large increases in cortical cross sectional area in experiments involving artificial loading of avian ulnae may reflect the unusual direction of the strain versus normal wing flapping (Lanyon, 1993). All of these factors should be considered when implementing a program designed to cause adaptation in bone. The less dramatic changes observed in traditional physical exercise programs such as running may be due to the more normal, physiological strains generated by such programs.

Short Duration Exercise

Bone is sensitive to only a few cycles of loading, providing the stimuli is beyond that normally experienced. Exposure of avian ulnae to a single loading event resulted in activation of the quiescent periosteum with flat inactive osteoblasts changing to more rounded active cells (Pead et al., 1988a). Rat tibia loaded for a single bout of 36 cycles increased periosteal woven bone formation along 40% of its surface (Forwood and

Turner, 1994). Four cycles per day of an externally applied load prevented bone loss in immobilized limbs, while 36 cycles resulted in an increase in bone mass above controls (Lanyon, 1984). Lanyon (1984) found no further increase in bone response between 36 or 1800 strains; thus, the response of bone appears to quickly become saturated. Extended durations of an activity are presumably not important as long as the threshold level is reached with only a few cycles of unusual strain being significant in a sedentary animal. Five jumps per day increased bone mass and mechanical strength of rat femora and tibia over sedentary controls, however an additional response was seen up to 100 jumps per day (Umemura et al., 1997) suggesting saturation had not occurred.

Strain in normal controlled locomotion such as walking may have little effect on bone architecture while sudden movements of very short duration, such as when an animal is startled or jumps create strains that may be five times greater than normal movement (Skerry and Lanyon, 1995). In sheep with an external fixator placed across the hock joint unloading the calcaneous, daily periods of walking were unable to prevent bone resorption (Skerry and Lanyon, 1995). The external fixator effectively suppressed the high-magnitude strains (1,150 microstrains) reported to occur during normal background loading. With the fixator in place, loading was minimal at only 150 microstrains (Skerry and Lanyon, 1995). Jumping in rats has been reported to cause loads of 2500 microstrains in comparison to 1200 microstrains due to running (Mosley et al., 1997) similar to recorded results in roosters as well (Judex and Zernicke, 2000a). In another study of treadmill-exercised roosters, a defined exercise bout resulted in strains generally greater than 500 microstrains while background loading (the load created by normal everyday activity-not training) resulted in intermittent high-magnitude strains

greater than 1,000 microstrains (Konieczynski et al., 1998). Therefore, while brief experiences of high strains may only account for 1% of the total loading history of an animal, they may be the largest contributor to the osteogenic response.

Exercise programs which create higher strains in the bone such as jumping or resistance training, appear to be more effective in causing osteogenesis. In young rats, a greater increase in femoral and tibia ash weight was observed in jump-trained versus run-trained rats (Umemura et al., 1995). In addition, while adult rats did not respond to run-training, an increase in bone mass was observed with jump-training. Jump-training would provide fewer, but higher, magnitude strains compared to run-training. In roosters which performed 200 drop jumps/d (roosters were lifted 50 to 60 cm off the ground, accelerated, and released) for 3 wk, bone formation rates (BFR) increased on both the periosteal and endosteal surface (Judex and Zernicke, 2000b), particularly in the regions experiencing the greatest strain rates. Bone formation rates in this study were much greater than in previous studies by the same laboratory using run-trained roosters. In pre-pubertal male and female children, after seven months of jump-training consisting solely of jumping off a 61 cm box 100 times, 3 d/wk , jumpers had a 4.5% and 3.1% increase in femoral neck and spine bone mineral content (Fuchs et al., 2001). As of yet, the threshold to elicit a cellular response is unknown and most likely is different for each system, being highly dependent on the loading history of the bone. The underlying factor is that those strains must be unusual to those normally experienced.

An alternative to increasing strain magnitude by resistance training or jumping is to increase the speed, which also will increase strain rate. Strain magnitude and rate has been reported to increase linearly with velocity (Davies and McCarthy, 1994; Biewener

and Betram, 1993). While excessive loading of the skeleton will certainly lead to fatigue damage or worse, some amount of high-speed exercise may have protective effects. Thoroughbreds which experienced injuries had less high speed exercise prior to injury than non-injured controls (Cohen et al., 2000). Horses which had greater cumulative exercise during the previous 1 to 2 months were at a decreased risk of injury (Cohen et al., 2000). A lack of high speed exercise may have led to a decreased bone density which predisposed the animal to injury, although the horses receiving less exercise may have already been sore and worked less. Therefore the population of horses with greater high speed exercise were those less predisposed to injury.

Disuse

As mentioned previously, disuse, such as occurs with micro-gravity, bed-rest, immobilization (Buckingham and Jeffcott, 1991a), or confinement (Marchant and Broom, 1986; Porr et al., 1998), results in unneeded mineral being resorbed in order to maintain the optimum strain environment and minimal bone mass. Immobilization of a single limb results in localized demineralization of the bone not observed in the contralateral control (Inman et al., 1999). This differs to bone resorption caused by hormonal imbalances or dietary deficiencies which can result in systemic demineralization of the skeleton.

Bone resorption due to immobilization occurs quickly, with decreases in bone mineral density, stiffness and ultimate load reported after only 6 wk in rats (Inman et al., 1999). In horses, 4 wk of immobilization of the forelimb increased the medullary cavity size (Buckingham and Jeffcott, 1991a). While it may take weeks to achieve a detectable decrease in bone density or altered bone geometry, the cellular resorption response occurs

within days. Markers of bone resorption including the cross-linked carboxyterminal telopeptide of type I collagen (ICTP), hydroxyproline, and deoxypyridinoline (DPD) increase within two days of bed rest (Lueken et al., 1993). This indicates that skeletal disuse results in an almost immediate response to begin degradation of "unneeded" bone. Concentrations of the carboxyterminal propeptide of type I procollagen (PICP) were found to decrease following three days of bed rest while osteocalcin (OC) values increased (Pedersen et al., 1995).

Confinement rearing of young animals over extended periods of time may lead to a decrease in the total bone mass able to be achieved at maturity and subsequently result in an inferior skeleton. The response to immobilization may be very different between ages of animals, both in degree of bone lost, as well location of resorption. Bone loss in immature rats was seen after 1wk of immobilization, versus 3 wk in mature rats (Steinberg and Trueta, 1981). Osteoclastic resorption occurred on trabecular and persiosteal surfaces in young dogs but occurred on the endosteal surface in mature animals (Uhtoff and Jaworski, 1978). Lack of physical exercise may also lead to decreased bone formation as opposed to bone resorption due to the over-riding process of growth. Sciatic neurectomy and tail-suspension in rats resulted in a decrease in bone mass as a result of decreased bone formation rather than increased resorption as determined by histomorphometry (Yeh et al., 1993, Kodama et al., 1991).

If confinement is coupled with Ca deficiency, the loss of bone is exacerbated. In 5-mo-old rats, immobilization of the hind-limb concurrent with Ca deficiency resulted in a 28% loss of BMC in the proximal tibia (Inman et al., 1999) and 32% bone loss in adult turkey ulnae (Lanyon et al., 1986). Application of a loading stimulus can only partially

compensate for the hormonal effects triggering bone resorption. Loading partially reduced the bone loss in Ca deficient animals (Lanyon et al., 1986), and attenuated the decrease in mechanical strength (Inman et al., 1999). Bone loss due to Ca deficiency becomes even more dramatic if imposed on the immature animal. A dramatic reduction of 46% of the femoral ash weight was observed after only 72 h of immobilization and Ca deficiency in 2-mo-old rats (Weinrab et al., 1991). Therefore, if young animals are confined while being on a diet that provides an insufficient amount of Ca, the owner or producer may experience greater problems with skeletal injuries.

Systemic or Hormonal Effects

The process of bone modeling/remodeling is not only governed by local laws of loading events, but also by systemic changes as well. When blood Ca concentrations drop, the skeleton serves as a large reservoir of available Ca. While bone remodeling does affect serum levels of minerals, minute-to-minute regulation of serum concentrations are not dependant on bone remodeling. However, long term changes in serum concentrations such as Ca defiency can alter bone metabolism.

Receptors for extracellular Ca are found in the main organs involved in Ca homeostasis: the parathyroid gland, thyroid and kidney. Low serum Ca results in the release of PTH from the parathyroid gland which increases bone turnover. However, the rapid increase in serum Ca is not due to bone remodeling, as it takes much longer to activate osteoclasts. Parathyroid hormone also acts directly on the kidney to increase reabsorption of Ca and to increase the hydroxylation of 25-OH $D_3$ to 1, 25-$(OH)_2$ $D_3$. The kidney also conserves Ca by altering renal blood flow, glomerular filtration rate, and

tubular resorption rate to reduce Ca excretion. Vitamin D increases Ca absorption from the small intestine and has similar effects of PTH on osteoclastic acitivity. Receptors for PTH and vit D are both found on osteoblasts, despite the fact that their main recognized role is to increase bone resorption (Takahashi et al., 2002). As discussed previously, the increased metabolic activity of osteoclasts as well as the increase in their numbers in response to PTH is believed to be mediated through osteoblasts. When ionized Ca concentrations increase, calcitonin is released from the thyroid gland which inhibits osteoclast activity and increases Ca excretion in the kidney (Becker et al., 2002).

Parathyroid hormone has been used as a therapy for bone loss, almost paradoxically with its known role in bone resorption. Sustained elevations in levels of PTH can lead to a dramatic decrease in bone mass. However, intermittent doses of PTH increased bone formation and bone mass (Gunness-Hey and Hock, 1984) and appears to act synergistically with mechanical loading (Chow et al., 1994; Ma et al., 1999). PTH administration increases the concentration of $2^{nd}$ messengers and increased intracellular Ca similar to effects of strain (Ryder and Duncan, 2001) and increased the sensitivity of osteoblasts to fluid sheer, perhaps explaining the effect of PTH in increasing bone mass. Administration of PTH helps regain bone mass following a period of immobilization (Ma et al., 1999).

Reproduction places large demands on the mineral homeostatic system of the dam for fetal development and later milk production. Mares (Glade, 1991), rats, monkeys (Ott et al., 1999) and humans (Heaney and Skillman, 1971) increase Ca absorption during late gestation in order to counterbalance the increase in Ca demand. Mares fed adequate Ca increased in metacarpal breaking strength during the last 12 wk of gestation, compared to

mares on a deficient diet which showed no change (Glade, 1993), suggesting a conservation of mineral for upcoming lactation. Adequate Ca stores in the bone are critical during lactation, as the skeleton is the mineral reserve mobilized during periods of high demand. For example, bone mass in humans decreased 5 to 20% during lactation, while monkeys lost 3 % of total body bone mineral between parturition and 3 mo of lactation (Ott et al., 1999). Metacarpal bone strength decreased in mares during the first 12 wk of lactation and was restored by 24 wk post-parturition in mares on the Ca-sufficient diet. However, those mares fed the deficient diet had not fully recovered 40 wk after foaling. More importantly, foals born to the Ca deficient mares had thinner cortical diameters and weaker third metacarpi (MC III; Glade, 1993). Therefore, during gestation and lactation, animals not only need to be on a Ca sufficient diet, but also to have an optimal bone mass for sustaining milk production and maintaining their own skeletal integrity.

As Ca deficiency results in an increase in systemic factors causing bone resorption, sufficient Ca in the diet is important for the exercising animal. Exercise could not prevent bone loss in Ca-deficient rats (Bauer and Griminger, 1983) due to the overriding hormonal stimulus directed towards bone resorption rather than bone formation. Porr et al. (2000) found that only horses conditioned while consuming a diet 2 times that of the NRC exhibited a small increase in BMC of the medial aspect of MCIII. Feeding increased concentrations of Ca above NRC recommendations may also enhance bone mass of the young racehorse during training (Nielsen et al., 1998; Nolan et al., 2001), but Ca supplementation of foals through long yearlings did not alter bone mineral content (Hoffman et al., 2001).

Bone Growth and the Young Animal

Much of the work done in the area of bone physiology has been primarily in the skeletally mature animal. However, most bone modeling occurs very early in life and mature bone may not be as sensitive to exercise-induced changes as younger, growing bone (Iwamoto et al., 2000; Loitz and Zernicke, 1992). In immature rats, cortical thickness (Steinberg and Trueta, 1981) and ash weight (Umemura et al., 1995) increased in response to running, but not in adult rats. Using the isolated turkey ulna model, increases in bone area and periosteal mineralizing surface were observed in 1-yr-old but not 3-yr-old turkeys (Rubin et al., 1992). However, at one year of age, turkeys should have reached skeletal maturity. While not conclusive evidence, thoroughbred racehorses which received their first start as two-year-olds had more starts and had a longer career in comparison to those which began racing at an older age (Bailey et al., 1999). However, this does not eliminate the possibility that these were the stronger horses better able to tolerate exercise in the first place, and those that did not start were delayed by injury.

Loading of the skeleton during this window of opportunity of active modeling may therefore be critical for achieving optimal bone mass or structure later in life, especially for the athletic animal. As human medicine has also focused more on the prevention rather than the treatment of the age related disease of osteoporosis, exercise during the growth period has been highly advocated (Bass, 2000; Fuchs et al., 2001). In human studies with targeted intervention trials, those which have reported the largest changes in bone mass have been in prepubertal children (Khan et al., 2000) and this appears to be the critical stage of development. In women recruited to play racquet sports

4 to 5 times per wk, those who began training before menarche achieved a 22% difference in BMC between arms, while this difference was only 10% in those who started after menarche (Kontulainen et al., 2001). Other studies have shown similar results in that the BMC of the dominant arm is 2 times greater if playing started before menarche (Kannus et al., 1995a). Children in the top quartile of physical activity had significantly higher BMC than their less active peers (Khan et al., 2000). Pre-pubertal elite female gymnasts were reported to have 30 to 85% greater BMD at the total body, spine and legs (Bass et al., 1998). However, with human studies, it is harder to separate effects of predisposition or selection bias in a sport. Bone differences may have existed prior to beginning the sport or differences in lifestyle (nutrition, genetics) may contribute as well. However, the arm-to-arm differences in tennis and racquetball players support mechanical loading effects.

In the young animal, the response of bone to exercise may be more complex due to the combination of loading along with normal biological growth. Results of similar loading trials between adult and immature animals cannot be directly compared. Even where and how new bone is formed in response to exercise may differ between ages and species of animals. In humans, exercise in the pre- or peripubertal years appears to cause an increase in periosteal expansion, while exercise late in puberty or after menarche results in endocortical bone formation (Bass, 2000). However, in an earlier study reported by Bass et al. (1998), endocortical diameter was reduced in prepubertal gymnasts. In young animals, the increase in bone mass due to exercise also appears to occur at the periosteal surface (Yeh and Aloia, 1990; Iwamoto et al., 2000; Notomi et al., 2001). However, others have reported that the dominant effect occurs on the endosteal

surface with an accompanying decrease in medullary cavity (Judex and Zernicke, 2000; Woo et al., 1981, Matsuda et al., 1986).

Training at a young age may alter geometric properties while at a more mature state volumetric bone density may increase instead. Tennis players who started playing at a young age had an increase in cross-sectional area of the distal radius, while those who started playing later increased the trabecular density (Ashizawa et al., 1999). The strain distribution may differ between adult and immature animals as bone was deposited in an anteromedial direction in adult roosters, but anterolaterally in growing animals (Loitz and Zernicke, 1992). Nutritional effects may also have more of an impact on growing bone, as Ca supplementation in young girls only increased bone mass in pre-pubescent but not pubescent girls (Slemenda et al., 1997).

Some discretion must be used in exercising the young animal. As the bone of young animals is more pliable, it may also be susceptible to adverse effects of prolonged loading or exercise. Conflicting results of positive and negative effects of exercise on long bone growth have been reported. Intense exercise inhibited longitudinal bone growth in immature animals (Matsuda et al., 1986; Kiiskinen, 1977; Li et al., 1991) and increased the amount of trabecular bone in relation to cortical bone in the femoral neck of rats (Hou et al., 1990). Li et al. (1991) also reported a concurrent decrease in tibial cross-sectional area and decreased mechanical strength. Artificial dynamic and static compressive axial loading of rat ulnae retarded longitudinal growth compared to the contra-lateral control limb (Mosley et al., 1997; Robling et al., 2001) in a dose dependant manner with strain magnitude, even though periosteal and endosteal bone formation rates were simultaneously increased in the dynamic loading group (Robling et al., 2001). In

humans, elite pre-pubertal gymnasts grew more slowly than controls (Bass et al., 1998). However, this could be in part due to the intense exercise schedule (15 to 36 h/wk) or the reduction in body fat (57% less compared to controls). Others have shown an increase in femoral length in run and jump-trained young rats (Umemura et al., 1995). Many of the studies showing adverse effects of exercise have used endurance running with immature rats as a model, an animal which did not evolve for endurance. Therefore, such negative effects may be more related to the species studied, rather than to just the animal being young.

Determinants of Bone Strength

While much discussion has been given to the adaptation of bone in response to loading, some understanding of the physics involved is helpful. When a force is applied to bone while it is fixed in place, deformation occurs, resulting in internal stress that resists that force (Einhorn, 1992). This resistance is referred to as stress, which is equal but opposite to the force applied. Numerically it is expressed as the units of force per unit area: $\sigma$ =force/area. The standard unit is the Pascal (Pa) which is 1 N of force over 1 $m^2$. The measurement of the deformation of the bone is known as the strain. Strain ($\varepsilon$) = the change in length/original length. As it is dimensionless, it can be expressed as a percentage. The stresses in bone can be described as three types: tension, compression and shear stresses. Normal loading of bone usually results in bending, which applies both tensile and compressive forces on opposite sides of the bone. Torsion or twisting produces shear stresses along the length of the bone, while tension and compression are also acting to shorten and lengthen the bone in different areas. Most stresses that occur in

bone cannot be resolved into one individual component, but are a complex combination of all three. The predominant stresses in bone as determined by in vivo strain gauge data are longitudinal normal stresses due to bending, and shear stresses generated from combined bending and torsion (Einhorn, 1992).

Physiological stresses applied to normal healthy bone generally produce small strains, or little deformation, while poorly mineralized tissue will yield to a greater degree, producing larger strains. These properties can be tested in a laboratory setting by applying a known force and measuring the subsequent deformation of the bone. At low levels of stress, this relationship is linear and is known as Young's modulus or the modulus of elasticity. It is a measure of the slope of the force vs. deformation line and can be calculated by dividing the stress by the strain at any point along the linear portion of this curve. The linear portion of the curve is known as the elastic region and provides information about the stiffness of the bone. When the curve becomes nonlinear, this portion is referred to as the plastic region. The point at which the curve becomes nonlinear is known as the yield point. Loading beyond this point causes permanent deformation of the bone. In the elastic region, bone will only deform while the load is applied and will return to its original shape when the load is removed. Measurement of the area under the curve up to the point of failure gives the total strain energy stored and is known as the toughness of the bone (Cullinane and Einhorn, 2002).

Bone is a highly anistropic material, meaning that its mechanical properties differ according to the direction it is loaded (Cullinane and Einhorn, 2002). Therefore, when conducting mechanical testing on bone and comparing data between experiments, different loading tests may produce different results for the same specimen.

Biomechanical properties can be tested at two levels: by either performing standardized testing on uniform sections, which provide information about intrinsic material properties, or by testing the bone as a whole with normal geometry in order to obtain its structural properties (Einhorn, 1992).

Bone not only varies in its mechanical properties according to which direction it is loaded, but also with the rate at which it is loaded. Therefore, strain rate and direction of load must always be specific when describing material properties of bone. As bone is a viscoelastic material, material flows internally due to applied stress. An increase in the strain rates increases the modulus of elasticity and ultimate strength of the bone, but decreases the ultimate strain (Einhorn, 1992). At low strain rates, the bone may show appreciable deformation, but at high strain rates, it behaves more like a brittle solid, resisting deformation. Equine cortical bone tested at a range of strain rates showed an increase in mechanical strength with increasing strain rates (Evans et al., 1992). A linear relationship exists between the velocity of gait and the strain rate (Rubin and Lanyon, 1982), therefore at higher speeds, bone is more able to resist applied stress. However at strain rates around 0.1/sec, equine cortical bone showed a lower strain to failure and energy absorbing capacity as bone acted more like a brittle solid (Evans et al., 1992). These strain rates were within the rates which would be expected *in vivo* in the galloping horse. In addition, at higher strain rates, the increasing cellular stiffness appears to render the bone cells more insensitive to loading (Jacobs et al., 1998).

Regions of the cortex within a single bone experience less loading than others. During bending, a region of bone defined as the standard neutral axis experiences no deformation and thus no strain at the instant of peak loading during running and walking

(Carter et al., 1981; Lanyon, 1987; Gross et al., 1992). However, the neutral axis can shift during different activities such that all parts of the bone will be loaded at some time. Mosley et al. (1997) showed a non-uniform increase in periosteal expansion in rat ulnae with more deposition occurring away from the neutral bending axis and towards regions of greater strain. Greater periosteal apposition rate was observed on the medial aspect of the rat tibia versus other areas of the bone corresponding with greater recorded strains during bending (Raab-Cullen et al., 1994b). In jump-trained roosters, both periosteal and endosteal BFR increased specifically in those sites where strain rates were increased (Judex and Zernicke, 2000b). Different aspects of a cross-section react differently to the same stress or loading stimulus, presumably due to the different distribution in strain magnitude across and along the bone (Rubin, 1984; Biewener et al., 1996). In rats, the number of osteons and osteocytes increased in the posterior and lateral aspect of the tibia in responses to exercise, while the posteromedial aspect increased in porosity compared to controls (Li et al., 1991).

Technologies

Numerous markers of bone metabolism have been used in a variety of species to monitor changes in bone metabolism and the balance between formation and resorption. Recently markers of bone metabolism have gained in popularity among equine researchers. Both markers of bone formation and resorption are available based on metabolism of the bone forming or resorbing cells. Bone resorption markers include

pyridinoline crosslinks, carboxy-terminal telopeptide of type I collagen, and tartrate resistant acid phosphatase, while markers of formation include bone specific serum alkaline phosphatase, propeptides of type I procollagen and osteocalcin.

Osteoblasts produce many proteins specific to their cell type including bone sialoprotein, osteopontin, osteonectin, and osteocalcin (OC). Of these bone specific proteins, OC is the most frequently used and commonly accepted to be a good marker for bone formation. Osteocalcin is believed to play a role in matrix mineralization, and is expressed late in the cell cycle. Also referred to as bone Gla protein due to the presence of three gamma carboxylic acid residues, OC is a small non-collagneous protein which is incorporated into the ECM. It is the most abundant non-collagenous protein in bone, accounting for 25% of the total of non-collagenous proteins (Azria, 1989). In the presence of Ca, the GLA residues cause conformational changes which allow OC to bind to hydroxyapatite and its subsequent accretion into the bone matrix. A portion of the OC produced is released into the circulation and can be assayed in the serum using radioimmunoassay (RIA; Delmas, 1990) or enzyme-linked immunosorbent assay (ELISA; Hyldstrup et al., 1989). In a study comparing the effectiveness of these two techniques on detection of equine OC, recovery of OC in a serial dilution was underestimated by the ELISA and overestimated by the RIA. In addition, the ELISA detected greater concentrations and differences between two horses of 82.65 and 77.08 ng/ml while differences between concentrations using the RIA assay on the same samples was 0.29 and 3.5 ng/ml (Hoyt and Siciliano, 1999). Depending on the assay used, whether it is a polyclonal or monoclonal antibody, some antibodies may recognize not only the intact osteocalcin protein, but also fragments of the protein that are released

following extracellular matrix (ECM) degradation (Gundberg and Weinstein, 1986). The RIA uses a polyclonal rabbit anti-bovine-OC antibody while the ELISA uses a monoclonal mouse anti-bovine-OC antibody.

Equine OC has just recently been isolated and characterized which will lead to greater specificity and confidence in the results of this assay (Carstanjen et al., 2002). Osteocalcin concentrations in horses have been shown to be affected by age decreasing with maturity (Price et al., 1995a; Black et al., 1999; Lepage et al., 1990), growth rate (Goyal et al., 1981; Petersen et al., 2001), type (Lepage et al., 1998) or breed (higher in Thoroughbred foals versus Quarter Horses, Reller et al., 2001), circadian rhythms (Lepage et al., 1991), season (Jackson et al., 1998, Price et al., 1997) and exercise (Jackson et al., 1998; McCarthy and Jeffcott, 1992; Fletcher et al., 2000). When both formation and resorption are coupled, OC is believed to be a good marker of bone turnover, and when the processes are uncoupled, it is a specific marker of bone formation (Delmas, 1993).

Many of the markers of bone resorption are based on the urinary excretion or serum concentrations of type I collagen degradation products, including hydroxyproline, and the pyridinium cross links – pyridinoline (PYD) and deoxypyridinoline (DPD). Urinary hydroxyproline was one of the first bone resorption markers to be developed due to the high concentration of hydroxyproline in collagen. However, collagen is ubiquitous and not specific to bone. Bone degradation is assumed to contribute to 50% of the urinary hydroxyproline, but this percentage may increase with increased bone turnover due to disease (Robins and Brady, 2002). One of the problems with the use of hydroxyproline is that its presence is not only due to collagen breakdown, but synthesis

as well. As the N- and C- terminal propeptide must be cleaved before incorporation into the collagen fibril, this presents an additional source of hydroxyproline. Further, hydroxyproline is extensively metabolized in the liver and dietary sources may further contribute to urinary concentrations. Therefore, the use of urinary hydroxyproline has fallen in popularity, especially as short term within-subject variablility is reported between 17 and 41% with longer term variability of 19 to 53% (Seibel et al., 2002).

Pyridinoline and DPD are two non-reducible pyridinium crosslinks present in the telopeptide domain of the mature form of collagen. As mentioned previously, they are created by the posttranslational action of lysyl oxidase linking lysine and hydroxylysine residues together, a process unique to collagen and elastin molecules. Deoxypyridinoline is derived from two hydroxylysines and one lysine residue, while PYD is derived from three hydroxylysine residues. The resulting aldehydes condense with lysyl or hydroxylysyl residues on adjacent collagen molecules to form the mature pyridinium crosslink. These interchain bonds strengthen and stabilize collagen molecules within the extracellular matrix (Robins et al., 1994).

When collagen is degraded during bone resorption, the pyridinoline cross-links are released into the circulation. These range in size from the free cross-linked amino acids, to those still bound to segments of N-telopeptide and C-telopeptide sequences. These small fragments are readily cleared by the kidneys and no evidence exists that they are themselves metabolically degraded (Calvo et al., 1996). In the human, free pyridinolines account for about 40% of the total pyridinolines found in urine (Robins et al., 1994) and most assays only test for the free form unless the samples are hydrolyzed. Increased bone turnover, as reflected by an overall increase in total DPD, resulted in a

preferential increase in the peptide-bound form and a relative decrease in the free form, although absolute values of both increased (Garnero and Delmas, 1996). Others found that only the free form of PYD increased with no increase in the peptide bound form in ovarectomized rats (Black et al., 1989). Free and peptide bound cross-links may be released from the action of different proteolytic enzymes which are under the regulation of different aspects of bone resorption. Therefore, as the proportion of cross-links that appear free vs. bound does not appear to be constant, it may be more useful to determine total pyridinoline concentrations rather than merely the free form.

In humans, the ratio in which pyridinolines appear in urine reflect their ratio in bone, thus bone is believed to be the primary contributor to their presence (Robins et al., 1994). However, in other soft tissues, even though the ratio of PYD:DPD is much higher, the actual concentration of DPD/mol of collagen is similar to that of bone (Calvo et al., 1996). Therefore, in certain diseases which may include muscle wasting, the DPD in urine may originate from non-bone sources (Eyre, 1995). In addition, while the ratio of PYD to DPD in humans is approximately 3:1, this ratio is variable between species (Garnero and Delmas, 1996). Data on the ratio of these two crosslinks in equine bone is currently unavailable and needs to be determined. Different forms of bisphosphonate therapy in osteoporotic rats resulted in an increase in content of PYD and DPD for some, while the ratio of PYD:DPD changed in cancellous but not cortical bone (Egger et al., 1994). The variability in the ratio of PYD:DPD needs to be identified across a large sampling of the horse population.

The most specific assay used for detection of bone resorption considered to be the gold standard by most clinicians and researchers is the total urinary excretion of

pyridinoline cross-links measured by HPLC (Robey, 1995). However, this method is tedious and time consuming and therefore simpler assays have been developed using immunoassays. Urinary free PYD is highly correlated with the total PYD excretion measured by HPLC, and increases with menopause (Garnero et al., 1996). The Pyrilinks (Metra Biosystems, Inc ,Mountainview, CA) assay detects the presence of both PYD and DPD while Pyrilinks-D (Metra Biosystems, Inc ,Mountainview, CA) is specific for only deoxypyridinoline, which is believed to be more specific for bone resorption. The ELISA of free-DPD uses a monoclonal antibody which does not cross react with free PYD and is also highly correlated with total DPD measured by HPLC (Garnero and Delmas, 1996). The original ELISA assays measure free pyridinolines unless they are modified to measure the total pyridinolines. To obtain total pyridinolines, the serum or urine must first be hydrolyzed to release the peptide bound fragments. However, this is also a potential source of error as acid hydrolysis can result in destruction of pyridinolines (Lepage et al., 2001). Within the last few years, an immunoassay was developed by Metra Biosystems which enables equine serum total DPD to be analyzed (Weitz et al., 1999).

There are also several assays of bone resorption based on the measurement of peptides associated with the cross-linked sections of collagen. The NTX assay is based on a cross-linked peptide from the N-terminal of the telopeptide of type I collagen and can be used on serum or urine. It is commercially available as an ELISA assay (Osteomark) from Ostex International (Seattle, WA). Other assays are based on the metabolism of the C-terminal of type I collagen. Previously known as ICTP, and now referred to as CTX-MMP, this assay measures the release into the serum of the trivalent

crosslink at the C-terminus following matrix metalloproteinase digestion of collagen (Seibel et al., 2002).

While these markers provide an idea of the balance between bone formation and resorption at a single time point, they do not provide information about the skeletal mass or architecture. Circulating concentrations of these markers can also be affected by factors other than changes in bone turnover, such as their metabolic clearance (liver uptake, renal excretion, and/or trapping on bone hydroxyapatite), and by the pattern of immunoreactive moieties recognized by the particular antibodies used. However, since blood or urine can be repeatedly sampled on a single subject and is relatively easy to obtain in the equine, changes in these concentrations may provide valuable information about systemic changes in bone metabolism. Known factors that affect biochemical bone markers, such as circadian rhythms, diet, age, gender, body and bone mass should be defined and adjusted when possible. Several studies have shown that with age, concentrations of bone markers decrease in the horse, reflecting advanced skeletal maturity (Lepage et al., 1990; Price et al., 1995a). Breed differences also occur in concentrations of markers as the concentration of osteocalcin and ICTP is higher in draft horses than in Thoroughbreds which may indicate different rates of bone turnover in different breeds (Grafenau et al., 2000). Similarly, racial differences exist in human bone mass, as blacks have higher BMD and lower bone marker concentrations than caucasians (Kleerekoper et al., 1994; Slemenda et al., 1997). Therefore, data for some breeds of horses may not necessarily be able to be extrapolated to others. Other factors such as season, and time of sampling need to also be considered as these influence concentrations of bone markers (Price et al., 1997; Jackson et al., 1998).

Bone markers can provide only a somewhat obscure picture of what is happening at the bone level. In human medicine, markers have been used extensively to identify patients at risk of osteoporosis as well as to monitor effectiveness of drug therapies. Single measurements provide only a static picture of a complex process, and higher concentrations of markers of bone turnover, including markers of both formation and resorption, have been interpreted by researchers as both a positive (Hiney et al., 2001) and a negative outcome of exercise (Jackson et al., 1997). Estrogen and bisphosphonate therapy in osteoporotic women reduces concentrations of bone markers of bone formation and resorption, indicative of reduced bone turnover (Garnero et al., 1996; Delmas, 1993). However, both high and low concentrations of OC are found in women diagnosed with veterbral osteoporosis (Delmas, 1993). Despite decreasing bone formation, these therapies result in a slowing of bone loss, or a small gain in bone mass. Lower concentrations of OC were correlated with higher bone mineral density in young girls (Slemenda et al., 1997). Some see high levels of bone markers such as PICP and ICTP as an indication that bone turnover is higher and contributing to bone loss while others proclaim the net result is greater bone deposition. Unless a more complete understanding of the physiological meaning of fluctuations is determined, and their relationship is correlated to actual structural changes, their usefulness in a practical, field situation will be limited. A single measurement will likely never be useful as changes in blood concentrations must be monitored over time. In addition, seasonal variations in blood markers may obscure the effects of exercise on these markers. Price et al. (1997) reported that biochemical markers are at their lowest concentration during the winter and increase in the spring.

The study of bone physiology is very complex due to the composite nature of the material and the various factors contributing to its strength. Not only is the structural integrity of bone determined by the state of mineralization and organic matrix organization, but also by geometry as well. Several non-invasive techniques currently available to the researcher, physician or veterinarian have been developed to study bone properties *in vivo* and predict or diagnose subjects with substandard bone. Several of these modalities include radiography, radiographic grammetry, single and dual photon absorptiometry, ultrasound velocity (Buckingham and Jeffcott, 1991b), radiographic absorptiometry (Meakim et al., 1981; Nielsen et al., 1998), dual energy x-ray absorptiometry (McClure et al., 2001), and quantitative computed tomography. All of these techniques have their advantages and disadvantages concerning ease of use, cost, precision, accuracy, and availability and ability to separate cancellous and cortical bone. These factors must be carefully considered when deciding upon the technique to be used in either a research setting or for diagnostic evaluation of metabolic bone disease and treatment effectiveness.

Ultrasonography has been used extensively (Buckingham and Jeffcott, 1991b; Jeffcott and McCartney, 1985; McCarthy et al., 1990), but is limited in its use as it is affected by limb temperature and soft tissue swelling, and can only be used in the frontal plane of the lower part of the limb. Therefore, changes in the dorsal aspect of third metacarpal bone (MCIII) cannot be assessed. In horses, adaptations in the dorsal cortex of MCIII are the most common training effect (Nunamaker et al., 1989; Stover et al., 1992; Sherman et al., 1995; Lawrence et al., 1994). Single photon absorptiometry coupled with ultrasound velocity detected a larger decrease in mineral content in horses

with immobilized forelimbs in comparison to the control limb, while radiographic photodensitometry found a larger decrease in the control limb (Buckingham and Jeffcott, 1991a). However, both the fortnightly single photon absorptiometry and weekly ultrasound measures of the bone showed considerable variation within the same animal, especially in the 12 wk following cast removal. In addition, single photon absorptiometry requires a long scan time of up to 1 min/site and decay of the radiation source affects long-term accuracy.

Dual energy x-ray absorptiometry (DEXA) offers the advantage of using x-rays of two different energy levels which are attenuated to a different degree by bone, fat and muscle. It is the technique most commonly used in human studies and provides an accurate assessment of site BMC. However, as it is a two dimensional technique, values of BMD are reported as an areal density rather than a true volumetric density. A DEXA scan provides areal BMD in units of $g/cm^2$ by giving the integral mass of bone mineral within the region of interest. Few studies examined the accuracy of this technique in predicting true BMD, as precision has been more the concern in monitoring changes in individuals. However differences of DEXA values with ashed samples have been within 0 and 15% (Blake et al., 1999). The sensitivity of DEXA is greater than radiographs as the smallest changes that can be observed in bone density are 4.5% and 7.5% for the spine and femoral neck respectively versus 10% changes which can be detected by radiographic bone aluminum equivalence (RBAE; Hodgson and Rose, 1994; Blake et al., 1999). Dual energy x-ray absorptiometry is used ex vivo in horses and correlates with bone mineral density as determined by the Archimedes' principle (McClure et al., 2001;

Hanson and Markel, 1994). Correlations improved significantly using a linear regression which accounted for differences in age, weight and the presence of soft tissue.

Radiogrammetry involves measuring bone diameters, medullary cavities and cortical widths directly off the radiograph with the aid of a ruler or caliper. It has similar advantages to standard radiographs in that it is simple to perform and inexpensive. However, separating cortical from cancellous bone with great accuracy is difficult when limited to visual inspection. Radiogrammetry can produce a rough estimate of cross sectional area if the bone is assumed to be symmetrically circular or elliptical. However, bone will adapt to place more mass in one direction to compensate for compressive forces (i.e. greater dorsal bone deposition in the equine); therefore, such assumptions should not automatically be made. Measurements made beyond 1 or 2 mm in definition will generally not be significant due to the loss of accuracy beyond this level. The sharpness of the x-ray, or the ability to define an edge, is critical when using this technique. When measuring cortical widths, the periosteal surfaces are usually easier to distinguish than the medullary cavity. Therefore, placement of the calipers becomes less certain when measuring the medullary cavity, especially with the gradual decrease in opacity of the radiograph due to the presence of the less dense, cancellous bone. In addition, margins of bone may be clouded by the unavoidable presence of penumbra, or the blurring of the edges of an image. Penumbra is created by the focal area (rather than a theoretical focal point) from which the x-rays emanate (Curry et al., 1990). Individual photons hit the object at slightly different angles, representing the many point sources of the x-rays. Radiogrammetry has been used previously in the equine, but was poorly correlated with bone mineral content (Meakim et al., 1981). More recently, radiogrammetry was used to

evaluate the effects of exercise and the use of exogenous equine somatotropin on young racehorses and did appear useful in showing treatment effects (Thomson et al., 2001).

Radiographic absorptiometry improves significantly on the technique of radiogrammetry as it is much more objective and provides quantitative data. It reduces the opportunity for manual mistakes and human error involved with visual inspection of radiographs. Although not as frequently used in human medicine, its ease of use and cost effectiveness make it very popular for use in animal studies. The optical density of the bone image is compared with a standard, typically an aluminum step wedge or penetrometer, which varies by a known thickness for each step (Meakim et al., 1981).

The radiographic image of the stepwedge and the selected site of the bone are scanned with a densitometer which transfers the information into a digital image with the aid of a computer software program. To compare data over time or between subjects, a specific site of the bone should be chosen which can be reliably and repeatedly selected between different sampling times. Background density due to film base, fog and scatter are subtracted from the images to provide a baseline of optical density. Logarithms of the percentage transmittance of the densitometer reading of the stepwedge are plotted against the known mm of Al of each step. This produces a linear regression equation which is used to calculate the percent transmission of the selected point on the bone scan. The data is now in the form of RBAE. These data are a relationship or an approximation of bone mineral content, not a direct measure. It is a comparison between the optical density of the bone and the optical density of the aluminum stepwedge. Radiographic bone aluminum equivalence has been shown to be a good estimator of bone mineral content with accuracy within 6% of mineral ash content of bone (Cohn, 1981). In equine

bone, RBAE values were highly correlated with BMC expressed as ash per 2 cm section of bone, but was not highly correlated when expressed on a per weight basis (Meakim et al., 1981). Radiographic bone aluminum equivalence does not provide information about bone mineral density which is a volumetric measure.

Total RBAE, a radiographic technique, describes volumetric changes in MCIII (Nielsen and Potter, 1997). However, scanning the bone at the same site using the dorsopalmar (d-p) view and lateromedial (l-m) view gives two different values, with the values from the d-p view being greater. Similarly when using DEXA to assess whole bone BMD across the width of the bone, the d-p and l-m view provide different numbers, with the lm view being greater, despite it "seeing" through the same amount of bone (McClure et al., 2001).

Quantitative computed tomography has the highest sensitivity to disease or age-related bone loss due to its three-dimensional abilities. Computed tomography (CT) operates using the same principle of photons being transmitted through the body and attenuation measurements made by a detecting source. The CT scan shows a value of the linear attenuation coefficient, $\mu$, at each pixel. The $\mu$ values are given in Hounsfield units (HU), where air = -1000 HU and water = 0 HU. A linear correlation is determined between the HU number and BMD by including a hydroxyapetite calibration phantom in the scan. The data are transferred by computer software to provide tomographic images that are slices of chosen cross-sections of the body. Quantitative CT provides information on bone density from cross-sectional rather than projectional images, thus it provides true physical density rather than projectional areal density, as does DEXA. This allows for areal measurements of the bone and gives true physical density in $g/cm^3$. Total

bone area, cortical area and medullary cavity can all be measured accurately with this method. The use of peripheral CT have revealed differences in cross-sectional area and trabecular density that DEXA is unable to determine (Adami et al., 1999). Peripheral CT allows the calculation of biomechanical properties such as the cross-sectional moment of inertia which is more indicative of bone strength. Computed tomography offers a further advantage as patient positioning does not have to be painstakingly duplicated between scans. However, the use of QCT is somewhat limited due to its high installation and running costs and the radiation dose is significantly higher with QCT than it is with DEXA.

# CHAPTER III

# BOVINE STUDY

## SUMMARY

The ability of short duration, high intensity exercise to increase bone formation in confined immature Holstein bull calves was investigated. Eighteen bull calves of 8 wk of age were assigned to one of three treatment groups – group housed (GR, which served as a control), confined with no exercise (CF), or confined with exercise (EX). The exercise protocol consisted of running 50 m on a concrete surface once per day, 5 d per wk. Confined calves remained stalled for the 42-d duration of the trial. At the completion of the trial, calves were euthanized, and both forelegs were collected. The fused third and fourth metacarpal bones were scanned using computed tomography for determination of cross sectional geometry and bone mineral density (BMD). Three point bending tests to failure were performed on metacarpal bones with a universal testing machine. Serum was analyzed for concentrations of markers of bone metabolism – deoxypyridinoline and osteocalcin. The exercise protocol resulted in the formation of a rounder bone in EX as well as increased dorsal cortex thickness, compared to the GR and CF calves ($P<0.05$). The exercised calves also had a significantly smaller medullary cavity than CF and GR and a larger percentage of cortical bone area than CF ($P<0.05$). Dorsal, palmar, lateral and total BMD was significantly greater in EX than in CF, and palmar and total BMD were also greater in EX than in GR ($P<0.05$). The bones from EX animals tended to have a higher fracture force than CF ($P<0.1$). Osteocalcin concentrations normalized from d 0 were higher in EX than CF ($P<0.05$)

Key words: bone development, confinement, exercise

# INTRODUCTION

The skeletal system is highly adaptive, able to sense alterations in its loading environment and respond in order to achieve a dynamic balance between skeletal strength and skeletal mass. Unfortunately, current management practices of many domestic livestock species may potentially jeopardize skeletal integrity. Confinement decreases skeletal strength due to the lack of exercise and loading placed on the bone (Marchant and Broom, 1996; Knowles and Broom, 1990). In the equine, stall confinement decreased bone mineral content in both weanlings and yearlings, compared to those left on pasture with access to free exercise (Hoekstra et al., 1999; Bell et al., 2001). This may be especially detrimental to the young equine if it is then placed into a strenuous training regime with a skeleton of compromised strength. Injury rates in two-yr-old racehorses exceed 50% (Rossdale et al., 1985); therefore, the relationship between confinement and bone strength and its culpability in skeletal injury needs to be explored.

Only a few loading cycles may be needed to stimulate an osteogenic response and ameliorate the reduction in bone mass observed with confinement. Rubin and Lanyon (1984) showed that only four cycles per day were needed to maintain bone mineral density in immobilized turkey ulnae, while 36 cycles prevented disuse osteoporosis in tibiae of rats (Inman et al., 1999). However, use of an externally applied load in the above experiments, while useful for establishing a very defined strain regime, creates an unusual, non-physiological strain on the bone. Few studies have examined the effectiveness of a limited number of physiological loading cycles created by normal locomotion. Daily periods of walking in sheep were unable to prevent decreased bone

mineral density of the calcaneus caused by immobilization with an external fixator placed across the tarsal joint (Skerry and Lanyon, 1995). Therefore, the stimulus placed on the bone must be of sufficient magnitude to elicit an adaptive response. Alternatively, the completely *in vivo* and practical approach of using sprinting exercise as the loading program is suggested. This would preclude any unusual or artificial effects due to pressure from the loading apparatus applied to the limb, a previous concern of researchers (Raab-Cullen et al., 1994b). Additionally, the non-invasive exercise approach subjects the bone to elevated, but physiologically normal strain patterns, which cannot be achieved through external mechanical loading. Therefore the goal of this study was to determine if 6 wk of stall confinement resulted in lowered bone mass compared to pen housed calves and to determine the potential benefits of short term, high intensity exercise in increasing bone quality.

## MATERIALS AND METHODS

### Animals and Management

Eighteen Holstein bull calves were obtained from the MSU Dairy Teaching and Research Center. Calves were age-matched in groups of three, such that each group began the project at an average age of 8 wk. Individuals from each group were randomly assigned to one of three treatments, resulting in six calves per treatment group. Calves were initially weaned from a milk replacer diet at 7 wk of age and thereafter had ad libidum access to a commercially available pelleted calf grower and allowed free access to water. Two groups were housed in tie stalls (0.65 m x 1.55 m) that allowed the calves only to stand and lie down. One group remained in the tie stalls for the 6 wk duration of

the project, with no access to exercise (CF), while 6 calves received controlled exercise (EX). The exercise regimen consisted of short duration, high intensity running 5 times per week. Calves were removed from their tie stalls, led to an adjoining barn 32 m from their stalls and verbally encouraged to run through a 50 m concrete alley once per day. This approximated 25 strides or cycles per sprint at approximately 4 m/s. They were then returned to their stalls, with the entire distance traveled being 164 m. The final treatment (GR) served as a control; these calves were housed in an 8.5 m x 7.3 m pen with free access to exercise and were allowed to interact with the other calves in this pen. Calves were weighed on d 0, 21 and 42. After 42 d, calves were euthanized, and both forelimbs were removed above the carpi to allow for collection of the fused third and fourth metacarpal bone. The left and right $9^{th}$ ribs were collected as representative of non-weight bearing bones.

Behavioral Observation

In order to determine the influence of voluntary activity on bone measures, observations of behavior were made over 24 h on d 0, 21, and 42. Calves were videotaped in either their tie- stalls or in the group setting with an extended play recorder which allowed 24 h to be recorded on one tape. Behaviors were observed and recorded for 4 randomly chosen 15 min periods per 24 h. As the primary objective of this project was to determine the influence of activity on bone parameters, behavior observations were limited to those activities which would load the bone, and therefore, impact bone strength. Therefore, no observations were made of ingestive or eliminative behavior or social interactions. Behavioral data was recorded with the Observer software (Noldus

Information Technologies, Sterling VA). Behaviors for stalled calves were defined as either standing or lying as they were primarily restricted to these activities. The behavior of the calves in tie stalls was analyzed for duration of the two activities as well as the frequency at which the calves altered from one posture to the next. The frequency of lying down/standing up was determined as the number of occurrences of the behavior pattern which occurred in the observation period. Duration was recorded as the cumulative number of minutes in each of the four 15-min periods for which the animal stood or was lying. The total duration of the behaviors was recorded as a proportion or percentage of time for which all occurrences of the behavior lasted over the observation session. Data were averaged over the six calves in each group. Similar observations were made for the group-housed calves. Voluntary activity of the group-housed animals was further defined to include bouts of standing, walking, lying, trotting, and jumping. Walking bouts were defined as four consecutive steps or one stride. If four consecutive steps were not taken, this behavior was characterized as simply standing. Trotting consisted of performing a definite two-beat gait and loping as a three-beat gait. Jumping was also included as a possible behavior, but was defined as an event versus a state, and thus was not timed.

Measurements and Sample Collection

Blood was collected via jugular venopuncture into non-heparinized vacutainers for analysis of concentrations of osteocalcin (OC) and deoxypyridinoline (DPD) – markers of bone metabolism. To determine how quickly bone responds to alterations of loading regimes, blood was drawn daily at 0800 for the initial 7 d, followed by once per

wk for the remainder of the project. In order to minimize the effects of diurnal variations (Black et al., 1999), daily sampling time was not varied. Blood was allowed to coagulate at 20°C and then centrifuged at 1340 g for 12 min for serum separation. Serum samples were frozen at –20°C for later analysis. Serum total deoxypyridinoline concentrations were analyzed using Pyrilinks-D enzyme-linked immunosorbent assay (ELISA). Serum samples were diluted in a 1:4 ratio with double distilled water. Osteocalcin concentrations were analyzed using Novacalcin, an ELISA kit obtained from Metra Biosystems, Inc (Mountainview, CA). Serum samples were diluted in a 1:30 ratio in order to obtain concentrations within the linear range of the standard curve. All assays were performed according to manufacturer's instructions.

Radiographic Bone Aluminum Equivalence

Radiographs were taken by a certified radiologist on d 0, 21, and 42 to determine radiographic bone aluminum equivalence (RBAE) values, measures of optical density. Radiographs of the dorsal-palmar and medial-lateral views of the left and right fused metacarpal bone (MCIII & IV) were taken at a focal length of 30 inches and an exposure of 60 kVp for .06 sec. An aluminum stepwedge was attached to the radiographic cassette to standardize readings and calculate RBAE values. Optical density of the bone was assessed using radiographic bone aluminum equivalence (RBAE) using a BIO-RAD Model 700 video densitometer (Hoekstra et al., 1999). Radiographs were scanned at 50% of the bone length of the fused MC III & IV. A linear regression was produced from the thickness of the steps on the aluminum penetrometer. Maximum optical density of each

cortex was expressed in millimeters of aluminum for both cortices in each view of MC III & IV.

Computed Tomography

Following euthanasia, intact limbs with the soft tissue intact were imaged by computed tomography for determination of cross sectional geometry and bone mineral density (BMD). Limbs were placed on a QCT phantom pad with hydroxyapetite bone standards for BMD analysis and scanned with a GE 9800 CT scanner (General Electric Medical Systems, Milwaukee, WI) at 80 KV, 70 mA, with a 2 sec scan time). An initial scout view was taken to determine the midpoint between the proximal margin of MC III & IV and the distal physis. A single 10-mm thick transverse image was then acquired at this selected location. This location was chosen as it corresponded with the smallest diameter of the bone and thus the location at which the break was predicted to initiate during the three-point bending test. This allowed evaluation of both bone density and cross sectional area at the same location.

The endosteal and periosteal margins of the bone were traced on the CT computer, which then calculated the area within the region of interest to provide total, cortical, and medullary cavity cross sectional area. The diameter of the bone was also measured, including the dorsopalmar (DP) bone diameter (D), DP medullary diameter (d), lateromedial (LM) bone diameter (B), and LM medullary diameter (b); (see Figure 1). These distances were measured at the line which bisected the bone for the DP diameter (minor diameter), and at the widest distance across the bone for the LM diameter (major diameter). Finally, the width of the individual cortices: dorsal (DC),

palmar (PC), medial (MC), and lateral (LC) were measured, again in the same plane as the previous measures were made.

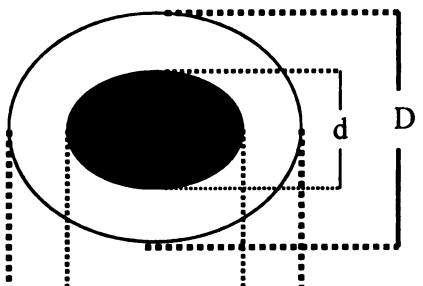


Figure 1. Schematic illustration of a cross-section of bovine MC III & IV showing cortical measurements. Measurements included :
B = outside major diameter, (lateromedial bone diameter)
b = inside major diameter, (lateromedial medullary diameter)
D = outside minor diameter, (dorsopalmar bone diameter)
d = inside minor diameter, (dorsopalmar medullary diameter)

From the cross sectional images, a region of interest (7 $mm^2$) in each cortex was selected with the cursor in the center of the cortex at approximately the same plane at which the width of the cortices were measured. Image brightness and contrast were standardized for the vertebral window. The bone mineral density (BMD; mg/cc equivalent) was then calculated by a software program which compares the linear attenuation coefficient of the bone to the BMD phantom for that region. Average BMD for the entire cross sectional area for each image was also calculated by surrounding the bone within the cursor as tightly as possible. Each measurement was performed in triplicate, with the mean value used for statistical analysis.

Mechanical Testing

Ultimate bending strength, modulus of elasticity, and fracture force of the metacarpi were determined using three-point bending tests conducted on a universal testing machine (Model 4202, Instron Corp., Canton, MA) according to ASAE Standards (2000). A cross-head speed of 10 mm/min was used with supports set at 10 cm apart. Three-point bending to failure was performed on left metacarpi alone, while deformation to 4 mm was also performed on the right metacarpi. Ultimate bending strength (stress) was calculated by:

$\sigma = FLC/4I$
where
$\sigma$ = ultimate bending stress, Pa
$F$ = applied force, N
$L$ = distance between supports, m
$C$ = distance from neutral axis to outer fiber (D/2), m
$I$ = moment of inertia, $m^4$

The moment of inertia (MOI) for a hollow ellipse was calculated as:

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

Modulus of elasticity was determined by taking the mean of the data from deformation tests performed on both the right and left limb. The force/deformation curve between 2 and 4 mm deformation was used to calculate the slope of the straight line ($F/\delta$). This portion was chosen as it fell in the linear portion of the curve, which provided an $r^2$ = 0.99. Modulus of elasticity (E) was calculated by :

$E=FL^3/48I\delta$
$\delta$ = deformation, m

The modulus of elasticity of the 9th rib was determined by three-point bending. The same cross-head speed and distance between supports was used as for the tests on the metacarpi. The curved portion of the ribs were cut 18 cm from the costochondral junction in order to provide a relatively flat sample for bending tests. Modulus of elasticity (E) was calculated by the same formula as above, with the exception that the moment of inertia was calculated using the equation for a quadrant of an ellipse:

$$I = 0.0549(B*D^3)$$

Ash Determination

One cm cross-sections of bone were cut at the midpoint between the proximal margin of MC III & IV and the distal physis in the same location as scanned for computed tomography. An additional 5 mm cross-section was made proximal to the carpus and further sectioned into 5 mm cubes to include the same cortical regions (dorsal, palmar, medial and lateral) analyzed for BMD with the CT. Bone volume was determined by suspending the intact bone slice within a beaker filled with ionized water. The volume of the bone was calculated as the difference of the weight of the beaker, water and sample, and the weight of only the beaker and water. Density of the bone was calculated using the formula: density = A/(A-B) * P, where P is the density of water, A is the weight of the bone out of water, B is the weight of the bone submerged in water, and A-B is the difference in weight, equivalent to the weight of the volume of water displaced by the bone which is equivalent to the volume of bone according to Archimedes' Principle. A one cm section from the midpoint of the left rib was removed and ashed as the metacarpal bone samples. Fat was removed from all bone samples via ether

extraction with a Soxhlet apparatus. Samples were then dried at 150° C for 8 h, weighed to determine the fat-free weight, placed in crucibles and ashed in a muffle furnace at 600° C for 12 h. Ash was then weighed and either expressed as a percentage of the dry fat-free weight, or based on the volume of the bone as determined with Archimedes' Principle.

Biochemical Analysis

After the original 1 cm section of bone was cut from the midsection of the metacarpal bone for purposes of ash determination, a 5 mm section of bone was sliced just distal to the midsection. These slices were then sectioned again to be representative of the dorsal, palmar, medial and lateral cortices of the bone, in the same location as BMD was determined. Samples were lyopholized for 24 h, weighed and then hydrolyzed in 1 ml of 6 M HCl for 18 h at 105° C, cooled, and stored at 4° C. For analysis of Ca and P, 500 ul samples were removed, and dried completely via an ATR Vacuum Concentrator (Appropriate Technical Resources, Laurel, MD). The dried samples were reconstituted in 1 ml of 6 N $HNO_3$, transferred to acid-washed volumetric flasks, and diluted to 25 ml with double-distilled water. Samples were diluted 200-fold in a 1% lanthanum chloride matrix. Calcium concentrations were determined by flame atomic absorption absorptiometry (Unicom 989 AA spectrophotometer, Thermo Elemental, Franklin, MA). For analysis of phosphorous concentrations, samples were diluted 10 fold in deionized water and measured against a standard curve of known phosphorous concentrations (Beckman Coulter DU 7400 spectrophotometer, Holton, CA).

Of the remaining 500 ul, 25 ul was removed for analysis of hydroxyproline concentrations. For collection of cross-links, 450 ul of the hydrolyzed sample was mixed with 500 ul of acetic acid, 500 ul of slurry (a cellulose and mobile phase mix), and 2.0 ml

of n-butanol. Samples were then vortexed and transferred to gravity-fed columns containing cellulose in mobile phase. After samples were transferred, 20 ml of mobile phase was added to elute all proteins except the cross-links. Collection tubes were placed under the columns following the wash. Cross-links were released from the cellulose with 7 ml double-distilled water followed by 1 ml mobile phase. Samples were dried using an ATR Vacuum Concentrator, and stored in the dark, at room temperature until analyzed by HPLC (high performance liquid chromatography).

Statistical Analyses

Data were analyzed for the effects of treatment according to the general linear method of SAS (2001). When treatment effects were significant, means were separated with the LSD method. A test for homogeneity of variance was performed using a Bartlett's Forsythe test. When variance was heterogeneous, the mixed procedure of SAS was performed on the data. Individual standard errors of the mean (SEM) for each treatment were then included in the tables. If the variance was homogeneous, pooled SEMs were provided. Statistical analysis of RBAE and serum data taken throughout the duration of the project was performed using the mixed procedure of SAS using a covariance test suitable for repeated measures. The covariance structure was autoregressive with calf within treatment used as the subject effect. In order to visualize changes over time in relation to initial values, some data were normalized or subtracted from d 0 values where appropriate. Correlations between BMD values, moment of inertia, and results from the mechanical testing were performed using the correlation procedure of SAS. Duration of behaviors averaged over the three observation periods were tested for differences between groups using a Fisher's exact test.

# RESULTS

## Weight Gain

The average age of the calves upon initiation of the project was 56.3 d. The group-housed calves began the project slightly, but significantly heavier than their stalled counterparts (Table 1). After 6 wk, while not gaining more weight than the CF calves (37.2 kg), EX calves tended to gain more weight than the GR calves, 43.9 kg versus 34.9 kg ($P<0.1$). Average daily gain over the entire period was not different between the treatments.

Table 1. Body weights (kg) and average daily gain (ADG).

| | EX | CF | GR | SEM |
|---|---|---|---|---|
| Body weight (kg) | | | | |
| Initial | 74.7 | 73.9 | 78.2 | 2.1 |
| Final | 118.6 | 111.1 | 113.1 | 2.8 |
| Gain | 43.9[a] | 37.2[ab] | 34.9[b] | 1.8 |
| ADG | 1.0 | 0.9 | 0.8 | 0.05 |

[ab] Treatments lacking a common superscript tend to differ ($P<0.1$)

## Computed Tomography

Computed tomography data did not differ between the right and left legs; therefore mean values were used for statistical analysis. Neither confinement nor exercise altered the total cross-sectional-area (CSA) of the bone; however, the medullary cavity of the bone was smaller in the forced-exercised calves versus CF or GR ($P<0.01$; Table 2). Absolute cortical bone areas were also not different between groups, but when the cortical area was expressed as a percentage of the total CSA, the confined animals had the smallest percentage of cortical bone compared to the GR and EX calves ($P<0.01$; Table 2).

Table 2. Cortical areas of MC III & IV ($cm^2$).

| | EX | CF | GR | SEM |
|---|---|---|---|---|
| Total bone | 4.44 | 4.38 | 4.25 | 0.07 |
| Medullary cavity | $1.64^{b}$ | $2.01^{a}$ | $1.88^{a}$ | 0.05 |
| Cortical bone | 2.61 | 2.56 | 2.37 | 0.06 |
| % cortical area | $0.61^{b}$ | $0.54^{a}$ | $0.58^{b}$ | 0.01 |

[ab] Treatments lacking a common superscript differ (P<.01)

Beyond a decrease in the medullary cavity area, the exercise protocol resulted in a rounder bone versus a more ovoid bone in the other two treatments as evidenced by bone measurements. Interestingly, the lateromedial bone diameter (B) was the widest in GR and smallest in EX (P<0.05), while CF animals were not different from either EX or GR (Table 3). Lateromedial medullary diameter (b) was greatest in CF (due to the overall larger medullary cavity) with the b of EX again being significantly smaller (P<0.05). Dorsopalmar bone diameter (D) did not differ between treatments, but dorsopalmar medullary diameter (d) was greater in CF versus EX (P<0.1). The group housed calves were intermediate between both groups and were not different from either group. Individual cortical diameters did not differ between groups with the exception of the dorsal cortex, which was greater in EX, 0.41 cm vs 0.31 and 0.35 cm in CF and GR respectively (P<0.05).

Table 3. Cortical diameters and widths of MC III & IV (cm).

| | | EX | CF | GR | SEM |
|---|---|---|---|---|---|
| Bone diameters (cm) | B | $2.58^{b}$ | $2.67^{ab}$ | $2.72^{a}$ | 0.03 |
| | b | $1.59^{b}$ | $1.81^{a}$ | $1.76^{a}$ | 0.04 |
| | D | 1.94 | 1.94 | 1.94 | 0.03 |
| | d | $1.13^{d}$ | $1.28^{c}$ | $1.20^{cd}$ | 0.04 |
| cortical widths (cm) | DC | $0.41^{a}$ | $0.31^{b}$ | $0.35^{b}$ | 0.02 |
| | PC | 0.38 | 0.34 | 0.34 | 0.01 |
| | MC | 0.50 | 0.43 | 0.49 | 0.02 |
| | LC | 0.48 | 0.42 | 0.47 | 0.02 |

[ab] Treatments lacking a common superscript differ (P<0.05)
[cd] Treatments lacking a common superscript tend to differ (P<0.1)

Overall, bone density, as determined by CT, was greatest in the exercised calves. Total BMD and BMD in the palmar cortex of EX was significantly greater than both GR and CF calves ($P < 0.05$ respectively) and was greater than CF in the dorsal cortex (Table 4). The EX and GR calves tended to have greater bone mineral density in the lateral cortex ($P<0.1$) while only medial BMD did not differ between groups.

Table 4. Bone mineral density as determined by computed tomography (mg/cc).

| | EX | CF | GR | SEM |
|---|---|---|---|---|
| dorsal | 1234 [a] | 1149 [b] | 1202 [ab] | 14 |
| palmar | 1144 [a] | 1072 [b] | 1083 [b] | 13 |
| medial | 1225 | 1190 | 1226 | 11 |
| lateral | 1228 [c] ± 10 | 1167 [d] ± 25 | 1225 [c] ± 18 | *** |
| total | 664 [a] ± 3 | 554 [b] ± 22 | 597 [b] ± 15 | *** |

[ab] Treatments lacking a common superscript differ ($P<0.05$)
[cd] Treatments lacking a common superscript tend to differ ($P<0.1$)

Mechanical testing

Treatment differences seen in bone geometry and density were not reflected in mechanical properties. Although exercised calves tended to have a higher fracture force (FF; $P<0.1$), when the ultimate bending strength (UBS) and apparent modulus of elasticity (ME) were calculated (which take into account geometric properties) there were no differences between groups (Table 5). Others have found bone mineral density (Les et al., 1994) or ash content (El Shorafa et al., 1979; Lawrence et al., 1994) to be predictive of mechanical properties such as failure stress. In this study, BMD was not correlated with FF, UBS or ME. However, if bone mineral density values were compared through regression analysis with the calculated moment of inertia (MOI), there was a significant positive correlation ($R^2 = 0.64$) between BMD in the medial cortex and MOI ($P<0.05$), while there was a weak correlation between total BMD and MOI ($P<0.1$;

$R^2 = 0.60$). As fracture force had a trend for differences between treatments ($P<0.1$), perhaps if the study had been extended beyond 6 wk, or greater numbers of calves were used, differences in other mechanical properties may have been more readily apparent.

Table 5. Mechanical properties of MC III & IV and 9th rib.

| | EX | CF | GR | SEM |
|---|---|---|---|---|
| Moment of inertia ($mm^4$) | $8.19 \times 10^{-9}$ | $7.78 \times 10^{-9}$ | $8.18 \times 10^{-9}$ | $2.9 \times 10^{-10}$ |
| Fracture force (N) | 5560[a] | 4746[b] | 5259[ab] | 152 |
| Ultimate bending strength (MPa) | 170.2 | 149.9 | 158.3 | 4.5 |
| Modulus of elasticity – metacarpi (MPa) | 3819 | 3286 | 3572 | 141 |
| Peak bending force (N) - ribs | 243 | 226 | 262 | 14 |
| Modulus of elasticity – ribs (MPa) | 4289[ab] | 4013[b] | 5074[a] | 214 |

[ab] Treatments lacking a common superscript tend to differ ($P<0.1$)

In order to determine if any treatment effects were present on whole body skeletal metabolism independent of loading stimuli, the 9th right rib was tested in three-point bending. Peak bending force sustained by the bones did not differ, but GR calves tended to have a higher value of ME (Table 5). However, as EX calves were no different from the CF calves in the ME of ribs, the higher value of FF of the fused third and fourth metacarpal bone is believed to be due to loading and not differences in management.

Radiographic Bone Aluminum Equivalence

Similar to the CT data, the left and right limb did not differ in RBAE measurements, thus the mean value for both limbs was used for statistical analysis. Radiographic bone aluminum equivalence (RBAE) in the lateral, medial, dorsal and palmar cortices was unaffected by treatment (Table 6). Values did increase significantly

Table 6. Radiographic bone aluminum equivalence over time (mm Al).

| | d 0[ab] | | | | d 21[bc] | | | | d 42 [c] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cortice | EX | CF | GR | SEM | EX | CF | GR | SEM | EX | CF | GR | SEM |
| dorsal | 10.3 | 10.3 | 10.4 | 0.2 | 10.8 | 10.4 | 10.6 | 0.1 | 11.2 | 10.7 | 11.3 | 0.2 |
| palmar | 10.5 | 10.4 | 10.2 | 0.2 | 10.5 | 10.2 | 10.5 | 0.2 | 11.0 | 11.1 | 11.7 | 0.2 |
| medial | 9.9 | 9.6 | 10.0 | 0.2 | 10.5 | 10.0 | 10.3 | 0.2 | 11.3 | 10.6 | 11.2 | 0.2 |
| lateral | 10.6 | 10.4 | 10.5 | 0.2 | 11.4 | 11.1 | 11.3 | 0.2 | 12.3 | 12.0 | 12.1 | 0.2 |

[abc] Superscripts indicate mean cortical RBAE values across treatments differ by day (P<0.05).

over time in all cortices (P<0.001), thus indicating that all calves were increasing in bone mineral content due to the normal growth process. In order to visualize changes in RBAE values from initial values, data were also normalized with respect to d 0 values. No differences existed between treatments in any cortice when data were normalized. Although bone density, as determined by computed tomography, did show differences in bone mineral density between treatments, the radiographic technique was not sensitive enough to detect any such changes.

Ca and P concentrations

Phosphorous or Ca concentrations in the bone sections did not differ with treatment. While Ca concentrations did not differ according to the region of the bone, P concentrations tended to differ by cortice (P<0.1), with the medial and lateral cortices having higher concentrations of P than the palmar region (Table 7). Ratios of Ca to P were not different between treatments or cortices of the bone. Similarly, bone density of cross sectional slices when calculated by Archimedes' Principle, percentage of ash expressed on a fat-free dry-weight basis, or density of bone calculated from ash weight and bone volumes did not differ with treatment. Differences between cortices in percentage ash were seen (P<0.05) as well as a trend for ash weight expressed in relation

to calculated bone volume (P<0.1; Table 8). Ash percentage was greater in the dorsal and medial cortice vs. lateral and palmar ash percentage, with the palmar ash percentage significantly less than any other aspect of the bone. When ash weight was expressed relative to bone volume, the dorsal cortical samples were greater in density vs. the palmar sections.

Table 7. Ca and P concentrations (mg/g) and Ca:P ratios of individual cortices within treatment groups.

| | dorsal | | | palmar | | | medial | | | Lateral | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ca | P [yz] | Ca:P | Ca | P [z] | Ca:P | Ca | P [y] | Ca:P | Ca | P [y] | Ca:P |
| EX | 270 | 115 | 2.36 | 254 | 108 | 2.34 | 264 | 116.2 | 2.27 | 262 | 119 | 2.21 |
| CF | 266 | 113 | 2.36 | 271 | 113 | 2.40 | 287 | 119.9 | 2.39 | 280 | 119 | 2.36 |
| GR | 266 | 116 | 2.29 | 255 | 115 | 2.21 | 269 | 119.5 | 2.25 | 277 | 118 | 2.35 |
| SEM | 6 | 3 | 0.03 | 9 | 3 | 0.04 | 5.8 | 1 | 0.05 | 6 | 1 | 0.05 |

[yz] Different superscripts indicate P concentrations tend to differ (P<0.1) between cortices.
SEMs given are pooled across treatments within day.

Table 8. Percent ash of cortical samples on weight and volume basis.

| | % ash | ash weight (g)/bone volume (ml) |
|---|---|---|
| dorsal | 0.68[a] | 0.58[y] |
| palmar | 0.66[c] | 0.51[z] |
| medial | 0.68[a] | 0.56[yz] |
| lateral | 0.67[b] | 0.56[yz] |

[abc]Cortices lacking common superscripts differ in percent ash (P<0.05)
[yz]Cortices lacking common superscripts tend to differ in ash weight/bone volume (P<0.1)

## Serum Total Deoxypyridinoline

Serum DPD has been used as an indicator of bone resorption as it is released into the bloodstream upon degradation of the organic matrix of bone (Delmas, 1990). As confinement results in resorption of bone (Hoekstra et al., 1999), stalling calves may result in higher levels of DPD. However, serum total DPD concentrations did not differ between treatment groups nor did these values differ over time (Table 9) or when normalized from d 0 values. Due to the large amount of variation between samples, any

biological differences created by the treatments were difficult to determine. The repeatability between duplicates was low, with duplicates accepted with a 15% error within the replication.

Serum Osteocalcin

Mean serum osteocalcin concentrations were not affected by treatment. However, if values were normalized from d 0, the EX group had higher concentrations of OC in relation to CF, indicative of greater bone formation as reflected by the changes seen in bone geometry ($P<0.05$). Even though the EX animals began the project with lower OC concentrations than CF, they later increased to almost consistently higher values than those seen in CF.

Behavioral observation

Analysis of behavioral tapes made on d 0, 21 and 42 revealed that the group-housed calves rarely underwent any high intensity exercise. When observational data were averaged over the three days, 63.4% of the time budget of the group-housed calves was spent lying down, 35.0% standing, while actual movement that would significantly load the bone was limited to 1.58% walking and only 0.02% trotting (Table 11). In comparison, CF calves spent 35% of the time standing and 65% laying down, with EX calves standing 27% and lying 73% of the day. The frequency of postural shifts decreased over time in the confined calves, further reducing the load placed on the bone (Table 12).

Table 9. Bovine serum deoxypyridinoline (ng/ml) over time.

| | d | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 14 | 21 | 28 | 35 | 42 |
| EX | 21.6 | 20.0 | 19.4 | 21.5 | 19.4 | 22.9 | 23.3 | 18.8 | 18.5 | 17.9 | 19.5 | 22.2 | 15.5 |
| CF | 23.1 | 19.9 | 21.7 | 18.4 | 20.8 | 25.9 | 19.0 | 15.8 | 14.9 | 17.2 | 19.2 | 17.0 | 18.6 |
| GR | 23.7 | 22.4 | 26.8 | 23.0 | 23.2 | 20.1 | 20.7 | 18.1 | 16.4 | 16.2 | 20.5 | 14.6 | 19.9 |
| SEM | 1.7 | 1.9 | 2.3 | 1.8 | 2.0 | 1.8 | 1.7 | 1.0 | 1.4 | 1.7 | 1.2 | 1.7 | 1.4 |



Table 10. Bovine serum osteocalcin (ng/ml) over time.

| | d | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 14 | 21 | 28 | 35 | 42 |
| EX | 147.2 | 135.8 | 158.8 | 174.3 | 145.8 | 166.5 | 143.9 | 136.9 | 152.7 | 156.4 | 134.4 | 135.5 | 134.1 |
| CF | 170.7 | 163.9 | 142.1 | 141.2 | 118.9 | 127.7 | 121.4 | 138.2 | 106.4 | 120.6 | 103.9 | 125.1 | 115.7 |
| GR | 165.5 | 136.5 | 148.5 | 140.7 | 140.7 | 142.5 | 149.5 | 146.4 | 126.2 | 122.0 | 131.9 | 134.1 | 118.4 |
| SEM | 15.2 | 14.9 | 13.3 | 15.4 | 10.2 | 12.7 | 11.6 | 11.5 | 13.3 | 10.7 | 10.7 | 7.8 | 7.9 |

Table 11. Total duration (%) of behavioral observations averaged over d 0, 21, and 42.

| | EX | CF | GR |
|---|---|---|---|
| standing | 35.1 | 27.5 | 35.0 |
| lying | 64.9 | 72.6 | 63.4 |
| walking | *** | *** | 1.6 |

Table 12. Frequency of postural shifts summed for each treatment on d 0, 21, and 42.

| | d 0 | d 21 | d 42 |
|---|---|---|---|
| EX | 14 | 9 | 6 |
| CF | 13 | 16 | 5 |
| GR | 7 | 17 | 12 |

## CONCLUSIONS

Bone resorption due to immobilization occurs quickly, with decreases in bone mineral density, stiffness and ultimate load after only 6 wk (Inman et al., 1999). In this study, stall confinement for 6 wk did not appear to adversely affect most properties of the bone when compared to group-housed calves. While bone geometry and density were the least favorable in the confined calves, they were not statistically different from the group-housed calves for most measurements, with the exception of the percentage of cortical area of the bone. Confinement of these calves probably did not alter the loading pattern of the bone much beyond that experienced by the calves allowed free exercise. Due to their own voluntary activity, the control animals (GR) did not differ greatly from the exercise-restricted animals (CF) in the amount of voluntary activity experienced. Thus the mechanical loading placed on the confined calves was not decreased greatly beyond the group-housed animals. While Friend and Dellmeier (1988) found that confined calves stood more and rested less than group-housed calves due to an increased motivation for locomotor activity, the calves in the present study did not differ in time spent lying down or standing. The group-housed calves voluntary activity was quite low,

with the majority of their time spent lying down. In fact, CF calves spent almost the same percentage of time lying down as GR calves, readily explaining the absence of relative differences in BMD. Even if the stalled calves had stood more, any skeletal effects likely would not have been observed, as static loading is ineffective in altering bone mass (Gross et al., 2002; Robling et al., 2001).

Short periods of walking in the GR calves would presumably not load the skeleton appreciably beyond that of standing in the stalled calves, due to the slow speeds at which they walked. This is similar to the results seen by Skerry and Lanyon (1995), where periods of walking did not maintain bone mass in otherwise immobilized limbs. In humans, as much as four hours of walking daily are needed to prevent bone loss (Anderson and Cohn, 1995). Therefore, in light of the limited activity of GR calves, conclusive statements in regards to our EX group compared to the control or "normal" condition are difficult to make. The environment of the GR calves may not have stimulated enough activity. The author suggests a pasture, rather than a pen setting, might elicit a greater treatment difference when compared to CF.

While the confinement protocol did not result in any reduction of bone mass, short duration, high intensity exercise appeared to positively influence both bone mass and structure even beyond the calves allowed free exercise. Short bouts of running exercise might improve bone mass beyond confined animals, but the improvement beyond that of the group-housed calves was unexpected, presumably due to the rather sedentary pattern of activity in the GR calves. Others have shown that sudden jumps or movements, while not being an appreciable percentage of daily activity, may be more important than the influence of standing or walking. Treadmill running in roosters

resulted in loads of 500 μstrains while intermittent high-magnitude strains greater than 1,000 μstrains were recorded during normal background activity (Konieczynski et al., 1998). Similarly in sheep, high-magnitude strains of 1,150 μstrains are recorded during startling responses (Skerry and Lanyon, 1995). Whalen and Carter (1988) predicted that bone mass is more dependent on stress magnitude than number of cycles, and as little as a single loading cycle applied to the foot of a sedentary individual equivalent to that produced by running could lead to a density change of 61%. Therefore, our hypothesis that daily running exercise as short as 50 m distance can result in bone hypertrophy appears to be substantiated.

The greater weight gain in the exercised calves could have contributed to the differences in bone mass and density as a larger body mass will, of course, produce more strain on the skeleton. Body weights of calves weakly but significantly correlated with BMD ($P<0.05$; $R^2= 0.28$) and % cortical area ($P<0.05$; $R^2=0.28$). In humans, bone density in the calcaneus is predicted to be proportional to the square root of the body weight (Whalen and Carter, 1988). In 5-mo-old foals subjected to confinement or confinement with sprint training, growth rate had a significant effect on BMD of the lateral radius, with the faster growing foals having greater BMD (Firth et al., 1999). The adaptations in EX were not likely due solely to weight gain, as body weight and medullary cavity were not correlated ($P=0.65$), and medullary cavity was significantly smaller in the EX calves. In data from mature cows, metacarpal weight or bone cortex composition were not different between cows with large differences in age and weight (Field et al., 1999). We did not anticipate differences in weight gain as calves were provided ad libidum feed. If similar studies are undertaken, controlling feed intake so

that the calves gain weight at a similar rate would be important. This would eliminate the question of a heavier overall body weight contributing to greater bone mass or density due to the increased loading.

The exercise protocol led to adaptation in MC III & IV, producing a rounder, more dense bone with less medullary cavity. In this study, the MC III & IV medulla of the CF and GR calves was larger than EX, yet the total cross-sectional area did not differ. Other studies have found geometric adaptation of bone to occur while not changing the cross-sectional area of the bone. In rats, immobilization did not change total cross-sectional area but did alter cortical width, increasing the marrow cavity and decreasing the percentage of cortical area (Ma et al., 1999). Such modification occurred through endocortical bone resorption in the sedentary groups accompanied by a decreased bone formation rate on both the endosteal as well as the periosteal surface (Ma, 1999). The normal cyclical process of bone remodeling was not entirely halted, as the increased resorption on the endosteal surface was accompanied by some new bone formation on the eroded surfaces. However, as mentioned previously, the confined calves were not very different from the GR animals, and thus an assumption of bone resorption occurring in the sedentary animals would be unsubstantiated.

Alternatively, and more likely, the greater cortical mass in EX is due to increased bone formation. Loitz and Zernicke (1992) found that exercised roosters did lay down bone endosteally along the anterior-posterior plane, which decreased the a-p endosteal diameter and increased cortical thickness, similar to our results. However, most studies have shown that new bone formation occurs on the periosteal surface of the bone rather than the endocortical surface (Raab-Cullen et al., 1994b). No attempt to resolve whether

this adaptation occurred via endocortical bone resorption or by greater bone formation was made, which would require labeling of the active surfaces or serial CT images. However, the increase in OC seen in the EX calves indicates greater bone formation, as mineral apposition rate increases in formerly sedentary sows subjected to exercise (Raab et al., 1991).

The data suggest that only short periods of high intensity exercise are needed to cause adaptation in the young growing animal. However, the changes in bone structure and density were not highly reflected by the mechanical testing. Woo et al. (1981) also found that while exercise caused a reduction of the medullary cavity of the femur of swine and increased ash content, there were no differences in the mechanical properties of the bone. Loitz and Zernicke (1992) also found that while exercise altered bone geometry in roosters, elastic modulus did not differ. Thus, exercise changed the quantity, but not the actual quality of the tissue. Perhaps had the study been continued for a longer period, it would have resulted in a mechanically stronger bone. Further more bone strength might have been enhanced in vivo in a manner which could not be measured by ex vivo testing methods. In addition, as values, while not statistically different, numerically increased with treatment from CF to GR to EX, a larger sample size may have lessened the effect of experimental error in the model.

Changing geometry of the bone does change its resistance to bending and thus alters the strain experienced. While true that bone density is the greatest factor governing strength of trabecular bone, the primary factor influencing strength of long bones is the moment of inertia or shape (Whalen et al., 1993). Therefore, changing the shape of bone may result in a stronger structure than merely an increase in bone density. Bone adapts to

decreased usage by reducing bone mass without overly affecting bone strength. The confined calves overall had a larger total bone area with more medullary area. This larger, more hollow cylinder appeared to be mechanically similar to the smaller, more dense bone of the exercised calves. Buckingham et al. (1992), found that although geldings had lower ultrasound speed velocity through MC III reflecting lower bone mineral density vs. mares or intact males, the geldings had larger cross-sectional areas. This adaptation may maintain mechanical integrity in the less dense bone.

Ca concentration as estimated by BMC or BMD using computed tomography, DEXA, or radiography increases with exercise. Calcium concentrations when analyzed biochemically have shown an increase in Ca content in all bones in older rats, and in the long bones of exercised immature rats (McDonald et al., 1986). Salem et al. (1993) found Ca concentrations in the femoral neck to be lower in 8-wk-old rats (234 μg/mg) but were similar after 10 wk between trained and untrained rats (254 and 252 μg/mg, respectively). Others have also found no differences in Ca concentration following training in immature rats (Tipton et al., 1972) or swine (Woo et al., 1981). In the present study, while BMD as predicted by CT differed between treatments, the Ca and P concentration were unaffected by treatment. This is somewhat difficult to explain as numerous studies have reported a high correlation between CT and analytical measurements of mineral concentrations. Phosphorous concentration did tend to differ between regions of the bone as did the percent ash. Similar to the CT BMD values, ash % was greatest in the cortex and the least in the palmar cortex. However, no differences between treatments in physical measurements were observed. Due to the small size of the cross sectional image of the bone, the region of interested selected in each cortice for

BMD determination was below the manufacturer's recommendations and could have influenced the accuracy of this technique. Therefore, while useful in many studies with larger animals, with small bones, physical measurements may provide more reliable assessment of mineral content of bone than CT. Why differences between treatments existed in CT data is unknown, but may be more related to differences in geometry between treatment groups.

Adaptations of bone can be limited to geometry with no changes in the composition of the bone. In mature roosters, exercise redistributed bone mass without altering total cortical area, or bone density (Loitz and Zernicke, 1992). While cross sectional area of the middiaphysis of the femur increased in dwarf rats treated with growth hormone, bone density, calcium and collagen content were smaller in the treated group (Martinez et al., 1996). Jarvinen et al., (1999) found an increase in failure load in exercised rats but no difference in BMC determined by DEXA, suggesting a redistribution of bone. Barengolts et al., (1993) also found an increase in mechanical properties of femurs of exercised rats despite no change in bone mass, cortical area or moment of inertia. Changes in collagen fiber orientation confer additional strength without changing mineral content or architecture of the bone (Turner, 1991). Similarly, in humans performing exercise intervention targeting the wrist area, BMD as determined by DEXA did not differ (Adami et al., 1999). However, when the same subjects were measured using computed tomography, training altered the cross-sectional area of the bone, and changed BMD, decreasing trabecular and increasing cortical BMD. Therefore studies focusing solely on changes in BMD may be missing important geometric changes that have a far greater impact on bending strength and moment of inertia. Thus, despite

the disparity in the data between the CT measurements and the direct chemical analysis of the bone, the observations of the change in bone geometry of the calves indicate that favorable changes truly were occurring in the EX calves.

While others have shown that stall confinement does result in an increase in DPD (Hoekstra et al., 1999) and a decrease in OC concentrations (Bell et al., 2000), these animals were of an older age compared to the 8-wk-old calves in this study. In the very young, rapidly growing animal, confinement may not result in an increase in bone resorption as would be observed by an increase in the bone resorption marker, DPD. Rather a lower bone mass in a sedentary group versus the exercised group is more likely due to a slower bone gain. Immobilization of the forelimb in young beagle dogs resulted in the experimental leg developing at a reduced rate compared to the control limb (Uhthoff and Jaworski, 1978). Immobilized legs had a smaller cross-sectional area than the normal leg, but both increased in size over time in both total area as well as medullary area due to normal growth. The total area was greater in the control legs while the increase in medullary area was greater in the experimental leg. The net result was a decrease in cortical area in the experimental limb (Uhthoff and Jaworski, 1978). Additionally, in young, growing rats, a period of deconditioning following exercise training resulted in bone mass similar to age-matched sedentary controls. Histomorphometry revealed the decrease in bone mass relative to the increased bone mass in comparison to the controls at the end of training was due to a decrease in bone formation, not an increase in resorption (Yeh and Aloia, 1990; Iwamoto et al., 2000). The change in osteocalcin concentrations seen in the current study also indicate that OC

may be more sensitive to increased activity and increased formation rather than an indicator of a slowing of bone formation.

Some caution may be necessary when implementing an exercise program in very young animals as some studies have reported an inhibition of bone growth. Dynamic axial loading suppressed longitudinal growth in rat ulnae (Ohashi et al., 2002). Running exercise also temporarily stunted longitudinal and radial growth of the tibiotarsus and femur of roosters (Maynard et al., 1995). In 3-wk-old roosters, moderate exercise caused the suppression of circumferential growth while at the same time enhancing mid-diaphyseal cortical thickening (Matsuda et al., 1986). This is similar to results of our bovine study in which 6 wk of exercise did not produce a significantly greater total bone area in comparison to the other groups but was effective in increasing cortical width. Similar to our study, they also did not see any change in cross-sectional area moments of inertia. However, where Matsuda et al. (1986) saw a decrease in bending stiffness, energy to yield and energy to fracture, our exercise protocol seemed to enhance these properties. While not significantly different, the EX calves appeared to have a mechanical advantage versus the other two groups, as evidenced by the trend for a higher fracture force. Again, perhaps if we had a larger number of calves in each treatment, we would have seen more statistically significant results.

Whether such an exercise system is useful for other species needs to be tested. In the equine, interest in promoting skeletal strength in the young animal to lessen the incidence of injuries is high. Using a short duration high intensity exercise at an early age may enhance skeletal strength beyond that of normal training regimens typically implemented in the equine industry. Additionally, one of the difficulties in performing

studies of bone physiology in the equine lies in the lack of accurate, non-invasive determinants of bone structure. While highly accurate, computed tomography is difficult to perform on the live animal due to the expense and risk involved with the anesthetization of large animals. Terminal studies would provide the most definitive information; however, the expense and negative social opinion of using the equine for terminal research limits this option. However, the usefulness of using a bovine model in studying the effects of exercise on bone physiology in the immature, rapidly growing animal appears promising. These animals may provide a better model for some investigations than rodents, as rodents are quite dissimilar morphologically and have a very different locomotion pattern. Among domestic ungulates, bull calves especially are more readily accessible and inexpensive compared to other production animals at many research institutions.

# CHAPTER IV

# EQUINE STUDY

## SUMMARY

In order to investigate the hypothesis that short duration exercise may ameliorate the reduction in bone mass witnessed with confinement, 18 Quarter Horses were weaned at 4 mo of age and placed into box stalls. After 5 wk, individuals were grouped by age and weight, and then divided randomly into three treatment groups – group housed (GR), confined with no exercise (CF), and confined with exercise (EX). The CF and EX groups were housed in 3.7 m x 3.7 m box stalls for the 56 d duration of the trial. The EX group was sprinted 82 m/d, 5 d/wk in a fenced grass alleyway. The weanlings were led down the alleyway, turned loose in a small pen, and then released and allowed to run back down the alley. The GR horses were housed together in 992 $m^2$ drylot with free access to exercise. On d 0, 28 and 56, dorsopalmar and lateromedial radiographs of the left third metacarpal bone were taken in order to estimate changes in bone mineral content and cortical widths. Mean values of medial, lateral, and total RBAE increased over time ($P<0.05$), while dorsal and palmar RBAE did not change significantly. Dorsal, medial and total RBAE, tended to differ by a treatment*day interaction, with values increasing over time only in the EX group. Normalized medial and total RBAE tended to differ ($P<0.1$) with treatment, with EX greater than CF. Dorsal-palmar cortical width in EX (29.3 mm) was greater than GR (27.2 mm) on d 56 (treatment*day; $P=0.07$). The dorsal palmar medullary cavity decreased in EX compared to GR ($P<0.05$), while dorsal and medial cortical width also increased only in the EX horses (treatment*day; $P<0.1$). Although results did not show strong treatment effects, there were indications that such a

short duration exercise protocol may be effective in improving bone mass and therefore skeletal strength.

## INTRODUCTION

Injuries to the skeletal system of the horse are a great concern to the racing industry. Early training in young horses may be beneficial to the longevity of their careers. In Australia, horses receiving their first starts as 2-yr-olds had more starts and raced longer than those which began their racing careers at a later date (Bailey et al., 1999). The very early training that these horses received in preparation to race as 2-yr-olds may have aided in modeling the skeleton for high speed activity. Most studies show that the greatest skeletal adaptations occur in very young animals or humans (Iwamoto et al., 2000; Loitz and Zernicke, 1992; Umemura et al., 1995). Thus, if horses received exercise at an even earlier age, well before traditional training began, greater benefits to the skeletal integrity of the horse may be seen.

We have recently shown that very short term sprinting exercise conducted 5 d/wk enhanced bone geometry in immature calves (Hiney, Chapter III Dissertation). Therefore, a similar protocol was then implemented for the equine study. However, some alterations were made to the study after examining results of the bovine study. In the calves, the RBAE technique did not reveal differences due to treatment that were detected using more sensitive and accurate computed tomography. However, due to the expense and risk involved with anesthetizing commercially marketable weanlings, computed tomography was not an available option for the equine study. Thus, the duration of the study was lengthened by 2 wk in order to extend the period of skeletal modeling, causing

greater differences that could be observed with radiographic absorptiometry. In addition, as these animals were older than the 8-wk-old-calves which had more readily adaptable bone, the distance the animals traveled during the exercise protocol was lengthened slightly.

## MATERIALS AND METHODS

### Animals and Management

Eighteen Quarter Horses, 9 colts and 9 fillies, were divided into two weaning groups according to foaling date and were weaned at approximately 4 mo of age. Twelve weanlings with earlier birth dates were used in the first group and 6 weanlings with later birth dates were used in the second group. Horses were weaned by removal from their dams and placed into box stalls (3.7 m x 3.7 m). The foals remained in the stalls for 5 wk prior to the initiation of the study. The stress due to weaning temporarily reduces feed intake and thus growth (Knight and Tyznik, 1985). Therefore, this time period allowed the foals to adjust from the stress of weaning prior to the beginning of the study as well as to adapt to handling. Individuals from each group were stratified according to age, weight, and gender as these factors influence growth (Hintz et al., 1979; McKeever et al., 1981; Pagan et al., 1996). The stratified horses were randomly assigned to one of three treatments resulting in 6 horses per treatment: confined without exercise (CF), confined with exercise (EX) and group housed (GR). Mean age of the first weaning group at commencement of the study was 166 ± 3 d and the mean age of the second weaning group was 172 ± 8 d.

The GR horses were housed together in 992 $m^2$ drylot with free access to exercise. The CF horses remained in box stalls for the 8 wk duration of the project with no access to exercise while the remaining EX horses received forced exercise 5 d per week. The EX horses were led from their stalls to the end of an 82-m grass alleyway and turned loose in a small pen. Horses were then released from the pen, and galloped down the alleyway back towards the barn and were caught in a small pen at the end of the alley. The average speed of the horses was between 6 to 8 m/s. They were then returned to their stalls with the entire distance traveled being approximately 264 m, including the distance walked from the barn to the alleyway.

Behavior Observations

Observations of behavior were made over 24 h on d 0, 28, and 56. For the 24-h period, horses were kept under constant lighting. Horses were videotaped in either their box stalls or in the group drylot with an extended play recorder which recorded 24 h on one tape. For all horses, 4 h of each 24-h period were randomly chosen for observation, and then 15 min of each hour was analyzed. Behavior observations were again limited to those activities which would load the bone, and therefore, impact bone strength. Durations of behaviors were recorded and summed for each 15 min period. The total duration of the behaviors was recorded as a proportion, or percentage, of time for which all occurrences of the behavior lasted over the observation session. Total duration of behaviors were summed for each observation day and over all three days. These behaviors included bouts of standing, walking, lying down, and trotting. In addition to the behaviors recorded as cumulative intervals, frequency or number of occurrences of

shifting of stance from lying to standing, walking bouts, pawing, startling (reaction in response to a sudden and novel stimulus) and jumping were recorded for each 15 min period.

Sample Collection

Blood was collected via jugular venipuncture into 10-ml non-heparinized vacutainers at 0800 on d 0 as well as daily for the initial 7 d in order to monitor any immediate changes in bone metabolism due to treatment. Blood sampling was then performed once per week for the remaining 7 wk of the project. Blood was allowed to coagulate at 20° C and then centrifuged at 1340 x g for 12 min for serum separation. Serum samples were frozen at –20° C for later analysis of osteocalcin (OC) and deoxypyridinoline (DPD)- markers of bone metabolism. Serum total deoxypyridinoline concentrations were analyzed using Pyrilinks-D (MetraBiosystems, Inc.) enzyme-linked immunosorbent assay (ELISA). Serum samples were diluted in a 1:3 ratio with double-distilled water. Osteocalcin concentrations were analyzed using Novacalcin, an ELISA kit obtained from Metra Biosystems, Inc (Mountainview, CA). which uses a monoclonal murine anti-bovine antibody. Serum samples were diluted in a 1:10 ratio in order to obtain concentrations within the linear range of the standard curve. All assays were performed according to manufacturer's instructions.

Radiographs were taken at d 0, 28, and 56 to determine radiographic bone aluminum equivalence (RBAE) values, measures of optical density and a reflection of bone mineral content (Meakim et al., 1981). Only the left metacarpal bone (MCIII) was radiographed for determination of bone mineral content as other studies in mature horses

have reported no difference in bone properties between forelimbs (Lawrence et al., 1994; Glade, 1993). Films of the dorsal-palmar and medial-lateral views of MCIII were taken at a focal length of 77 cm and an exposure of 65 Kvp for 0.1 s. An aluminum stepwedge was attached to the radiographic cassette to standardize readings and calculate RBAE values. Horses were also weighed on a livestock scale on d 0, 28 and 56.

Radiographic Bone Aluminum Equivalence

Optical density of the bone was assessed using radiographic photodensitometry to determine RBAE according to the method of Meakim et al. (1981) using a Bio-Rad Model GS 700 imaging densitometer (Bell et al., 2001). Radiographs were scanned distal to the nutrient foramen of MC III. A linear regression was produced from the thickness of the steps on the Al penetrometer. Maximum optical density of each cortex was expressed in mm of Al for both cortices in each view of MC III. Total RBAE was measured by taking the area under the curve of the bone scan and expressing it in relation to a known volume of Al (Nielsen and Potter, 1997).

Cortical Widths

The dorsal-palmar radiographic view was used to measure the width of the medial (MC) and lateral (LC) cortices, the inner medullary diameter (b) and outer cortical diameter (B). The beginning of the curve of the bone image as developed by the Multi-Analyst software to the highest point of the curve was measured for the width of each individual cortice, and the medullary diameter (or medullary cavity) was measured from the distance between the two peaks of the curve. The outer cortical diameter was

measured as the entire distance of the curve. The similar procedure was used for the lateral-medial view for determination of dorsal (DC) and palmar (PC) cortical widths, and the inner medullary diameter (d) and outer cortical diameter across the dorsopalmar (D) aspect of the bone. The regions of bone measured by this method are depicted in Figure 2 and 3.

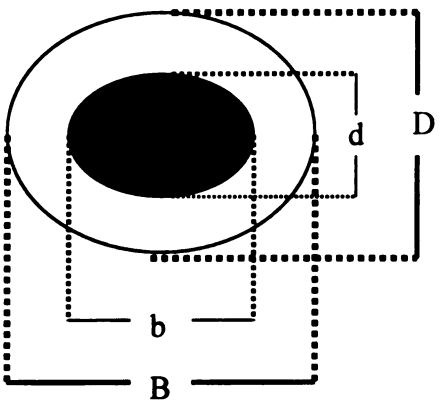


Figure 2. Schematic illustration of a cross-section of equine third metacarpal showing cortical measurements.
Measurements included :
B = lateromedial bone diameter
b = lateromedial medullary diameter
D = dorsopalmar bone diameter
d = dorsopalmar medullary diameter

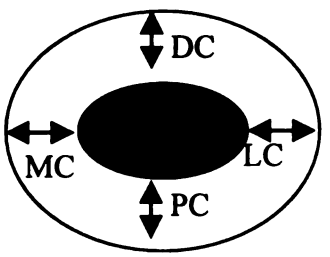


Figure 3. Schematic illustration of a cross-section of equine third metacarpal showing cortical measurements.
Measurements include:
DC = dorsal cortical width
PC = palmar cortical width
MC = medial cortical width
LC = lateral cortical width

Using the digital image potentially limited the human error involved with visual inspection of the radiographs and caliper placement. An additional study was performed to examine the validity of this technique, comparing the cortical widths obtained from the radiographic method to those developed from computed tomography on legs obtained postmortem. Data from this study is presented in Appendix Chapter A.

Statistical analysis

Statistical analysis of RBAE and serum data which were taken throughout the duration of the project was performed using the mixed procedure of SAS using a covariance test suitable for repeated measures. When variables were measured at equal intervals, such as the RBAE data, the covariance structure was first order autoregressive with horse within treatment used as the subject effect. As serum markers were taken at unequal intervals, daily for the first 7 d followed by weekly sampling, the covariance structure used was a spatial power. The model tested for treatment, day, and day*treatment interactions. In addition, because there were two separate groups of horses due to differences in foaling date, a test for period effect was also included in the model statement. When main effects were significant, post hoc comparisons were used to separate differences between means. Least square means were separated by the Tukey's method. Individual standard errors of the mean (SEM) for each treatment are included in the tables and figures. To aid in visualizing changes over time within an individual, data were also normalized by subtracting d 0 values from all subsequent values. Behavior data were analyzed with a multinomial distribution which predicted the probability of behavior occurrence between groups. Frequency behaviors, such as postural shifts, walking bouts and pawing, were analyzed using a Poisson distribution. For all analyses, P values less than 0.05 were considered significant while values less than 0.1 were discussed as trends.

# RESULTS

## Weight Gain

As horses were balanced and assigned to treatments according to weight, body weights were similar at the initiation of the study and remained similar throughout (Table 13). However, there was a significant effect of period ($P<0.05$) on weight gain, with the foals in the 2nd weaning group gaining more weight over the 56 days than the earlier born foals, 48.8 kg vs 36.7 kg respectively.

Table 13. Body weight data (kg) and average daily gain (ADG) of horses separated by treatment.

| | EX | CF | GR | SEM |
|---|---|---|---|---|
| Body weight (kg) | | | | |
| Initial | 226 | 226 | 227 | 4 |
| Final | 270 | 269 | 263 | 4 |
| Gain | 44 | 43 | 36 | 3 |
| ADG | 0.8 | 0.8 | 0.6 | 0.1 |

## Radiographic Bone Aluminum Equivalence

Dorsal and palmar RBAE values did not differ according to time or treatment, but there was a trend for a trt*day interaction ($P = 0.09$) in the dorsal cortex, with values increasing to d 56 only in the EX group (Table 14). The dorsal cortex of MC III in the CF and GR animals remained essentially unchanged from the initiation of the trial. While dorsal and palmar values did not increase over time, medial and lateral RBAE values increased significantly over the duration of the study ($P< 0.0005$).

Table 14. Mean cortical RBAE (mm Al) of treatment groups over time.

| | dorsal | | | palmar | | | medial | | | lateral | | | total | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | d 0 | d 28 | d 56 | d 0 | d 28 | d 56 | d 0[a] | d 28[b] | d 56[c] | d 0[a] | d 28[b] | d 56[c] | d 0[a] | d 28[a] | d 56[b] |
| EX | 15.0[y] | 15.1[y] | 16.1[z] | 14.6 | 14.3 | 14.7 | 16.9[y] | 18.9[z] | 18.9[z] | 15.6 | 17.2 | 18.1 | 123[y] | 168[y] | 260[z] |
| CF | 15.5 | 15.6 | 15.7 | 15.1 | 14.7 | 14.6 | 17.3 | 17.9 | 18.9 | 16.6 | 17.1 | 18.0 | 186 | 156 | 224 |
| GR | 15.6 | 15.3 | 15.3 | 15.3 | 14.9 | 14.8 | 17.8 | 17.5 | 19.0 | 16.5 | 16.8 | 18.1 | 176 | 170 | 184 |
| SEM | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.3 | 0.4 | 0.3 | 0.3 | 0.3 | 15 | 13 | 25 |

[abc] Days lacking a common superscript differ (P<0.05).
[yz] Different superscripts indicate a trend (P<0.1) for trt*day interactions in EX only.
SEMs given are pooled across treatments within day.

Furthermore, in the medial RBAE, the only increase in RBAE found was in the EX group (treatment*day interaction; P = 0.05), similar to the dorsal RBAE data. Total RBAE also increased over time (P<0.005) and similar to both dorsal and medial RBAE data, total RBAE values tended to increase in only the EX animals (P=0.09). When total RBAE values were normalized there was a significant effect (P<0.05) of treatment with EX horses gaining more (137 ± 44 $mm^2$ Al) than either CF (38 ± 40) or GR horses (8 ± 25).

Table 15. Mean dorsal-palmar cortical diameters (mm) according to treatment groups over time.

| | D | | | d | | | DC | | | PC | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | d 0[a] | d 28[a] | d 56[b] | d 0 | d 28 | d 56 | d 0 | d 28 | d 56 | d 0 | d 28 | d 56[w] |
| EX | 27.0[yz] | 26.6[y] | 29.3[z] | 15.0 | 14.2 | 13.5 | 7.7[y] | 8.1[y] | 9.8[z] | 4.7 | 4.9 | 5.9 |
| CF | 26.9[yz] | 26.4[y] | 28.6[z] | 14.1 | 13.3 | 14.2 | 8.5 | 8.6 | 8.7 | 4.8 | 5.1 | 5.8 |
| GR | 26.9 | 27.3 | 27.2 | 13.7 | 14.4 | 13.9 | 8.5 | 8.3 | 8.2 | 5.1 | 5.1 | 5.0 |
| SEM | 0.4 | 0.4 | 0.5 | 0.4 | 0.3 | 0.3 | 0.3 | 0.3 | 0.4 | 0.2 | 0.2 | 0.2 |

[abc] Days within a cortical measurement lacking a common superscript differ (P<0.05).
[w]Indicates a trend for d 56 to be greater than d 0 and 28 (P<0.1).
[yz] Means within a cortical measurement and within a treatment with different superscripts indicate a trend (P<0.1) for trt*day interactions.

Cortical Widths

Dorsal-palmar cortical diameter (D) increased over time (P<0.05; Table 15), with values on d 56 greater than either d 0 or d 28. In addition, EX (29.3 mm) was greater than GR (27.2 mm) on d 56 (treatment*day; P=0.07). Both CF and EX had greater dorsal-palmar bone diameters on d 56 in comparison with d 28. When data were normalized by examining the changes in values from d 0, the treatment*day interaction was significant as EX had increased by 2.3 mm (P=0.07), CF had increased by 1.7 mm (P=0.02), and GR did not change (P=0.91). Overall, the medullary dorsal-palmar diameters (d) did not change over the duration of the trial, but treatment*day interactions were significant (P<0.05). When data were normalized, the medullary cavity decreased

in EX (-1.5 mm) compared to GR which gained slightly (0.2 mm; $P<0.05$). The change in medullary diameter of CF was not different from either EX or GR. These data seem to indicate a greater expansion of MC III in EX compared to GR as the width of the bone increased in the dorsal-palmar direction while the medullary cavity was being reduced. This would suggest the presence of more bone via either a decrease in the normal expansion of the medullary cavity with growth or by endosteal bone formation.

Dorsal cortical width (DC) averaged over all treatments did not change over time, but an increase did occur in the EX horses (treatment*day; $P=0.09$). When dorsal cortical width was normalized in relation to d 0, EX was greater than both CF and GR ($P<0.05$). The average change in the EX was 2.0 mm vs 0.2 mm in CF and –0.3 mm in the GR foals. Palmar cortical widths (PC) tended to increase over time ($P=0.08$), but did not differ between treatments.

The medial-lateral bone diameter (B) increased over time ($P<0.05$) but was not different between treatments (Table 16). Conversely, the medial-lateral medullary diameter (b) did not change over the duration of the trial but again, the horses in the 2nd weaning group had a larger medullary cavity diameter measured from the medial to the lateral aspect of the bone ($P=0.07$). Mean medial (MC) and lateral (LC) cortical widths increased over time ($P<0.05$), and in the normalized medial widths, EX gained more width in the medial cortex (2.1 mm) compared to GR (0.4 mm) ($P=.09$). Finally, the lateral cortex was not different by treatment, but the earlier born foals gained more bone in the lateral cortex (1.1 mm) vs. the foals weaned later (-0.4 mm; $P<0.05$).

Table 16. Mean medial-lateral cortical diameters (mm) according to treatment groups over time.

| | B | | | b | | | MC | | | LC | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | d 0[a] | d 28[a] | d 56[b] | d 0 | d 28 | d 56 | d 0[a] | d 28[b] | d 56[b] | d 0[a] | d 28[ab] | d 56[b] |
| EX | 35.0 | 35.9 | 38.3 | 22.4 | 22.2 | 21.1 | 7.0 | 8.1 | 9.1 | 6.8 | 7.1 | 7.4 |
| CF | 35.2 | 35.5 | 38.1 | 21.8 | 21.4 | 21.5 | 6.9 | 7.4 | 7.9 | 7.2 | 7.1 | 8.0 |
| GR | 34.7 | 35.9 | 36.3 | 21.0 | 20.9 | 20.4 | 7.5 | 8.0 | 7.9 | 6.5 | 7.7 | 7.7 |
| SEM | 0.7 | 0.7 | 0.6 | 0.7 | 0.7 | 0.5 | 0.3 | 0.3 | 0.3 | 0.2 | 0.3 | 0.3 |

[abc] Days within a cortical measurement lacking a common superscript differ (P<0.05).

Table 17. Total duration (%) of behaviors averaged over all days of recording for each treatment.

| | EX | CF | GR | SEM |
|---|---|---|---|---|
| lying | 34.2[a] | 20.2[b] | 11.6[b] | 4.5 |
| standing | 63.5[b] | 77.9[a] | 83.5[a] | 4.3 |
| walking | 2.3 | 2.8 | 4.9 | 1.0 |

[ab]Treatments lacking common superscripts differ (P<0.05).

## Behavior

Duration of behaviors differed between treatments, but not to the extent expected, especially in horses allowed free access to exercise (Table 17). Despite their greater opportunity for movement, the GR horses differed slightly from the box-stalled horses. The percent of observed time spent walking averaged over all days did not differ between treatments, 2.3% and 2.8% for EX and CF respectively with the GR horses averaging slightly more walking at 5% of the observed time. The EX foals spent less time standing (64% of the observed time), compared to the CF foals (78%) and GR (84%; P<0.05). The difference in time budget, or how the foals proportioned their activities over time, for the EX foals was mainly in preference for lying down. The EX foals spent a greater proportion of time (34%) lying down compared to CF (20%) or GR (12%; P<0.05). The

incidence of pawing in the CF foals was greater (Table 18; P<0.05) than in EX or GR, perhaps showing greater frustration in CF at the lengthy period of confinement. The frequency in shifting stance between standing and lying was greater in CF foals than GR foals (P<0.05). The number of walking bouts were similar between treatments. As animals were never observed startling or jumping during the observation periods, these behaviors are not reported in the tables. Incidence of trotting bouts were very infrequent. Thus, duration of trotting was very low, did not provide sufficient data for statistical analysis, and therefore is also not reported in Table 18.

Table 18. Total number of occurrences of behaviors summed over all observations and days for each treatment group.

| | Number of occurrences (#) | | |
|---|---|---|---|
| | EX | CF | GR |
| paw | $13^{b}$ | $96^{a}$ | $2^{b}$ |
| walking bouts | 315 | 410 | 288 |
| stance change | $10^{ab}$ | $15^{a}$ | $4^{b}$ |

$^{ab}$Treatments lacking common superscripts differ (P<0.05).

Osteocalcin

Mean serum OC differed by day (P<0.05; Table 19) and values changed much the same over time in all three treatments, with an unexplained increase in values on d 7. Post hoc analysis revealed a trend for the OC concentrations of the CF animals to be greater than in the GR horses (69.5 vs. 58.2 ng/mL, respectively) while EX was intermediate (62.8 ng/ml; P = 0.07).

Deoxypyridinoline

Serum DPD differed by treatment ($P<0.05$) with mean concentrations in the GR horses lower than either EX or CF (Table 20). Deoxypyridinoline concentrations tended to differ by day of trial ($P<0.1$) but with no discernible pattern of change.

Table 19. Serum osteocalcin (ng/ml) over time.

| | d | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $0^{a}$ | $1^{a}$ | $2^{a}$ | $3^{a}$ | $4^{a}$ | $5^{a}$ | $6^{a}$ | $7^{b}$ | $14^{ab}$ | $21^{ab}$ | $28^{a}$ | $35^{ab}$ | $42^{a}$ | $49^{a}$ | $56^{a}$ |
| $EX^{yz}$ | 53.8 | 49.1 | 60.4 | 50.0 | 55.1 | 53.3 | 50.1 | 80.2 | 73.2 | 71.7 | 68.0 | 84.2 | 74.2 | 50.1 | 67.9 |
| $CF^{z}$ | 60.3 | 71.6 | 65.9 | 67.2 | 69.4 | 54.5 | 59.9 | 88.1 | 79.1 | 80.7 | 62.7 | 66.2 | 77.2 | 64.6 | 74.5 |
| $GR^{y}$ | 48.5 | 49.1 | 53.9 | 52.8 | 62.0 | 52.0 | 54.2 | 84.2 | 77.7 | 66.9 | 64.9 | 66.5 | 45.3 | 44.9 | 49.7 |
| SEM | 4.5 | 5.1 | 8.3 | 5.7 | 6.4 | 4.1 | 4.1 | 6.4 | 5.1 | 4.2 | 4.8 | 4.7 | 7.7 | 5.4 | 5.2 |

$^{ab}$ Days lacking common superscripts differ ($P<0.05$).
$^{yz}$ Indicates treatments differ ($P<0.1$).

Table 20. Serum deoxypyridinoline (ng/ml) over time.

| | d | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $0^{yz}$ | $1^{z}$ | $2^{yz}$ | $3^{xyz}$ | $4^{z}$ | $5^{yz}$ | $6^{x}$ | $7^{yz}$ | $14^{z}$ | $21^{xyz}$ | $28^{xyz}$ | $35^{z}$ | $42^{xy}$ | $49^{z}$ | $56^{xyz}$ |
| $EX^{a}$ | 22.6 | 22.8 | 25.1 | 20.2 | 23.6 | 20.8 | 15.9 | 18.8 | 23.2 | 16.6 | 20.0 | 22.2 | 19.2 | 21.1 | 18.0 |
| $CF^{a}$ | 17.8 | 23.5 | 14.6 | 21.9 | 21.4 | 19.0 | 16.7 | 22.6 | 21.6 | 23.3 | 20.1 | 21.7 | 19.0 | 23.3 | 26.1 |
| $GR^{b}$ | 21.1 | 22.6 | 21.9 | 14.6 | 19.1 | 20.6 | 12.0 | 18.4 | 19.6 | 16.2 | 13.3 | 19.9 | 10.2 | 18.6 | 11.5 |
| SEM | 1.3 | 2.0 | 2.1 | 1.8 | 1.3 | 1.4 | 1.4 | 2.2 | 1.8 | 1.8 | 1.4 | 1.5 | 1.4 | 2.0 | 2.3 |

$^{xyz}$ Days lacking common superscripts differ ($P<0.1$).
$^{ab}$ Indicates treatments differ ($P<0.05$).

## DISCUSSION

Many studies of weanling and yearling horses have shown an increase in RBAE values over time, with increasing mineralization of the skeleton occurring with maturation (Buckingham and Jeffcott, 1987; McCarthy and Jeffcott, 1992; Raub et al., 1989) with the majority of increase in mineral content of the young horse limited to the first year and a half of life (Hiney, 1998; Nielsen et al., 1997). In the present study,

medial, lateral and total RBAE values increased in all groups over 56 d, but dorsal or palmar RBAE values did not change.

The increase in RBAE may not have been due solely to increased BMC, but rather the normal expansion of MC III due to growth. While BMD as determined by ultrasound velocity and single photon absorptiometry did not change, the RBAE of MC III increased with growth in weanlings (Buckingham and Jeffcott, 1987). One of the difficulties of determining changes in mineralization with radiographic photodensitometry is that it does not specifically measure density. As the animal grows, bone increases in size, thus the x-rays pass through a thicker, but not necessarily more dense tissue, appearing more dense on the film.

As the largest recorded strains during galloping occur in the dorsal and medial cortex of MC III (Gross et al., 1992), exercise typically causes greater mineralization to occur in the dorsal and medial cortex. While overall medial and total RBAE values increased over time, only the EX group increased in medial, dorsal, and total RBAE values, as shown by post hoc analysis. Although only trends, the short-term exercise appeared to be causing more mineral deposition in those areas of the bone experiencing the most strain during galloping. Again, these changes may have been due to formation of new bone, rather than increased mineralization of pre-existing bone.

As one of the greatest adaptations created by exercise is in the geometry of bone, a method of measuring widths from radiographs was attempted. Radiogrammetry is a method used to measure cortical thickness directly off the film with the aid of a ruler or digital calipers and this method has been used by others (Meakim et al., 1981; Thomson et al., 2001). However, while the periosteal surface of the bone may be easier to discern,

the gradual reduction in opacity in the medullary cavity makes placement of the calipers difficult. Greater detail can be observed with radiogrammetry which may aid in cortical measurements, but must involve the use of either optical or radiographic magnification. Ordinary medical radiographic film prevents maintenance of fine detail, but finer grain film can increase the detail able to be seen at greater magnification levels. However, fine grain film requires longer exposure times (with the patient remaining stationary longer), and may require hand-processing rather than machine-processing. The processed fine detail radiograph can then be observed under a stereoscopic microscope (Cohn, 1981). The higher definition of bone surfaces with this technique allows for more accurate measurement of cortical widths. Therefore rather than using fine grain films, an alternative strategy of measuring widths from the image scanned into the Multi-Analyst software was used.

The main effects of growth in just 56 days appear to be more related to the size and shape of the bone rather than mineral content. While only EX increased in dorsal, medial, and total RBAE, CF and EX increased in d-p bone diameters and all three treatments increased in palmar, medial, and lateral cortical widths, as well as the m-l bone diameter, over the 56 d. However, the tendency of the EX group to increase in mineral content was reflected in changes in width in the same aspects of the bone.

As changes in shape of the bone may be the dominant loading adaptation occurring during early life, the changes in the shape of MCIII may be the best indicator of increased mechanical integrity. The total circumference of the bone (as estimated from measuring across the medial-lateral and dorsal-palmar widths of the bone) increased in all groups over the 56 d of the trial, indicating normal periosteal expansion with

growth reported previously (Buckingham and Jeffcott, 1992). Periosteal expansion in the d-p direction was greater in EX in relation to GR and normalized data showed both CF and EX to be greater than GR. While no mechanical testing was performed in this study, others have shown that stiffness of equine bone increases with increased bone diameter (Hanson et al., 1995). As the moment of inertia varies with the $4^{th}$ power of the outer diameter of the bone, the EX horses with greater bone diameter may potentially be at a mechanical advantage.

The size of the medullary cavity did not change with time, but endosteal expansion may not occur until later in skeletal development. Even so, in the normalized data, the EX group tended to have less medullary cavity than GR, but whether this was due to contraction of the endosteal space due to exercise, or an expansion of the endosteal space in GR is impossible to determine without histomorphometric studies. Mature roosters strenuously exercised also decreased dorsopalmar medullary diameter, and increased in dorsal and medial cortical diameters (Loitz and Zernicke, 1992), similar to the results here. In contrast, in young animals exercise increased periosteal formation with no change in endosteal space (Kiuchi et al., 1998; Biewener and Betram, 1994). The location of bone formation, endosteal vs. periosteal, may be due to species or stage of maturity of the animal at the initiation of exercise, as Woo et al. (1981) found a significant decrease in endosteal diameter but no change in bone diameter in immature swine exercised for 1 yr.

Medial and lateral cortical widths increased significantly over time, but palmar cortical width only tended to increase. Normalized medial cortical width also showed a trend for an increase in EX vs. the GR horses. While dorsal width didn't change, the EX

group tended to increase. Therefore, the exercise protocol did appear to be causing some adaptation of MC III, but usually only in comparison to the GR horses. Why the GR horses would have shown less periosteal expansion than either of the box-stalled groups, especially as they underwent no less activity than that performed by the CF horses is unclear.

While the estimation of cortical widths from radiographic images is not as precise as data that can be obtained from modalites such as computed tomography, it does at least provide an additional tool of monitoring changes in bone that may not be related to density or mineral content changes. In the young animal, adaptation of the architecture of bone is the predominant response to exercise. Exercise increased the dorsal periosteal apposition rate in young Thoroughbreds (McCarthy and Jeffcott, 1991; McCarthy and Jeffcott, 1992). Race training also increased dorsal, medial, and lateral cortical diameters as well as dorsopalmar and mediolateral widths and decreased the medullary cavity, similar to the results here (Thomson et al. 2001). Thus the data, while only showing trends for improvement in the EX, corresponds with alterations in bone geometry due to exercise previously reported in horses. In addition, the foals receiving the short-term exercise responded in a manner comparable to calves performing a similar exercise program (Hiney, Chapter II Dissertation).

Stalling the weanlings for 2 mo did not result in bone loss in CF and would not be expected in such rapidly growing animals. However, the CF horses were not at any disadvantage when compared to GR horses. The behavioral observations made of the horses aid in explaining the lack of differences in bone measures between the CF horses and GR horses allowed freedom of movement. The CF horses did not have lower RBAE

or the less favorable bone geometry compared to GR. Rather, the GR horses tended to have the lowest bone measurements which was unexpected, and most treatment differences were seen between EX and GR. However, the GR horses did not differ in the time spent performing activities which would significantly load the bone compared to CF.

However, the imposed confinement did seem to increase frustration in the CF horses as they pawed more than either EX or GR. Presumably this is due to the increase in motivation for locomotor behavior following a period of behavior deprivation, leading to a variety of abnormal behaviors in horses (Dellmeier, 1989). Even the EX horses, which were out of the boxed stalls for only a very short time period 5 d per wk, exhibited less frustrated behaviors. While not a large amount of activity, how much strain on the bone could result from activities such as pawing is unknown. In addition, while infrequent, the CF horses were the only animals to ever be observed jumping or startling in their stalls. Both of these activities have been reported to result in very large strain magnitudes (Skerry and Lanyon, 1995; Konieczynski et al., 1998). These infrequent strains may play a significant role in the adaptation of bone and may explain why CF were not different from GR.

One of the major criticisms of this study is the lengthy stalling period due to weaning before the initiation of the study. As mentioned previously, weaning does result in a slowing of the normal growth of the foal and reduced feed intake, due to the stress of separation from their dam, which the duration of the stalling period would have eliminated. It would have been of value to have radiographic information from the foals when they were weaned in comparison to the beginning of the project. However, due to

unforeseen management issues, we did not anticipate that the initial stalling period was going to be 5 wk before the initiation of the project.

In this study, a different question from that originally proposed was raised due to these unforeseen and unavoidable difficulties. Although not the original design, the study in part became an examination of the effects of remobilization after a period of confinement. Remobilization does improve bone parameters but this recovery may be incomplete, especially if the duration of immobilization is lengthy (Minaire, 1993). The duration of remobilization may need to exceed that of the initial immobilization period, as 20 weeks of remobilization of rats immobilized for 18 weeks only partially restored the minimal cortical width of the tibia (Ma et al., 1999).

In addition, normal reambulation is not sufficient to restore bone mass in previously immobilized animals. In rats with one hindlimb temporarily immobilized for 3 wk, 8 wk of free remobilization only partially restored BMC and BMD compared to both the control limb and age-matched controls. However, low and high intensity exercise, while not alleviating the side-to-side differences between limbs, increased BMC such that the previously mobile limbs were greater than age-matched controls, while the previously immobilized limbs were equivalent to controls (Kannus et al., 1994). It may therefore be necessary to load the skeleton to a greater extent than normal voluntary activity to successfully recover from periods of disuse. Therefore, the GR horses may have needed greater stimulation to "recover" from confinement, if indeed deleterious effects to the skeleton had taken place.

As mentioned previously, while body weights did not differ across treatments at any time point, weaning period had a significant effect on weight gain, with the 2$^{nd}$ group

gaining more weight. However, the results of the changes in cortical width would be opposite of the expected observations if increased weight, and thus greater skeletal loading, occurred in the more rapidly growing group causing a greater skeletal adaptation. Unfortunately, additional measurements such as wither and hip height, or body condition scores were not taken which could have aided in explaining this phenomena. However, these differences in cortical widths may be merely artifacts of random individual variation, especially with the unequal animal numbers between the two periods.

Osteocalcin and DPD did not appear to be useful indictors of differences in bone modeling between treatments, especially in such young animals undergoing rapid growth. Similar to this study, Bell et al. (2001) reported no change in stalled and pastured horses in serum markers of bone formation, other than an age-related decrease in concentration.

The greatest mean OC concentrations in this study were seen in the CF animals. It would seem unlikely that the greater serum OC in CF horses was due to osteoblastic activity especially in light of the fact that most changes in bone as determined by radiography were occurring primarily in the EX group. Serum DPD, a marker of bone resorption, was lowest in GR horses, but this group was consistently the lowest in terms of gain in BMC or presumably advantageous geometric changes. The combination of biochemical and radiographic data seems difficult to resolve, especially without the aid of more definitive information such as could be gained by histomorphometry.

The imposition of the different treatments did not result in any observable changes in the initial 7 d of the study. One of the unanswered questions in the use of makers of bone metabolism in research studies is how soon are changes in exercise,

nutrition or other factors reflected in serum markers. In humans, bed rest results in alterations of bone markers within two days of bed rest (Lueken et al., 1993) indicating that skeletal disuse results in an almost immediate response to begin degradation of "unneeded" bone. Concentrations of PICP were found to decrease following three days of bed rest while OC values increased (Pedersen et al., 1995). Others have reported that serum OC changes within only a few hours of moderate-intensity exercise (Nishiyama et al., 1988). Hiney (1998) reported a decrease in markers of bone turnover in long yearling horses after 2 d confinement in box stalls. Michael et al. (2001) also reported a similar effect of stalling as OC and ICTP concentrations were significantly reduced following 4-d total collection periods. However, the absence of any change in concentrations of serum markers in this study may be related to the younger age of the animals, and thus the more rapid growth rate with accompanying higher bone turnover. There was an unexplained increase in OC concentrations on d 7 of the study but animals were treated no differently on this day, nor was the sampling protocol altered. As CF animals also increased on this day, it is doubtful that this is a real physiological effect as they were already acclimated to their environment for 6 wk prior to that day.

## CONCLUSIONS

Results of this study seem to indicate that very short periods of exercise increased bone mineral content of stalled weanling horses in comparison to those kept in small paddocks and altered bone geometry Confinement of at least 8 wk duration did not appear to cause any dramatic effects of disuse osteopenia, however this is difficult to determine without pre-weaning RBAE values. The exercise protocol was quite easy to

implement and was of minimal stress to the young animals. Therefore, similar exercise protocols might be suggested in order to enhance skeletal strength prior to more traditional under-saddle training.

# CHAPTER V

## SUMMARY AND CONCLUSIONS

The short periods of daily sprinting in both the equine and bovine studies appeared to positively influence the geometry of the bone. As strain rate is linearly proportional to strain magnitude and frequency, and as strain rate appears to be the major factor in controlling osteogenesis, sprinting should be effective exercise for stimulating the skeleton. Loading at only 1,000 to 1,400 microstrains induces a bone modeling response in rats, turkeys, dogs and sheep. Strain magnitude experienced by the equine third metacarpal during galloping may be much greater (3,200 microstrains; Rubin, 1984). Studies of strain magnitude in the immature calf have not yet been performed and thus no information in this animal is available. It would be interesting to compare the strains created by the exercise protocol in the forelimbs of both of these species.

Regardless of the exact magnitude of the strains caused by sprinting, the threshold stimulus was likely reached using that intensity of work. Generally, gains in mechanical strength are associated with periosteal expansion versus a reduction in medullary cavity (Notomi et al., 2001). The moment of inertia, and thus weight bearing capacity, varies as a function of the diameter of the bone raised to the $4^{th}$ power. Therefore, mathematically, the easiest way to increase bone strength is to increase the diameter of the bone through periosteal expansion. In both the equine and the bovine studies, however, the response to training appeared to be a reduction in the medullary cavity. The reduction in medullary cavity seen in both calves and horses in response to exercise is difficult to explain. Altering bone shape in a manner that does not dramatically improve strength would seem a major disadvantage in energy expenditure to model the bone in that fashion.

The suggested endosteal bone formation in the calves and horses may be related to the age of the animals as the manner in which bone adapts may differ with stage of maturity. Changes in bone geometry appear to differ between loading and normal ageing. In humans and roosters, periosteal deposition and endosteal resorption occur simultaneously with ageing after the cessation of long bone growth (Loitz and Zernicke, 1992). However, when exercised, mature roosters deposited bone endosteally, but showed no increase in persiosteal apposition. Similar changes have also been observed in swine (Woo et al., 1981).

Contrary to the original hypothesis of the studies, confinement did not appear to adversely affect either CF calves or foals in comparison to GR animals. In the young, rapidly growing animal, confinement may not result in bone resorption. Suspension of activity in the young, growing animal may lead to decrease in bone mass via a reduction of bone formation rather than bone resorption as seen in the adult animal. Unloading induces resistance in osteoprogenitor cells to such anabolic agents as insulin-like growth (IGF-1), growth hormone (GH) and parathyroid hormone (PTH; Kostenuik et al., 1999). In fact, skeletal unloading in the tail suspension rat model increases IGF-1 in bone and receptor expression while at the same time decreasing bone formation (Bilke et al., 1994). Therefore, the function of osteoblasts may be decreased with unloading or confinement. Without histomorphometric analysis and labeling of active surfaces of the bone, it is impossible to ascertain if the observed changes were due to endosteal bone formation in EX or bone resorption on the endosteal surface in GR or CF.

While no obvious bone loss was observed in the CF calves or weanling horses, substantial evidence links bone loss with reduced activity. Therefore, confinement of

young animals may be a concern when trying to optimize skeletal strength. The more severe the disuse conditions or the longer the duration, the longer lasting the effects. For example, in the extreme case of microgravity, Tilton et al. (1980) reported decreased BMC of the calcaneus of Skylab crew members 5 yr after the flights compared with pre-flight values. Further, how long a period of immobilization must be before a new steady state is established and no further bone loss occurs is unknown. Bone loss appeared to still be occurring 12 wk following stall confinement in previously conditioned mature horses (Porr et al., 1998). The process of bone resorption must cease or reach a steady state with bone formation before the skeleton is completely resorbed or structural integrity is lost, presumably determined by the genetic baseline (Rubin, 1984). Even within the normal active animal, bone cells close to the neutral axis in long bones experience minimal strains, yet maintain mineral density.

Whether short-term confinement of growing animals results in long-term adverse affects is unclear. The young animal may be more capable of recovery than older animals placed in confinement. Studies show a greater potential for recovery in young animals versus mature animals (Jaworski and Uhtoff, 1986; Tuukkanen et al., 1992). Firth et al. (1999) showed that bone mineral density (BMD) of foals confined to box stalls for the first 5 mo of life was depressed compared to controls, but were no different from pasture-reared controls at 11 mo of age, indicating that bone density due to periods of confinement can be easily compensated for without long term effects. Therefore, the skeletal system of young animals may recover completely in time, but the time taken to recover skeletal mass is unknown.

The time to recover may be longer than the initial immobilization period. In 9 – 11 wk rats subjected to 3 wk of single limb immobilization, followed by 8 wk of free exercise, BMD, while improved, did not return to values seen in controls (Kannus et al., 1994). In humans, BMD in casted limbs was still lower than the contralateral limb 10 yr following immobilization (Kannus et al., 1994). Apparently if immobilization results in the disappearance of trabecular bone, it cannot be restored. Jaworski and Uhtoff (1986) also found that while remobilization caused marked improvement in bone tissue, this restoration was incomplete.

One of the important questions remaining to be answered is how long will exercise-induced adaptations persist. While bone density was higher in retired athletes who trained at an early age compared to non-athletes (Bass, 2000), cessation of activity or a training program may result in an immediate reversal of effect. Studies in young rats and humans have shown that gains in BMD or BMC due to exercise are subsequently lost once the exercise protocol was terminated (Vuori et al., 1994; LeBlanc et al., 1990; Yeh et al., 1993). In young, growing rats deconditioning following an exercise protocol resulted in bone mass similar to age-matched sedentary controls. The reduction in bone mass back to control levels was due to both a decrease in bone formation and increased resorption rates compared those recorded during exercise (Yeh and Aloia, 1990; Iwamoto et al., 2000). Urinary excretion of pre-labeled $^{3}$H-tetracycline, a marker of bone resorption, was significantly higher 4 to 11 d after rats ceased exercise versus those that continued to train (Yeh and Aloia, 1990), thus the detraining effects may begin rather rapidly. Six mo of detraining in foals previously receiving sprint exercise for the initial 5 months of life, resulted in bone density lower than pastured foals (Barnevald and van

Weeren, 1999). Similarly, Ca supplementation increased bone mass in young girls, but when discontinued, showed no long-term benefits (Slemenda et al., 1997). Therefore, whatever measures are taken to increase bone mass must be continued to maintain the positive effects.

Nevertheless, some encouraging evidence suggests that exercise-induced bone adaptations persisting after training has ceased. Following an average of 8 yr of retirement, elite gymnasts maintained a 6 to 16% increased bone mass over controls. However these former athletes might have continued to lead a slightly more active lifestyle than controls (Bass et al., 1998). Elite raquet ball players maintained higher bone mass after 5 yr of a reduced training schedule from 4 to 5 d/wk to only 2 d/wk (Kontulainen et al., 2001). Male tennis players also maintained the same side-to-side differences in limbs in a 4 yr follow-up after reducing training frequency (Kontulainen et al., 1999). Therefore if some amount of equivalent stimulation is performed periodically, bone mass may be maintained even if training frequency is reduced.

Benefits of exercise lasting decades following training have been suggested in humans. In postmenopausal women, historical activity between ages of 14 to 21 provided a stronger correlation with bone area than any other activity (Kriska et al., 1988). In ballet dancers who had ceased training for 26 yr, there was no association between current activity and hip BMD, but there was a positive correlation between BMD and hours of ballet training at 10 to 12 yr of age (Khan et al., 1998). Karlsson et al. (2000) found that soccer players retired for 25 yr had increased bone mass compared to controls, but these benefits were not maintained beyond that time of retirement.

In animal studies of shorter duration, ten wk of exercise training in 4-wk-old rats increased bone length, cross-sectional area, and BMC and these advantages were not lost after 10 wk of detraining (Kiuchi et al., 1998). Nelson and Bouxsein (2001) suggested that alterations in skeletal geometry, similar to those observed in these studies, may be more resistant to a lessening of training intensity than skeletal benefits that are due only to increased bone density. Therefore, if exercising the animal at a young age alters bone geometry, these advantages may persist longer, especially is the skeleton is still subjected to periodic stimulation.

An important question for the producer who wishes to implement an exercise program into the management of young animals is how often must such exercise be conducted. While many studies have indicated a low number of effective cycles stimulate bone hypertrophy, what the effective frequency at which the stimulus must be applied in order to maintain the increased bone mass is currently unknown. In mice subjected to external loading for 5 d, followed by three wk of no loading, cortical bone area increased compared to controls (Gross et al., 2002). Apparently just 5 d of loading was sufficient to maintain a response up to 3 wk later.

The osteogenic response to loading does appear to saturate after a few cycles (Rubin and Lanyon, 1984) but an increase from five jumps to 100 jumps per day in rats resulted in greater bone formation in the 100 jump group (Umemura et al., 1997). Others have suggested that shorter, more frequent bouts with the same total number of cycles may cause a larger response. When rats were subjected to four separate loading bouts of 90 cycles versus a single bout of 360 cycles, there was a 71% increase in mineral apposition rate, an 80% increase in bone formation rate, and a 94% increase in

mineralizing surface on the endocortical surface of the tibia in the multiple bout animals (Robling et al., 2001). These changes were observed after only 3 d of loading separated by 24 h or on d 1, 3, and 5 of the study. Raab-Cullen et al. (1994c) also found an equivalent response in periosteal mineral apposition in mature rats loaded 3 or 4 d/wk or daily, indicating exercise or loading every day is probably unwarranted.

Bone cells may become insensitive to repeated cycles at the same strain and thus exercise bouts might need to be shortened and performed more frequently. Robling et al. (2000) assumed a linear association between bone formation and the number of loading days and suggested that 24-h separation between loading appears a sufficient time span for the bone cells to recover full mechanosensitivity. Robling et al. (2000) suggested that 2 to 3 h separation between 60 loading bouts 6 times/d and 90 bouts 4 times/d appeared adequate for cells to regain their mechanosensitivity. In a subsequent study of recovery intervals from 0.5 to 8 h, while an increase in bone formation rate/bone surface was observed in animals allowed 4 h between loading bouts, the optimal recovery time appeared to be 8 h intervals when compared to bone formation rates of studies with 24 h recovery periods (Burr et al., 2002). Raab-Cullen et al. (1994c) suggested that the signal or intercellular response to loading may continue up to 48 h, while [3H] uridine incorportation of osteocytes persisted up to 24 h following a single loading bout (Pead et al., 1988a). Chow et al. (1997) found that maximal gene expression of bone matrix proteins did not occur until 72 h post-loading of tail vertebrae in rats, while bone-lining cells stimulated to differentiate to osteoblasts returned to their original state 120 h post-loading. Others have suggested that the maximum interval between repeated bouts in

order to have an additive effect is probably 7 days (Kimmel, 1993), suggesting at least the need for weekly loading.

Whether these theories apply to physiological versus externally-applied loading remains to be tested. This avenue of research does appear promising, however, at some point, the practicality for implementing such a system does become limiting. For use in optimizing the skeletal system of domestic animals, the benefits of a possibly slightly improved skeletal strength or mass must be weighed against the operative time taken to implement such a system. The studies above suggest that the exercise regimen used here could be reduced in frequency (perhaps only 3 d/wk) as long as the intensity remained sufficient and would thus be a valuable line of future research.

Although changes in geometry and bone distribution occurred in response to exercise in both the equine and bovine studies, actual mechanical properties may not have changed. It is unknown how large a change in bone geometry is needed to be a physiological advantage to the animal. In the bovine study, there were definite changes in cross sectional geometry in the EX calves, yet there was no difference in breaking strength. In mature roosters, exercise decreased the dorsopalmar endosteal diameter and increased the diameter of the dorsal and medial cortices of the tarsometatarsus, mirroring the results of the present equine study, but no differences in mechanical properties were observed (Loitz and Zernicke, 1992). Woo et al. (1981) also found an increase in femoral cross-sectional area in exercised swine, but no change in mechanical or material properties. Alterations in mineral distribution may offer advantages to the animal which cannot be observed by the ex vivo three-point-bending tests which do not load the bone in a physiological manner.

Single site selection for sampling of BMD or BMC, as used in both the bovine and equine studies, may have limited the complete picture of changes occurring throughout the entire bone. In horses, modeling affects different areas of the bone (Sherman et al., 1995; Warren et al., 1998). In addition, some techniques for measuring bone density such as dual energy x-ray absorptiometry (DEXA) correlate more with true BMD in one region of the bone versus another (McClure et al., 2001) and thus may obscure changes occurring within the bone. While a more thorough investigation along the length of the entire bone may have yielded a more definitive picture of bone adaptation, for practicality, the approach taken still provided valuable information.

Furthermore, while most studies tend to focus on changes within a particular long bone, exercise may affect bones within the same limb differently. Strenuous exercise in the immature rat resulted in smaller tibial cross-sectional geometry and structural properties compared to controls, while 2$^{nd}$ metatarsal geometry and structural properties remained the same (Li et al., 1991). While not equivocal to the equine or bovine due to the different anatomical site of the bones in rat, it does suggest that different modifications between bones occur and it may be useful to examine other bones.

Bone response to altered stimuli may be very site specific with differences in response not only between bones within the skeletal system but also even within the bone being loaded. In rat ulnae loaded in axial compression, Mosley and Lanyon (1998) found a reduced rate of periosteal bone formation proximally while an increase in bone formation occurred distally. Alteration in bone shape can also occur with a simultaneous increase in periosteal formation and endosteal resorption. Therefore, bone deposition due to loading may not increase throughout and along the entire bone, but discreet

populations of cells within a localized region may increase or decrease activity or even convert from a bone forming population to an area undergoing bone resorption (Mosley and Lanyon, 1998). Such a localized response would greatly diminish the likelihood of observing differences in systemic blood markers.

The results of both studies did not show great promise in the ability of biochemical markers to detect altered rates of bone turnover due to exercise in the young rapidly growing animal. Saastamoinen et al. (1994) and Black et al. (1997a) found considerable inter-individual variation in weanling horses when using a radioimmunoassay produced by Incstar (Stillwater, MN) due to the amount of bone activity during growth. Reller et al. (2001) reported similar issues of repeatability in OC of nursing foals measured with NovoCalcin immunosassays produced by Metra Biosystems. In the present studies, an enzyme linked immunosorbent assay (ELISA) was used, which yielded higher OC concentrations in weanlings horses (mean: 64 ng/ml) vs. traditional radioimmunoassay; (30 to 40 ng/mL; Saastamoinen et al., 1994; Black et al., 1997a). Differences between assays may be due to the ability of the antibodies to recognize equine OC, and the possibilities of binding to different OC fragments as both intact OC and OC fragments appear in the circulation. Development of an assay which specifically measures intact equine OC may aid in the applicability of this assay.

The ability of serum markers to reflect changes in bone density may be of some question, especially with young animals. In young adult Oriental males, OC and alkaline phosphatase increased with resistance training, and DPD was reduced, but no change in total body, femoral neck, lumber spine, or midradius BMD was observed despite the elevation in bone formation markers persisting for 4 mo (Fujimura et al., 1997).

Conversely in equine studies, OC transiently decreased with exercise (Jackson et al., 1998; McCarthy and Jeffcott, 1992; Fenton et al., 1999, Nielsen et al., 1998). In addition, no change in serum OC occurred with confinement in mature animals, despite an observed decrease in bone mineral content (Porr et al., 1998), while others report a decrease in OC (Hoeskstra, 1999). Here again a considerable range in values was observed (4 to 31 ng/ml). Without additional information from direct histomorphometry studies, it is hard interpret alterations in OC concentrations.

## IMPLICATIONS

The results of these studies indicate the potential value of implementing an exercise program in immature animals. By stimulating the skeleton at an early age to model for intense activity, these animals may be better adapted for training than those which begin training at later age. Beyond athletic endeavors, increasing bone mass may produce animals with greater longevity in such production systems as the dairy and swine industry. Animals with higher bone mass may be better prepared to meet the mineral demands for multiple cycles of gestation and lactation, as well as reducing their elimination from the herd due to lameness. Currently, more research needs to be performed on the long term benefits of these exercise programs as well as the frequency at which they should be employed.

In order to determine whether such a training protocol was truly useful, it may be necessary to perform longer longitudinal studies and monitor "performance" of animals such as incidence of bone trauma during exercise, as well as issues related to production. If bone mass is truly a concern for the lactating dairy cow, potentially useful information

could be gathered concerning bone mass in heifers and if any correlations exist between duration, total production during lactation, and numbers of lactation before removal from the herd. Such information concerning the effects of short-term exercise on skeletal soundness and increasing time spent in production could also be of value for intensively-managed swine.

More specifically, information on the frequency at which exercise must occur are most easily addressed using dynamic histomorphometry involving labeling the actively bone-forming surfaces. This would provide information on site specific changes as well, and answer the questions concerning whether exercise-induced adaptations in this age of animal are related to increased periosteal or endosteal bone formation, a reduction to activation of resorption, or if increased resorption occurred in the medullary cavity of CF animals. Certainly how frequently exercise must be performed is of critical information for animal producers as well as those involved in human medicine. Potential studies could involve graded levels of exercise and monitor bone response using a linear regression model to determine where or if a plateau in response occurs. The bovine model should prove the most useful in such studies which facilitate direct tissue analysis. As the numbers of calves in this study may not have been enough to show significant differences in mechanical properties, perhaps increasing the number of calves to eight per treatment might be necessary. If the desired is to pursue responses in horses, a more sensitive technique of monitoring bone changes should be employed.

# APPENDICES

# APPENDIX A

## INTRODUCTION

The shape of the bone is one of the main determinants of skeletal strength. Therefore, it is extremely important that the geometry of the bone, in addition to mineral content, are included in the assessment of bone adaptation to exercise. In the bovine study, the use of computed tomography (CT) allowed the measurement of cortical and medullary cross-sectional areas, and cortical widths. Computed tomography provides a cross sectional image with clearly discernible boundaries between the cortical bone and the medullary cavity. As significant differences were seen in bone geometry between treatments in the calves, it was important to attempt measuring bone geometry in the horses. However, due to the expense and risk involved with anesthesia necessary to use CT, the only technology available was radiography. The radiographic image of MC III as scanned by the BioRad Densitometer and translated by Multi-Analyst software, produces a graphic representation of the optical density of the bone. Typically, this curve has two peaks corresponding to the maximal mineral content of the bone, between which is an area of lower opacity representing the medullary cavity. The opacity of the object seen on the radiographic image is not only a function of the density and atomic number of the object through which the x-rays pass, but also the thickness, with more x-rays attenuated by a thicker object. Therefore, the longest path of the x-ray through the cortical bone, and thus potentially the highest point of the peak on the curve, should be just before the opacity diminishes due to the medullary cavity. Thus measuring cortical diameter from the initiation of the curve to its highest peak should provide an estimate of an individual cortical diameter. Similarly, the entire width of the curve should represent the diameter

of the bone, with the width of the medullary cavity being the distance between the two peaks.

Others have used radiogrammetry, the practice of measuring bone widths from radiographs, in equine studies (Hanson and Markel, 1994; Larkin and Davies, 1996; Thompson et al., 2001). However, the exact boundaries of cortical bone and medullary cavity may be difficult to discern with the human eye, and could be a potential source of measurement errors. The sharpness of an image, and thus its ability to define an edge, is affected in part by the very object that is filmed. Since MC III is a round object, the edge of the bone will be unsharp, not only due to the presence of penumbra, or the edge gradient, but simply because the bone does not have sharp edges. Increasing the contrast of the radiographic film with a lower kVp setting and higher mAs will produce a radiograph with more visibility of fine detail. However, this also increases the exposure time, a definite disadvantage when using unsedated animals. Additionally, lower kVp results in short-scale contrast with most objects appearing in black and white and few shades of gray. Therefore, the contrast in thickness of Al of the stepwedge required for RBAE determination is unable to be discerned, eliminating this possibility if only one radiograph is to be taken. Others have used a detail screen with the bone placed directly on the cassette to decrease distortion and penumbra, thereby improving detail and precision of measurements (Hanson and Markel, 1994). Hanson and Markel used a lower kVp than that used for the present studies. Larkin and Davies (1996) used a similar technique of detail screens when assessing radiographic indexes of racehorses. This equipment was not available for this study. Thomson et al., (2001) measured cortical widths directly from radiographs that were taken for use in assessing RBAE values, using

a digital caliper and a magnifying lens. On low contrast films, the ability to detect cortical margins is diminished, thereby increasing the margin of error. Cortical width measurements taken from the curve, produced by the Multi-Analyst software, decreases this human error.

The objective of this preliminary study was to compare the accuracy of cortical width measurements of the equine MC III on radiographs and CT images.

## MATERIALS AND METHODS

Thirty forelimbs were obtained post-mortem from the MSU Veterinary Teaching Hospital and from a commercial slaughter facility. Limbs were radiographed and CT scanned with skin and soft tissues in place to simulate field conditions. Dorsopalmar and lateromedial radiographs were taken (65 kVp for 0.1 s) as typically performed by this laboratory, necessary to provide enough gray scale when using the aluminum stepwedge. The dorsal-palmar view was used to measure the width of the medial and lateral cortices, the inner mediolateral medullary diameter and outer mediolateral cortical diameter.

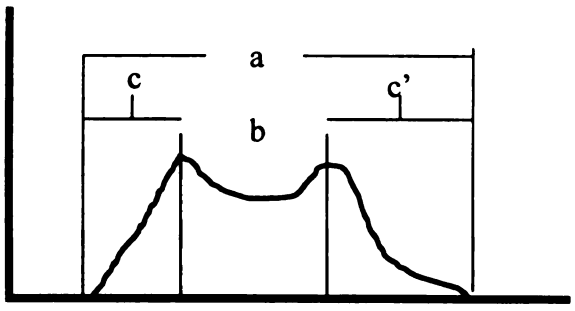


Figure 4. Schematic illustrations of cortical measurements created from Multi-Analyst software.
a = bone diameter
b = medullary diameter
c and c' = individual cortical diameters

The initial rise of the curve above baseline to the highest point was used as the width of each individual cortice, and the medullary diameter was measured from the distance between the two peaks of the palmar cortical widths curve. The outer cortical diameter was measured as the entire distance of the curve. The same procedure was used for the lateral-medial view for determination of dorsal and palmar cortical widths.

Computed tomography

Limbs were scanned with a GE 9800 CT scanner (General Electric Medical Systems, Wilwaukee, WI) at 80 kV, 70 mA, 2 sec scan time. An initial scout view was taken to determine the location of the nutrient foramen. A single 10-mm thick transverse image was then acquired just distal to this location, to correspond with the same site used for RBAE measurements in this and previous studies. The diameter of the bone was measured, including the dorsopalmar (dp) bone diameter, dp medullary diameter, lateromedial (lm) bone diameter and lm medullary diameter. Finally, the width of the individual cortices: dorsal, palmar, medial, and lateral were measured in the same plane as the previous measurements.

Statistical Analysis

The general linear model of SAS with the correlation procedure was used to estimate the predictability of radiographic measurements in comparison to those obtained from computed tomography. A P value less than 0.05 was considered significant.

## RESULTS

All measures from the radiographs were significantly correlated to those obtained from computed tomography. However, the linearity between the two modalities did vary considerably between bone measurements (Table 1). It appeared that radiographs more closely correlated with CT for the dp bone diameter ($R^2 = 0.66$), ml bone diameter ($R^2 = 0.59$), and the dorsal ($R^2 = 0.77$) and medial cortical widths ($R^2 = 0.76$). Measurements that involved the transition from cortical bone to the medullary cavity were more variable, and thus the fit of the line was much lower for medullary diameter measurements. The correlation for palmar cortical widths was also poor, presumably due to the presence of MC II and IV. The difference in repeatability between medial and lateral cortical widths is more difficult to explain. As most modeling of the equine MC III involves an increase of bone in the dorsomedial direction, the greater width of the medial cortex produces a better fit than measuring the lateral cortex, whose thinner diameter may produce greater variability.

## DISCUSSION

The technique of measuring cortical dimensions from radiographs appears to be a reasonable alternative to the very costly computed tomography method. The radiographic technique may detect differences in weanlings caused by short term high intensity exercise, as previously reported (McCarthy and Jeffcott, 1992; Larkin and Davies, 1996). The presence of splint bones (MC II and IV) in the mediolateral radiographs increased the error. In the young horses, the splint bones were not yet fused

and could be discerned on the radiograph. As the animal ages, this is no longer possible and increases the error involved, especially in measurements of the palmar cortex. Piotrowski et al. (1983) found that the fused MC II and IV only alter geometry of the bone in the proximal 20 to 30% of the bone. Measurements taken further distally on MC III, where MC II and IV diminish, have less superimposition and may be more accurate.

Patient positioning can easily effect cortical measurements taken from radiographs. Slight errors from the true dorsopalmar or lateromedial direction can greatly change the measurements. In addition, conformation influences the radiographic appearance of the bones and may require adjustment of film placement. Larkin and Davies (1996) stated that deviation from a true mediolateral radiograph considerably altered cortical measurements, but the extent and correction of this effect was not discussed.

Although the technique described above is not optimal for assessing bone geometry, it does appear to provide a reasonable compromise between cost, convenience, and accuracy. Using the digital image ideally minimized the human error component as compared to visual inspection of the radiographs and caliper placement. In future studies radiographs may be taken at different kVp exposures, one at higher kVp for RBAE determination, and one at lower kVp for cortical measurements.

Table 1A. Regression coefficients and significance levels for cortical measurements as compared by computed tomography and radiography.

| | $R^2$ | P value |
|---|---|---|
| d-p bone | 0.66 | 0.0001 |
| d-p medullary cavity | 0.24 | 0.0048 |
| dorsal | 0.77 | 0.0001 |
| palmar | 0.28 | 0.002 |
| m-l bone | 0.59 | 0.0001 |
| m-l medullary cavity | 0.39 | 0.0001 |
| medial | 0.76 | 0.0001 |
| lateral | 0.26 | 0.0026 |

Appendix B
Bovine Data

Calf Diets for Bovine Study
Appendix Table 1B.

MSU Calf Special
Active drug ingredient
Lasalocid (as lasalocid sodium).....30 G/Ton

Guarantee Analysis

| | |
|---|---|
| Crude Protein | MIN 18.00% |
| Crude Fat | MIN 1.50% |
| Crude Fiber | MAX 22.00% |
| Acid Detergent Fiber | MAX 26.00% |
| Calcium | MAX 1.00% |
| Calcium | MIN 0.50% |
| Phosphorus | MIN 0.60% |
| Selenium (as sodium selenite) | MIN 3.00 ppm |
| Vitamin A | MIN 4,000.00 IU/LB |

Ingredients: roughage products, processed grain by-products, plant protein products, forage products, molasses products, calcium phosphate, salt, bicarbonate of soda, ground limestone, sodium selenite, zinc sulfate, zinc oxide, manganous oxide, copper sulfate, ferrous sulfate, cobalt carbonate, calcium iodate, vitamin A acetate, vitamin D-3 supplement, vitamin E supplement

Appendix Table 1C.
Bovine cortical areas of MC III & IV.

| Calf ID | total area ($cm^2$) | medullary area ($cm^2$) | cortical area ($cm^2$) | % medullary cavity | %cortical bone |
|---|---|---|---|---|---|
| 9797 | 4.84 | 2.14 | 2.70 | 44.2% | 55.8% |
| 9801 | 4.68 | 2.03 | 2.65 | 43.3% | 56.7% |
| 9802 | 4.47 | 1.84 | 2.64 | 41.1% | 58.9% |
| 9812 | 4.37 | 1.69 | 2.68 | 38.7% | 61.3% |
| 9817 | 3.97 | 1.88 | 2.09 | 47.4% | 52.6% |
| 9821 | 4.32 | 1.72 | 2.60 | 39.7% | 60.3% |
| 9798 | 4.58 | 1.82 | 2.76 | 39.7% | 60.3% |
| 9799 | 4.46 | 1.72 | 2.74 | 38.6% | 61.4% |
| 9804 | 3.92 | 1.44 | 2.48 | 36.7% | 63.3% |
| 9811 | 4.54 | 1.61 | 2.93 | 35.5% | 64.5% |
| 9819 | 3.72 | 1.44 | 2.29 | 38.65 | 61.4% |
| 9820 | 4.30 | 1.82 | 2.48 | 42.3% | 57.7% |
| 9796 | 4.53 | 1.88 | 2.65 | 41.4% | 58.6% |
| 9800 | 4.37 | 2.01 | 2.36 | 45.9% | 54.1% |
| 9805 | 4.46 | 2.31 | 2.15 | 51.8% | 48.2% |
| 9813 | 4.72 | 1.88 | 2.84 | 39.8% | 60.2% |
| 9818 | 4.03 | 2.05 | 1.98 | 50.6% | 49.4% |
| 9822 | 4.17 | 1.95 | 2.22 | 46.7% | 53.3% |

Appendix Table 2C. Bovine cortical widths of MC III& IV.

| | | | | | cortical width | | | |
|---|---|---|---|---|---|---|---|---|
| Calf ID | d-p outer | d-p inner | m-l outer | m-l inner | palmar | dorsal | medial | lateral |
| 9796 | 1.93 | 1.22 | 2.73 | 1.79 | 0.36 | 0.335 | 0.455 | 0.46 |
| 9800 | 1.99 | 1.25 | 2.635 | 1.765 | 0.4 | 0.34 | 0.415 | 0.42 |
| 9805 | 2.03 | 1.435 | 2.655 | 1.905 | 0.315 | 0.245 | 0.39 | 0.355 |
| 9813 | 2.03 | 1.29 | 2.755 | 1.66 | 0.365 | 0.335 | 0.555 | 0.54 |
| 9818 | 1.78 | 1.225 | 2.67 | 1.935 | 0.25 | 0.305 | 0.375 | 0.38 |
| 9822 | 1.885 | 1.24 | 2.6 | 1.785 | 0.335 | 0.305 | 0.41 | 0.39 |
| 9798 | 2.01 | 1.24 | 2.68 | 1.73 | 0.39 | 0.4 | 0.495 | 0.41 |
| 9799 | 2.035 | 1.155 | 2.645 | 1.635 | 0.46 | 0.4 | 0.53 | 0.455 |
| 9804 | 1.81 | 1.06 | 2.56 | 1.54 | 0.34 | 0.385 | 0.495 | 0.48 |
| 9811 | 1.99 | 1.265 | 2.615 | 1.455 | 0.335 | 0.36 | 0.56 | 0.585 |
| 9819 | 1.83 | 0.97 | 2.435 | 1.555 | 0.35 | 0.495 | 0.455 | 0.45 |
| 9820 | 1.97 | 1.095 | 2.535 | 1.63 | 0.375 | 0.42 | 0.455 | 0.47 |
| 9797 | 2.035 | 1.275 | 2.815 | 1.89 | 0.365 | 0.365 | 0.435 | 0.45 |
| 9801 | 2 | 1.28 | 2.815 | 1.825 | 0.34 | 0.31 | 0.535 | 0.46 |
| 9802 | 1.925 | 1.14 | 2.735 | 1.76 | 0.36 | 0.365 | 0.51 | 0.485 |
| 9812 | 1.865 | 1.14 | 2.72 | 1.66 | 0.35 | 0.345 | 0.525 | 0.505 |
| 9817 | 1.885 | 1.305 | 2.525 | 1.695 | 0.26 | 0.31 | 0.41 | 0.425 |
| 9821 | 1.905 | 1.085 | 2.685 | 1.72 | 0.365 | 0.415 | 0.51 | 0.475 |

Appendix Table 3C.

Bovine Computed Tomography Bone Mineral Densities

| calf ID | total | medial | lateral | dorsal | palmar |
|---|---|---|---|---|---|
| 9796 | 611.5883 | 1252.565 | 1236.51 | 1230.333 | 1131.252 |
| 9797 | 568.925 | 1247.098 | 1254.518 | 1241.938 | 1111.925 |
| 9798 | 656.5483 | 1280.127 | 1270.765 | 1287.445 | 1211.943 |
| 9799 | 663.5783 | 1239.103 | 1237.888 | 1248.065 | 1138.485 |
| 9800 | 564.1183 | 1227.265 | 1206.872 | 1198.54 | 1100.945 |
| 9801 | 611.1117 | 1272.947 | 1283.135 | 1211.093 | 1110.318 |
| 9802 | 622.965 | 1217.953 | 1201.713 | 1156.565 | 1089.847 |
| 9804 | 674.31 | 1225.228 | 1220.738 | 1247.688 | 1087.187 |
| 9805 | 491.5017 | 1176.795 | 1090.372 | 1022.058 | 1055.742 |
| 9811 | 669.8083 | 1212.528 | 1212.177 | 1213.035 | 1125.813 |
| 9812 | 614.2617 | 1228.715 | 1237.485 | 1196.075 | 1061.452 |
| 9813 | 608.18 | 1209.793 | 1214.215 | 1181.537 | 1112.428 |
| 9817 | 538.03 | 1174.452 | 1159.865 | 1187.973 | 1005.707 |
| 9818 | 491.87 | 1076.957 | 1109.962 | 1080.122 | 962.0683 |
| 9819 | 654.7233 | 1180.305 | 1203.385 | 1195.965 | 1154.237 |
| 9820 | 667.3633 | 1210.68 | 1224.045 | 1209.29 | 1148.687 |
| 9821 | 626.9433 | 1216.712 | 1212.472 | 1219.618 | 1118.808 |
| 9822 | 557.2467 | 1199.07 | 1146.473 | 1182.673 | 1067.443 |

Appendix Table 4C. Bovine mechanical testing data.

| calf | moment of inertia | fracture force | ultimate bending strength |
|---|---|---|---|
| 9796 | 8.02412E-09 | 5098 | 153.4446 |
| 9800 | 8.48627E-09 | 4413 | 130.5159 |
| 9805 | 8.12347E-09 | 3986 | 123.2507 |
| 9813 | 9.566E-09 | 5915 | 166.881 |
| 9818 | 5.63762E-09 | 3887 | 146.9578 |
| 9822 | 6.86342E-09 | 5178 | 178.3223 |
| 9798 | 9.04852E-09 | 5752 | 161.7665 |
| 9799 | 9.68843E-09 | 5293 | 140.9845 |
| 9804 | 6.53874E-09 | 5298 | 188.6756 |
| 9811 | 8.65436E-09 | 6286 | 179.1754 |
| 9819 | 6.61679E-09 | 5212 | 181.9416 |
| 9820 | 8.5839E-09 | 5520 | 168.4385 |
| 9797 | 9.71483E-09 | 5641 | 142.697 |
| 9801 | 9.15848E-09 | 4943 | 136.849 |
| 9802 | 8.28292E-09 | 5504 | 162.6589 |
| 9812 | 7.46038E-09 | 5090 | 172.1181 |
| 9817 | 6.44595E-09 | 4554 | 161.9687 |
| 9821 | 8.01654E-09 | 5820 | 173.5353 |

Appendix Table 5C. Bovine modulus of elasticity and rib mechanical data.

| calf | modulus of elasticity | rib modulus of elasticity | rib peak force |
|---|---|---|---|
| 9798 | 3614862666 | 5254251245 | 245 |
| 9799 | 2989975408 | 4961875146 | 252 |
| 9804 | 4598095119 | 3949426846 | 203 |
| 9811 | 3994419697 | 2957095789 | 240 |
| 9819 | 4608447831 | 5223934015 | 248 |
| 9820 | 3107639103 | 3388264665 | 269 |
| 9796 | 3779387253 | 3640899441 | 294 |
| 9800 | 2590123618 | 4081136514 | 131 |
| 9805 | 2525662006 | 3573151515 | 212 |
| 9813 | 3343614207 | 4445149303 | 361 |
| 9818 | 3529392004 | 5380507391 | 161 |
| 9822 | 3949949445 | 2960135074 | 199 |
| 9797 | 3229415858 | 5067429911 | 345 |
| 9801 | 2907364969 | 4737066859 | 252 |
| 9802 | 3896793470 | 4247729859 | 313 |
| 9812 | 3857142622 | 5501925379 | 182 |
| 9817 | 3988832060 | 6003893940 | 222 |
| 9821 | 3550515610 | 4886716491 | 255 |

Appendix Table 6C. Bovine palmar radiographic bone aluminum equivalence (mm Al).

| calf ID | 6wk | D0 | D21 | D42 |
|---|---|---|---|---|
| 9798 | 8.84770965 | 11.2588099 | 9.13393893 | 10.2060435 |
| 9799 | 9.06720146 | 9.57659539 | 10.6891958 | 11.9148236 |
| 9804 | 8.08378827 | 10.8394762 | 9.81880031 | 10.4916397 |
| 9811 | 12.2883339 | 11.4351889 | 11.814378 | 11.190622 |
| 9819 | 9.23295792 | 10.2016501 | 10.7523485 | 11.1263928 |
| 9820 | 8.77995654 | 9.46734795 | 10.602998 | 11.1613525 |
| 9796 | 11.3199872 | 11.3302304 | 10.3791455 | 11.7144336 |
| 9800 | 11.3215572 | 9.71415629 | 7.85015627 | 9.53590215 |
| 9805 | 8.41117937 | 8.82526603 | 11.2695735 | 10.4763835 |
| 9813 | 10.187752 | 10.9186393 | 11.0694055 | 12.1900471 |
| 9818 | 9.36886192 | 10.8839083 | 11.0924414 | 12.0964286 |
| 9822 | 9.86856805 | 10.6862092 | 9.71023116 | 10.432032 |
| 9797 | 10.6404758 | 10.1653577 | 9.06385784 | 10.4906101 |
| 9801 | 10.7028538 | 10.2427717 | 9.7206352 | 12.0885591 |
| 9802 | 9.29599912 | 9.30476308 | 10.2261078 | 11.2260728 |
| 9812 | 10.7258232 | 10.0730093 | 12.0297439 | 13.2967719 |
| 9817 | 9.44006035 | 9.42213749 | 10.4267218 | 10.7519364 |
| 9821 | 9.26662477 | 12.084318 | 11.4003613 | 12.4429729 |

Appendix Table 7C. Bovine dorsal radiographic bone aluminum equivalence (mm Al).

| Calf ID | 6wk | D0 | D21 | D42 |
|---|---|---|---|---|
| 9798 | 9.92128435 | 11.3382256 | 10.4013259 | 11.5941014 |
| 9799 | 9.78227515 | 9.139692 | 10.5971969 | 11.5652253 |
| 9804 | 8.69559017 | 9.99885806 | 10.8500287 | 10.0479074 |
| 9811 | 11.5701152 | 11.280422 | 11.3575526 | 11.6156056 |
| 9819 | 9.01959889 | 10.3450241 | 10.746433 | 10.6851194 |
| 9820 | 9.27536183 | 9.95828256 | 10.7883508 | 11.5886367 |
| 9796 | 10.2174629 | 11.0331623 | 10.7941454 | 10.9411228 |
| 9800 | 10.3036195 | 9.64273943 | 9.24213735 | 10.8750378 |
| 9805 | 8.46616897 | 8.52824158 | 9.51626967 | 8.75943504 |
| 9813 | 11.4802129 | 11.3382576 | 11.0016342 | 11.4185295 |
| 9818 | 9.31115682 | 9.88998933 | 10.9578897 | 10.7012316 |
| 9822 | 10.7037676 | 11.2553545 | 10.6678794 | 11.7273204 |
| 9797 | 10.8017127 | 10.8415264 | 9.86577034 | 10.852873 |
| 9801 | 9.86574118 | 9.92747902 | 10.0575195 | 11.4394631 |
| 9802 | 9.36534053 | 9.41004929 | 10.1511368 | 10.6373298 |
| 9812 | 10.864762 | 10.6716791 | 11.5704013 | 11.6510793 |
| 9817 | 9.17054486 | 9.51982988 | 11.0061628 | 10.5424362 |
| 9821 | 9.8675844 | 11.7709384 | 11.1449957 | 12.8061963 |

Appendix Table 8C. Bovine lateral radiographic bone aluminum equivalence (mm Al).

| Calf ID | 6wk | D0 | D21 | D42 |
|---|---|---|---|---|
| 9798 | 9.54518461 | 10.8610051 | 10.6515231 | 11.9644705 |
| 9799 | 9.49616895 | 9.07116472 | 9.80692719 | 11.3745735 |
| 9804 | 9.03259498 | 10.3282131 | 12.3968631 | 12.3733245 |
| 9811 | 12.9070837 | 13.0590031 | 12.8659712 | 13.2970919 |
| 9819 | 9.55237546 | 9.69419445 | 10.823212 | 11.4629994 |
| 9820 | 9.81817909 | 10.6854874 | 11.5755349 | 13.2041922 |
| 9796 | 9.66390581 | 10.5031364 | 10.5027553 | 11.3254503 |
| 9800 | 10.2395088 | 9.79876729 | 10.8293589 | 12.3091944 |
| 9805 | 9.3679233 | 9.38812892 | 11.2809143 | 11.4937132 |
| 9813 | 11.8456389 | 12.2779738 | 12.1243647 | 13.243664 |
| 9818 | 9.51827754 | 9.67030196 | 10.5206676 | 10.6211006 |
| 9822 | 10.5515965 | 10.793034 | 11.1193825 | 12.7734495 |
| 9797 | 10.8949082 | 10.9600226 | 10.0262978 | 12.0880596 |
| 9801 | 10.585776 | 10.0472443 | 10.9964976 | 12.4651118 |
| 9802 | 9.18426121 | 9.05040678 | 11.809963 | 12.2192036 |
| 9812 | 10.6833097 | 11.4467547 | 11.6684461 | 11.6505355 |
| 9817 | 9.37209956 | 10.2656745 | 10.5158806 | 11.5642891 |
| 9821 | 9.21060822 | 11.0022692 | 11.0347046 | 12.6696055 |

Appendix Table 9C. Bovine medial radiographic bone aluminum equivalence (mm Al).

| Calf ID | 6wk | D0 | D21 | D42 |
|---|---|---|---|---|
| 9798 | 8.88014055 | 10.6493806 | 10.0918561 | 11.8701347 |
| 9799 | 9.54371121 | 9.04303247 | 9.96033161 | 11.5086136 |
| 9804 | 8.32428565 | 8.99247112 | 10.9014865 | 10.6914555 |
| 9811 | 11.5986308 | 11.0926599 | 11.124595 | 11.7253027 |
| 9819 | 8.84839597 | 9.11586 | 10.2130017 | 9.98441201 |
| 9820 | 8.70126667 | 10.2270701 | 10.9983254 | 12.0739268 |
| 9796 | 9.37199587 | 10.2737995 | 9.47743691 | 10.6956646 |
| 9800 | 9.39435605 | 8.32916512 | 8.41530879 | 9.6820386 |
| 9805 | 8.69353892 | 8.82537302 | 10.2748374 | 10.1012423 |
| 9813 | 10.9244661 | 11.2330087 | 11.3920652 | 12.1708455 |
| 9818 | 8.72760147 | 9.2006018 | 10.3714846 | 10.1226485 |
| 9822 | 9.58954426 | 10.0115517 | 10.2994541 | 10.6323256 |
| 9797 | 10.3280208 | 10.5490027 | 9.3521611 | 11.2938104 |
| 9801 | 10.4093207 | 10.3769919 | 10.7688738 | 11.9869141 |
| 9802 | 8.38500515 | 8.17619497 | 10.1239486 | 10.7743459 |
| 9812 | 9.8184613 | 10.7022906 | 10.8375394 | 10.7531023 |
| 9817 | 8.39078181 | 9.49452068 | 9.81693025 | 10.3396095 |
| 9821 | 8.58982058 | 10.5389211 | 10.6993482 | 11.8495637 |

Appendix Table 10C. Bovine Ca and P concentrations (mg/g) by cortices.

| | dorsal | | palmar | | medial | | lateral | |
|---|---|---|---|---|---|---|---|---|
| Calf ID | P | Ca | P | Ca | P | Ca | P | Ca |
| 9798 | 120.50 | 282.8559 | 117.02 | 280.2082 | 114.62 | 247.2494 | 117.36 | 293.6941 |
| 9799 | 121.18 | 258.661 | 115.16 | 287.3128 | 117.91 | 258.278 | 128.10 | 246.6087 |
| 9804 | 119.75 | 280.5267 | 100.95 | 217.0426 | 113.29 | 235.8008 | 116.33 | 232.5234 |
| 9811 | 121.96 | 282.4392 | 115.14 | 266.1921 | 118.11 | 281.7288 | 112.90 | 254.8084 |
| 9819 | 111.09 | 283.8298 | 113.77 | 283.9798 | 121.64 | 294.2654 | 120.64 | 250.3715 |
| 9820 | 96.20 | 234.3303 | 87.00 | 190.6979 | 111.39 | 266.1654 | 117.55 | 294.8814 |
| 9796 | 95.74 | 221.2418 | 105.77 | 247.6323 | 115.31 | 258.9095 | 111.90 | 261.6117 |
| 9800 | 116.13 | 276.2619 | 108.22 | 251.4645 | 117.43 | 333.0842 | 117.77 | 274.8709 |
| 9805 | 94.39 | 227.8165 | 111.47 | 247.1362 | 116.67 | 268.9172 | 121.12 | 249.221 |
| 9813 | 122.60 | 293.0541 | 118.39 | 270.9783 | 125.27 | 286.4856 | 119.88 | 279.2062 |
| 9818 | 123.96 | 297.5126 | 119.11 | 307.1737 | 122.76 | 266.6637 | 125.63 | 273.3889 |
| 9822 | 123.93 | 277.2163 | 113.55 | 300.6514 | 122.12 | 304.7299 | 116.73 | 339.667 |
| 9797 | 119.40 | 281.849 | 98.81 | 217.1594 | 120.19 | 249.3683 | 118.01 | 258.326 |
| 9801 | 125.11 | 253.5981 | 115.78 | 255.1473 | 120.46 | 240.6781 | 117.60 | 280.7288 |
| 9802 | 112.79 | 290.874 | 146.80 | 340.9481 | 118.26 | 274.2467 | 119.05 | 274.1463 |
| 9812 | 127.56 | 286.1493 | 114.95 | 260.7644 | 121.35 | 270.3678 | 121.28 | 288.3396 |
| 9817 | 113.05 | 237.3885 | 108.30 | 227.0087 | 121.72 | 281.3749 | 118.49 | 299.1609 |
| 9821 | 100.68 | 243.3787 | 104.01 | 226.1912 | 115.02 | 297.7698 | 113.10 | 258.9109 |

## Appendix Table 11C. Bovine serum deoxypyridinoline (ng/ml).

| Day | 9796 | 9797 | 9798 | 9799 | 9800 | 9801 | 9802 | 9804 | 9805 | 9811 | 9812 | 9813 | 9817 | 9818 | 9819 | 9820 | 982 | 9822 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 wk | 33.171 | 42.001 | 28.613 | 27.308 | 37.93 | 25.982 | 29.216 | 22.185 | 13.437 | 24.751 | 19.558 | 16.802 | 24.843 | 7.575 | 11.602 | 16.31 | 14.64 | 14.871 |
| D0 | 29.879 | 20.33 | 16.398 | 25.758 | 28.654 | 27.668 | 36.172 | 30.539 | 22.511 | 18.189 | 12.882 | 27.113 | 32.988 | 17.186 | 18.52 | 19.953 | 12.03 | 13.534 |
| D1 | 26.363 | 27.039 | 9.008 | 39.072 | 17.043 | 22.66 | 32.242 | 19.152 | 25.928 | 13.694 | 20.979 | 12.033 | 14.163 | 13.345 | 19.234 | 15.847 | 17.41 | 24.747 |
| D2 | 28.858 | 49.867 | 26.353 | 14.214 | 31.663 | 26.086 | 26.425 | 26.046 | 28.926 | 21.056 | 17.766 | 21.276 | 12.116 | 11.062 | 16.278 | 12.478 | 28.42 | 8.512 |
| D3 | 15.048 | 31.673 | 14.423 | 32.376 | 18.628 | 22.723 | 26.463 | 24.285 | 33.039 | 25.064 | 29.594 | 16.423 | 6.09 | 15.016 | 15.627 | 17.007 | 21.26 | 12.232 |
| D4 | 28.366 | 28.358 | 23.612 | 24.159 | 26.056 | 26.186 | 40.938 | 23.621 | 19.983 | 11.965 | 12.166 | 23.543 | 15.066 | 12.678 | 13.697 | 20.311 | 16.37 | 16.77 |
| D5 | 31.86 | 15.795 | 22.829 | 33.777 | 25.698 | 23.635 | 21.379 | 23.728 | 40.993 | 22.463 | 22.712 | 19.431 | 22.245 | 21.81 | 17.996 | 16.095 | 16.9 | 15.347 |
| D6 | 25.119 | 12.904 | 26.296 | 22.07 | 27.041 | 28.329 | 21.03 | 28.304 | 12.751 | 27.656 | 27.171 | 18.813 | 14.143 | 15.894 | 12.166 | 7.408 | 11.73 | 11.33 |
| D7 | 18.297 | 22.894 | 24.564 | 22.088 | 15.2 | 24.154 | 17.615 | 22.458 | 18.198 | 13.563 | 13.706 | 12.662 | 15.608 | 14.63 | 15.096 | 15.068 | 14.89 | 16.819 |
| D14 | 20.469 | 27.938 | 23.834 | 25.727 | 15.939 | 13.625 | 24.042 | 21.071 | 13.41 | 11.989 | 10.608 | 11.843 | 8.761 | 12.937 | 11.196 | 17.298 | 13.45 | 16.849 |
| D21 | 19.697 | 25.51 | 16.336 | 23.372 | 14.397 | 10.95 | 13.563 | 29.642 | 26.089 | 9.018 | 17.962 | 19.061 | 11.773 | 73.022 | 67.112 | 10.951 | 17.16 | 6.754 |
| D28 | 27.287 | 17.126 | 25.262 | 14.43 | 20.077 | 28.05 | 19.592 | 29.904 | 19.758 | 19.063 | 15.66 | 11.996 | 22.406 | 20.297 | 14.816 | 13.264 | 20.11 | 15.496 |
| D35 | 28.387 | 19.329 | 13.122 | 18.036 | 17.8 | 13.04 | 13.672 | 31.664 | 12.952 | 12.357 | 14.741 | 14.741 | 11.982 | 11.264 | 21.591 | 26.566 | 29.2 | 12.769 |
| D42 | 12.533 | 26.274 | 16.942 | 14.769 | 23.834 | 19.449 | 10.646 | 16.193 | 15.938 | 13.786 | 26.686 | 26.686 | 11.808 | 9.104 | 19.258 | 11.995 | 16.23 | 23.606 |

Appendix Table 12C. Bovine serum osteocalcin (ng/ml).

| Day | 9796 | 9797 | 9798 | 9799 | 9800 | 9801 | 9802 | 9804 | 9805 | 9811 | 9812 | 9813 | 981 | 9818 | 9819 | 9820 | 9821 | 9822 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 wk | 88.23 | 198.93 | 213.495 | 186.597 | 344.52 | 259.125 | 135.017 | 147.461 | 75.973 | 179.226 | 136.13 | 208.84 | 328.53 | 191.587 | 200.535 | 245.406 | 68.296 | 206.856 |
| D0 | 104.211 | 79.125 | 70.464 | 124.334 | 284.113 | 169.943 | 151.312 | 73.896 | 101.914 | 192.99 | 206.354 | 222.049 | 220.74 | 115.775 | 156.953 | 264.634 | 165.516 | 196.342 |
| D1 | 105.658 | 105.664 | 106.01 | 92.92 | 245.099 | 93.912 | 101.265 | 74.469 | 83.527 | 236.672 | 211.825 | 209.005 | 203.26 | 88.359 | 157.536 | 147.282 | 103.318 | 251.772 |
| D2 | 123.398 | 122.788 | 113.658 | 113.38 | 264.038 | 131.524 | 114.54 | 107.215 | 56.833 | 234.675 | 164.882 | 171.6 | 219.40 | 63.079 | 202.92 | 181.037 | 137.779 | 173.524 |
| D3 | 138.513 | 134.426 | 110.757 | 123.356 | 205.139 | 130.167 | 98.313 | 95.674 | 45.974 | 200.739 | 143.628 | 234.032 | 211.94 | 71.591 | 198.317 | 316.731 | 125.463 | 151.848 |
| D4 | 120.152 | 69.063 | 116.708 | 119.223 | 128.707 | 174.111 | 84.459 | 96.554 | 82.349 | 173.985 | 168.505 | 151.293 | 212.70 | 74.471 | 197.84 | 170.479 | 135.451 | 156.294 |
| D5 | 162.031 | 171.509 | 149.625 | 84.667 | 175.037 | 169.17 | 111.276 | 169.277 | 84.807 | 169.509 | 177.489 | 174.391 | 179.66 | 61.669 | 271.255 | 154.726 | 45.637 | 108.424 |
| D6 | 106.401 | 147.829 | 121.165 | 94.22 | 201.957 | 109.647 | 112.866 | 117.298 | 115.894 | 180.302 | 173.441 | 176.539 | 244.78 | 44.59 | 188.957 | 161.247 | 108.422 | 82.855 |
| D7 | 107.605 | 112.93 | 70.532 | 88.165 | 176.296 | 100.668 | 107.002 |  | 75.003 | 178.544 | 148.727 | 170.624 | 227.87 | 118.535 | 140.502 | 206.787 | 181.443 | 181.319 |
| D14 | 76.256 | 134.026 | 129.686 | 95.551 | 133.789 | 75.683 | 127.742 | 83.843 | 102.716 | 90.484 | 88.249 | 120.613 | 168.68 | 78.712 | 219.644 | 296.939 | 162.871 | 126.064 |
| D21 | 80.383 | 148.687 | 177.315 | 69.435 | 143.727 | 108.494 | 112.812 | 136.656 | 99.142 | 111.748 | 101.289 | 156.945 | 137.70 | 129.425 | 273.677 | 169.661 | 123.087 | 113.789 |
| D28 | 91.859 | 119.389 | 67.459 | 100.986 | 100.055 | 82.771 | 98.446 | 130.8 | 99.323 | 211.97 | 93.049 | 101.797 | 245.87 | 103.805 | 137.257 | 158.183 | 151.959 | 126.456 |
| D35 | 110.778 | 89.701 | 152.519 | 94.95 | 117.554 | 116.143 | 100.156 | 100.771 | 96.293 | 156.349 | 166.956 | 180.37 | 199.51 | 103.08 | 150.681 | 157.907 | 132.378 | 142.232 |
| D42 | 91.146 | 113.461 |  | 98.477 | 110.511 |  | 86.665 | 133.67 | 103.247 | 127.501 | 75.574 | 102.205 | 164.15 | 128.5 | 119.517 | 191.154 | 152.313 | 158.599 |

# Appendix D

## Appendix Table 1D. Equine radiographic bone aluminum equivalence (mm Al).

| | dorsal | | | palmar | | | medial | | | lateral | | | total | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Horse | D0 | D28 | D60 | D0 | D28 | D60 | D0 | D28 | D60 | D0 | D28 | D60 | D0 | D28 | D60 |
| 1 | 15.65592 | 16.31542 | 15.5221 | 15.34544 | 16.34956 | 14.81816 | 16.08947 | 16.38122 | 17.9486 | 15.21161 | 16.11943 | 17.40257 | 184.5987 | 278.5518 | 168.6382 |
| 2 | 13.9968 | 13.73302 | 14.3383 | 14.62427 | 13.02492 | 12.94054 | 16.65585 | 16.34595 | 17.100 | 14.46029 | 14.71182 | 16.93229 | 72.01307 | 88.23832 | 123.7981 |
| 3 | 15.278 | 14.83191 | 15.643 | 15.34504 | 14.32592 | 15.27615 | 18.12511 | 16.15363 | 18.5787 | 16.33187 | 16.65709 | 16.13864 | 109.4946 | 98.45976 | 150.4744 |
| 4 | 17.00733 | 15.22792 | 15.79259 | 16.89144 | 15.76411 | 15.58926 | 18.28669 | 17.85003 | 19.1376 | 17.96155 | 18.27959 | 19.28053 | 237.9557 | 228.9911 | 305.5886 |
| 5 | 15.39064 | 15.82056 | 17.05077 | 14.77192 | 14.99061 | 16.03397 | 17.79687 | 22.02443 | 22.0870 | 16.733 | 21.0588 | 20.54836 | 202.3233 | 266.5121 | 533.9042 |
| 6 | 15.96248 | 14.45723 | 15.86873 | 15.48069 | 13.61031 | 14.83881 | 17.60574 | 18.70677 | 19.5308 | 14.60104 | 16.71762 | 18.38352 | 106.5663 | 191.9796 | 258.0511 |
| 7 | 14.18321 | 15.31052 | 16.32563 | 13.12786 | 14.07491 | 14.73651 | 16.68437 | 18.25406 | 19.1520 | 15.73327 | 16.76766 | 17.97228 | 161.4662 | 165.6727 | 259.227 |
| 8 | 14.83369 | 14.59104 | 16.00379 | 15.22708 | 14.34741 | 14.77929 | 16.57692 | 17.3566 | 17.963 | 15.46602 | 16.70898 | 17.9634 | 80.52043 | 172.1078 | 215.9546 |
| 9 | 16.15776 | 15.79416 | 15.87576 | 15.81976 | 15.68905 | 14.89005 | 16.2382 | 17.47681 | 18.3315 | 16.24687 | 17.58746 | 17.87038 | 165.4233 | 151.1443 | 183.5107 |
| 10 | 15.34502 | 15.60021 | 16.05448 | 14.98663 | 14.45731 | 14.55914 | 16.61371 | 18.30158 | 19.6612 | 16.3122 | 17.67431 | 17.87962 | 208.0421 | 201.7372 | 268.6296 |
| 11 | 15.65076 | 15.84342 | 15.84425 | 15.48355 | 14.87806 | 15.34896 | 17.62009 | 17.34649 | 21.3996 | 16.1325 | 17.32997 | 20.18519 | 105.4094 | 152.2714 | 262.49 |
| 12 | 15.48799 | 16.22243 | 15.37394 | 14.79874 | 15.49 | 14.3448 | 17.37135 | 18.49931 | 18.8459 | 16.72544 | 17.34196 | 17.5472 | 236.7113 | 172.8366 | 364.1803 |
| 13 | 15.44755 | 15.79692 | 15.85962 | 14.68779 | 14.9165 | 14.04309 | 18.61669 | 17.92345 | 20.3008 | 17.84255 | 17.36544 | 20.91942 | 212.3281 | 128.2229 | 112.6913 |
| 14 | 15.94065 | 16.13028 | 14.4817 | 14.67268 | 15.10419 | 16.21709 | 19.2528 | 20.58011 | 20.7366 | 17.14325 | 17.79819 | 18.03329 | 239.4618 | 196.0886 | 241.2841 |
| 15 | 13.78617 | 14.8558 | 14.97034 | 12.99093 | 14.05634 | 12.89153 | 16.47393 | 18.03472 | 17.0499 | 15.15956 | 14.81214 | 15.71811 | 107.3788 | 94.14673 | 120.4885 |
| 16 | 15.69847 | 15.6887 | 16.53524 | 15.71591 | 14.89793 | 15.12445 | 15.97016 | 16.98758 | 17.4945 | 15.83971 | 17.13506 | 17.97397 | 78.32051 | 119.3165 | 173.5065 |
| 17 | 15.64908 | 15.247 | 16.5597 | 14.19323 | 13.89868 | 14.76613 | 18.94018 | 19.04223 | 17.5085 | 18.43079 | 17.2993 | 17.94141 | 269.0003 | 155.0833 | 171.1196 |
| 18 | 14.71045 | 15.09436 | 14.60332 | 15.24131 | 13.65267 | 13.54003 | 17.09359 | 16.84859 | 17.4025 | 15.75972 | 15.40598 | 16.73765 | 129.1729 | 101.0265 | 92.07383 |

## Appendix Table 2D. Equine cortical widths for d 0 and 28.

| | D 0 | | | | | | | | D 28 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| horse | dp outer | dp inner | dorsal | palmar | ml outer | ml inner | medial | lateral | dp outer | dp inner | dorsal | palmar | ml outer | ml inner | medial | lateral |
| 1 | 27 | 13.8 | 7.9 | 4.7 | 39.6 | 24.4 | 7.8 | 7.3 | 29.2 | 16.4 | 8.3 | 5 | 39.5 | 23.1 | 7.9 | 9.2 |
| 2 | 24.4 | 13.2 | 8.1 | 4.4 | 29.3 | 19.4 | 5.8 | 5.1 | 24.1 | 13.7 | 7.2 | 4.1 | 30.6 | 18.9 | 6.9 | 6.3 |
| 3 | 27.1 | 14.1 | 8 | 5.4 | 32.7 | 20.3 | 6.3 | 5.9 | 27.2 | 13.8 | 8.4 | 5.4 | 34.5 | 20.3 | 7.4 | 8.1 |
| 4 | 29.4 | 15.1 | 8.8 | 6.1 | 34.9 | 20.8 | 8.3 | 6.6 | 28 | 14.4 | 8.7 | 5 | 38 | 20.6 | 8 | 9.8 |
| 13 | 25.9 | 13.2 | 8 | 5.1 | 36.3 | 21.1 | 7.7 | 7 | 26.4 | 13.9 | 8 | 4.8 | 36.4 | 22.5 | 7.7 | 6.3 |
| 14 | 27.5 | 12.8 | 10.2 | 5 | 35.5 | 19.7 | 8.9 | 6.8 | 29 | 14.3 | 9.4 | 6 | 36.4 | 19.7 | 10.3 | 6.7 |
| 5 | 29.3 | 13.4 | 10.2 | 5.5 | 33.5 | 17.7 | 9.5 | 7.2 | 28.2 | 12.9 | 9.1 | 6.6 | 34.7 | 20.3 | 9.6 | 8.6 |
| 6 | 25.9 | 14.5 | 9.4 | 3.6 | 36.2 | 22 | 6.4 | 6.1 | 27.2 | 13.7 | 8.9 | 5.1 | 34.7 | 19.8 | 8.1 | 7.1 |
| 7 | 25.1 | 13.3 | 7.4 | 4.6 | 32 | 19.2 | 6.8 | 7 | 27.3 | 13.8 | 9.5 | 5.3 | 31.7 | 18.1 | 7.4 | 6.9 |
| 8 | 28.6 | 17.1 | 6.2 | 6 | 35.7 | 22.5 | 5.9 | 7.3 | 25 | 15.4 | 6.3 | 4 | 37.9 | 24.4 | 7.3 | 7.4 |
| 15 | 25.1 | 15 | 6.6 | 3.5 | 33.2 | 27.5 | 7 | 5.2 | 25.4 | 13.5 | 7.3 | 4.3 | 35.8 | 22.6 | 6.8 | 6.2 |
| 16 | 27.8 | 16.7 | 6.5 | 4.9 | 39.3 | 25.2 | 6.3 | 8.1 | 26.5 | 16.1 | 7.2 | 4 | 40.3 | 27.8 | 9.3 | 6.1 |
| 9 | 28.9 | 16.8 | 7.8 | 6.3 | 35.9 | 22.8 | 5.6 | 8.3 | 26.8 | 14.7 | 8.6 | 4.1 | 36.8 | 23.2 | 7.2 | 7 |
| 10 | 26.1 | 12 | 9.4 | 4.1 | 33.8 | 19.9 | 7.5 | 6.9 | 26.8 | 12.6 | 10.3 | 4.7 | 34.3 | 20.6 | 7.1 | 6.6 |
| 11 | 25.7 | 12.9 | 9.2 | 4.1 | 32 | 19.6 | 5.6 | 7.1 | 24.6 | 12.4 | 9.1 | 4.1 | 33.4 | 19.6 | 7.2 | 8.1 |
| 12 | 25.4 | 12.2 | 8.6 | 5.3 | 34 | 18.3 | 8.8 | 7.8 | 25.6 | 12.3 | 8.3 | 5 | 34.3 | 17.8 | 9 | 8 |
| 17 | 26.6 | 12.5 | 9.5 | 5 | 35 | 20.6 | 8 | 7.3 | 25.9 | 12.6 | 9 | 4.7 | 33.1 | 18.2 | 7.6 | 7 |
| 18 | 28.4 | 18.2 | 6.2 | 3.7 | 40.6 | 29.3 | 6.1 | 5.9 | 28.6 | 14.9 | 6.2 | 7.7 | 41.1 | 28.7 | 6.1 | 6.1 |

## Appendix Table 3D. Equine cortical widths of MC III for d 56 (mm)

| | | | | | D 60 | | | |
|---|---|---|---|---|---|---|---|---|
| horse | dp outer | dp inner | dorsal | palmar | ml outer | ml inner | medial | lateral |
| 1 | 27.2 | 14.9 | 7.3 | 4.1 | 39.7 | 22.7 | 7.9 | 8.5 |
| 2 | 24.5 | 12.4 | 7.6 | 4.9 | 32.9 | 19.1 | 7.4 | 5.9 |
| 3 | 25.1 | 13.8 | 6.2 | 4.7 | 35.6 | 19.4 | 7.7 | 7.9 |
| 4 | 29 | 14.7 | 9.3 | 4.7 | 37.9 | 20.8 | 8 | 9.1 |
| 13 | 28.4 | 14.3 | 8.4 | 5.4 | 36.5 | 21.1 | 7.3 | 7.6 |
| 14 | 29.2 | 13.3 | 10.5 | 5.9 | 34.9 | 19.1 | 8.9 | 6.9 |
| 5 | 34.3 | 13.4 | 14 | 7.5 | 39.9 | 19 | 12.2 | 8.1 |
| 6 | 28.4 | 13.3 | 9.7 | 5.2 | 36.5 | 20.5 | 7.9 | 8.5 |
| 7 | 28.4 | 12.2 | 9.2 | 6.1 | 35.7 | 19.5 | 7.8 | 8.5 |
| 8 | 28.2 | 12.6 | 10.2 | 5.8 | 43.5 | 23 | 10.1 | 6.5 |
| 15 | 26.4 | 14.3 | 7.7 | 4.3 | 35.5 | 21.5 | 8.1 | 5.7 |
| 16 | 30.1 | 15 | 7.7 | 6.4 | 38.9 | 23.2 | 8.4 | 6.9 |
| 9 | 31 | 15.5 | 8.7 | 7.1 | 39.1 | 22.8 | 6.8 | 10 |
| 10 | 29.7 | 14 | 11.1 | 5.5 | 35.9 | 21.5 | 7.8 | 7.1 |
| 11 | 26.2 | 12.9 | 7.5 | 5.5 | 38.9 | 20.1 | 8.3 | 8.3 |
| 12 | 28.6 | 13.2 | 9.2 | 6 | 37.4 | 18.4 | 10.3 | 9 |
| 17 | 27.3 | 12.3 | 9.2 | 5.2 | 36.6 | 20 | 8.5 | 7.1 |
| 18 | 28.5 | 17.4 | 6.2 | 5.3 | 40.4 | 25.9 | 5.6 | 6.3 |

## Appendix Table 4D. Equine osteocalcin (ng/ml).

| | Horse | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| 0 | 52.1 | 31.47 | 25.599 | 30.537 | 29.171 | 42.797 | 50.84 | 46.834 | 65.723 | 36.835 | 67.397 | 69.391 | 76.631 | 74.672 | 80.37 | 72.668 | 45.647 | 76.543 |
| 1 | 48.984 | 58.63 | 38.381 | 35.975 | 27.022 | 46.566 | 43.118 | 57.5 | 83.442 | 31.708 | 78.593 | 53.177 | 63.291 | 49.079 | 44.582 | 76.023 | 68.308 | 114.411 |
| 2 | 36.593 | 75.745 | 41.08 | 30.035 | 65.545 | 44.269 | 33.555 | 43.721 | 50.927 | 28.937 | 56.322 | 39.875 | 74.083 | 65.817 | 112.259 | 62.825 | 44.532 | 174.923 |
| 3 | 40.99 | 69.84 | 40.998 | 38.376 | 35.888 | 27.367 | 42.8 | 58.084 | 42.666 | 71.842 | 57.222 | 45.76 | 62.693 | 64.125 | 44.161 | 91.438 | 55.471 | 130.284 |
| 4 | 40.691 | 78.338 | 34.148 | 37.065 | 47.79 | 38.067 | 72.805 | 44.299 | 64.712 | 46.4 | 91.697 | 44.076 | 129.799 | 51.769 | 54.18 | 73.733 | 54.535 | 114.771 |
| 5 | 62.923 | 77.676 | 49.37 | 43.379 | 44.136 | 46.872 | 58.249 | 39.932 | 31.381 | 30.548 | 56.094 | 45.654 | 45.443 | 33.465 | 56.004 | 74.82 | 65.681 | 97.935 |
| 6 | 55.275 | 81.853 | 39.998 | 35.141 | 41.157 | 35.35 | 57.403 | 41.963 | 53.236 | 34.174 | 45.252 | 70.879 | 69.597 | 43.367 | 51.861 | 72.923 | 62.155 | 93.675 |
| 7 | 152.03 | 96.06 | 75.516 | 70.257 | 56.718 | 57.966 | 135.7 | 82.822 | 83.337 | 60.359 | 91.256 | 105.047 | 61.706 | 49.472 | 64.667 | 83.575 | 95.613 | 93.044 |
| 14 | 124.667 | 62.896 | 91.004 | 47.511 | 95.67 | 56.096 | 62.116 | 69.363 | 65.578 | 60.595 | 84.705 | 71.528 | 99.236 | 40.604 | 85.612 | 70.592 | 107.022 | 85.211 |
| 21 | 75.517 | 81.057 | 62.993 | 96.357 | 86.606 | 54.292 | 61.208 | 85.249 | 77.763 | 60.766 | 89.944 | 106.206 | 49.053 | 36.712 | 65.695 | 77.1 | 62.09 | 87.604 |
| 28 | 64.083 | 91.781 | 111.086 | 56.051 | 72.846 | 42.241 | 86.056 | 78.981 | 45.882 | 55.51 | 56.325 | 76.595 | 31.673 | 34.767 | 52.239 | 75.464 | 71.732 | 70.143 |
| 35 | 74.921 | 60.841 | 99.745 | 83.822 | 79.887 | 92.046 | 74.418 | 72.454 | 58.065 | 57.687 | 64.812 | 61.379 | 40.639 | 38.738 | 65.809 | 120.371 | 74.066 | 81.183 |
| 42 | 27.259 | 31.84 | 77.69 | 63.578 | 77.28 | 43.761 | 153.14 | 52.294 | 107.949 | 45.964 | 84.371 | 98.013 | 40.082 | 31.521 | 54.184 | 64.32 | 39.429 | 87.705 |
| 49 | 64.354 | 48.394 | 41.315 | 39.244 | 62.842 | 23.209 | 27.809 | 57.76 | 56.144 | 22.555 | 120.567 | 60.211 | 42.437 | 33.611 | 56.77 | 72.15 | 53.331 | 74.877 |
| 56 | 42.095 | 97.813 | 19.38 | 55.006 | 72.63 | 60.365 | 42.303 | 93.581 | 98.615 | 60.236 | 79.073 | 62.708 | 47.611 | 36.325 | 58.94 | 79.614 | 65.101 | 81.192 |

## Appendix Table 5D. Equine total deoxypyridinoline (ng/ml).

| | Horse | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Day | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| 0 | 29.5 | 17.9 | 30.7 | 18 | 30.8 | 22.3 | 19.5 | 21.6 | 9.7 | 21.9 | 16.8 | 20.7 | 16.9 | 13.1 | 18.8 | 22.8 | 19.2 | 18.7 |
| 1 | 20.9 | 10.4 | 22.2 | 34.1 | 39.3 | 15.8 | 16.1 | 14.6 | 40.2 | 18.2 | 22.8 | 28.9 | 18 | 30 | 26 | 24.9 | 15.3 | 15.3 |
| 2 | 20.1 | 21.8 | 21.2 | 21.5 | 26.5 | 40.3 | 31.3 | 6.7 | 16.2 | 6.6 | 5.7 | 22.3 | 24.3 | 22.3 | 23.8 | 21.7 | 26.1 | 10.5 |
| 3 | 12.9 | 15.6 | 18.2 | 14.9 | 15.2 | 35.4 | 24.4 | 15.7 | 19 | 14.9 | 15.3 | 15.8 | 13.9 | 11.9 | 15.1 | 15.6 | 38.8 | 27.4 |
| 4 | 15.9 | 24.9 | 16.5 | 16.8 | 19.5 | 21.5 | 18.4 | 26.8 | 19.8 | 19.1 | 25.9 | 25 | 21.4 | 19 | 21.6 | 33.9 | 10.8 | 27.6 |
| 5 | 16.6 | 31.3 | 25.7 | 14.1 | 34.7 | 17.9 | 17.3 | 18 | 18.8 | 19.4 | 27.3 | 19.9 | 19.6 | 16.5 | 15.7 | 21.2 | 15.1 | 13.5 |
| 6 | 19.5 | 13.8 | | 8.3 | 11.9 | 16.8 | 14.8 | 13 | 18.7 | 12.9 | 14.1 | 13.8 | 9.2 | 9.2 | 11.7 | 27.2 | 27.7 | 12.8 |
| 7 | 19.5 | 18.1 | 32.3 | 15.3 | 16 | 23.4 | 16 | 20.7 | 49.3 | 14.8 | | 23.1 | 12.4 | 12.6 | 14.4 | 22.4 | 11.2 | 14.8 |
| 14 | 17.7 | 20.5 | 20.8 | 17.6 | 15.7 | 16.4 | 17.9 | 20.6 | 22.5 | 22 | 22.3 | 27.1 | 21.1 | 19.7 | 21.3 | 47 | | 14.2 |
| 21 | 15.7 | 10.1 | | 19.8 | | | 11.9 | 10.2 | 32.4 | 16.8 | 24.2 | | 12.9 | 22.3 | 17.8 | 26.5 | 18.8 | 24.2 |
| 28 | 13.8 | 16.1 | 11.6 | 14.7 | 18 | 17.1 | 17.7 | 18.5 | 18.2 | 16.9 | 17.6 | 16.1 | 11.9 | 11.6 | 18.8 | 29.7 | 17 | 34.9 |
| 35 | 14.5 | 36.6 | 13.2 | 27.9 | 28 | 20.1 | 20.9 | 17.8 | 24.6 | 23.8 | 17.8 | 17.6 | 13.7 | 13.3 | 25 | 21.2 | 19.4 | 26.7 |
| 42 | 17.2 | 9.6 | 3.2 | 4.6 | 13.6 | 15.5 | 21.1 | 24.1 | 18.4 | 15.9 | 15.7 | 24.4 | 13.4 | 13 | 16.2 | 24.4 | 18.6 | 21 |
| 49 | 15.9 | 30 | 18.2 | 22.6 | 18.1 | 18.6 | 25.5 | 22.5 | 39.2 | 31.1 | 9.4 | 12 | 11.1 | 13.8 | 32.3 | 9.8 | 20.7 | 27.4 |
| 56 | 12.9 | 9.88 | 7.4 | 14.3 | 9.6 | 13 | 27.8 | 19.4 | 44.8 | 25.7 | | 26.5 | 13.5 | 10.8 | 11.1 | 27.3 | 18.4 | 14.9 |

# LITERATURE CITED

Adami, S., P. Gatti, V. Braga, D. Bianchini, and M. Rossini. 1999. Site-specific effects of strength training on bone structure and geometry of ultra-distal radius in postmenopausal women. J. Bone Min.Res. 14(1):120-124.

Anderson, S.A., and S.H. Cohn. 1985. LSRO Report: Bone demineralization during space flight. Physiologist. 28:212-217.

ASAE. 2000. Shear and three-point bending test of animal bone. ASAE STANDARDS. ANSI/ASAE Standard S459. Amer. Soc. Agri. Eng. St. Joseph, MI.

Ashizawa, N., K. Nonaka, S. Michikami, T. Mizuki, H. Amagai, K.Tokuyama, and M. Suzuki. 1999. Tomographical description of tennis-loaded radius: Reciprocal relation between bone size and volumetric BMD. J. Appl. Phys. 86(4):1347-1351.

Azria, M. 1989. The value of biomarkers in detecting alterations in bone metabolism. Calc.Tissue Inter. 45:7-11.

Bailey, C.J., S.W.J. Reid, D.R. Hodgson, and R.J. Rose. 1999. Factors associated with time until first race and career duration for Thoroughbred racehorses. Amer. J. Vet. Res. 60(10):1196-1200.

Barengolts, E.I., D.J. Curry, M.S. Bapna, and S.C. Kukreja. 1993. Effects of endurance exercise on bone mass and mechanical properties in intact and ovariectomized rats. J. Bone Min. Res. 8(8):937-942.

Barnevald. A., and P.R. van Weeren. 1999. Conclusions regarding the influence of exercise on the development of the equine musculoskeletal system with special reference to osteochondrosis. Equine Vet. Supp. 31:112-119.

Bass, S., G. Pearce, M. Bradney, E. Hendrich, P.D. Delmas, A. Harding, and E. Seeman. 1998. Exercise before puberty may confer residual benefits in bone density in adulthood: Studies in active prepubertal and retired female gymnasts. Journal of Bone and Mineral Research. 13(3):500-507.

Bass, S.L. 2000. The prepubertal years: A uniquely opportune stage of growth when the skeleton if most responsive to exercise? Sports Med. 30(2):73-78.

Bauer, K.D., and P. Griminger. 1983. Long term effects of activity and calcium and phosphorous intake on bones and kidneys of female rats. J. Nutr. 113(10):2011-2021.

Becker, K.L., B. Muller, E.S. Nylen, R. Cohen, J.C. White, and R.H. Snider, Jr. 2002. Calcitonin gene family of peptides: Structure, molecular biology, and effects. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 619-639.

Bell, R.A., B.D. Nielsen, K.Waite, D. Rosenstein, and M. Orth. 2001. Daily access to pasture turnout prevents loss of mineral in the third metacarpal of Arabian weanlings. J. Anim. Sci. 79:1142-1150.

Biewener, A.A., and J.E.A. Bertram. 1993. Skeletal strain patterns in relation to exercise training during growth. J. Exper. Bio. 185:51-69.

Biewener, A.A., and J.E.A. Bertram. 1994. Structural response of growing bone to exercise and disuse. J. Appl. Phys. 76(2):946-955.

Bilke, D., J. Harris, B. Halloran, and E. Morey-Holton. 1994. Skeleton unloading induces resistance to insulin-like growth factor. J. Bone Min. Res. 11:1789-1796.

Black, D., C. Farquharson, and S.P. Robins. 1989. Excretion of pyridinium cross-links of collagen in ovariectomized rats as urinary markers for increased bone resorption. Calc. Tissue Inter. 44:343-347.

Black, A., S.L. Ralston, S.A. Shapses, L. Suslak-Brown, and P.A. Schoknecht. 1997a. Skeletal growth patterns in Standardbred foals from birth to 1 year. Proc. 15th Equine Nutr. Phys. Symp. Ft. Worth, TX. p 326-327.

Black, A., P.A. Schoknecht, S.L. Ralston, and S.A. Shapses. 1999. Diurnal variation and age differences in the biochemical markers of bone turnover in horses. J. Anim. Sci. 77:75-83.

Blake, G.M, H.W. Wahner, and I. Fogelman. 1999. The Evaluation of Osteoporosis: Dual Energy X-ray Absorptiometry and Ultrasound in Clinical Practice. 2nd edition. Martin Dunitz. London

Buckingham, S.H.M., and L.B. Jeffcott. 1987. Changes in bone strength and density in Standardbreds from weanling to onset of training. Equine Exercise Physiology 2: Proc. 2nd International Conference on Equine Exercise Physiology, San Diego, CA, August 7-11, 1986. ICEEP Publications. 631-643.

Buckingham, S.H.W., and L.B. Jeffcott. 1991a. Osteopenic effects of forelimb immobilization in horses. Vet. Rec. 128:370-373.

Buckingham, S.H. W., and L.B. Jeffcott. 1991b. Skeletal effects of a long-term submaximal exercise programme on Standardbred yearlings. Equine Exercise Physiology. 3: 411-418.

Buckingham, S.H.W., R.N. McCarthy, G.A. Anderson, R.N. McCartney, and L.B. Jeffcott. 1992. Ultrasound speed in the metacarpal cortex- a survey of 347 Thoroughbreds in training. Equine Vet. J. 24(3):191-195.

Burr, D.B., R.B. Maring, M.B. Schaffler, and E.L. Radin. 1985. Bone remodeling in response to *in vivo* fatigue microdamage. J. Biomech.18(3):189-200.

Burr, D.B., A.G. Robling, and C.H. Turner. 2002. Effects of biomechanical stress on bones in animals. Bone. 30(5): 781-786.

Calvo, M.S., D.R. Eyre, and C.M. Gundberg. 1996. Biological markers of bone turnover. Endo. Rev. 17(4):335-368.

Carstanjen, B., H. Amory, B.Wattiez, O.M. Lepage, and B. Remy. 2002. Isolation and characterization of equine osteocalcin. Annales de Medecine Veterinaire. 146(1):31-38.

Carter, D.R., W.E. Caler, D.M. Spengler, and V.H. Frankel. 1981. Fatigue behavior of adult cortical bone: The influence of mean strain and strain range. Acta Orthop. Scand. J. Orth. Res. 52:481-490.

Chow, J.W.M., and T.J. Chambers. 1994. Indomethacin has distinct early and late actions on bone formation induced by mechanical stimulation. Am. J. Physiol. 267:E287-E292.

Chow, J.W.M., A.J. Wilson, T.J. Chambers, and S.W. Fox. 1997. Mechanical loading stimulates bone formation by activation of bone lining cells. J. Bone Min. Res. 12(suppl. 1):S111.

Cohen, N.D, S.M. Berry, J.G. Peloso, G.D. Mundy, and I.C. Howard. 2000. Association of high-speed exercise with racing injury in Thoroughbreds. J. Amer. Vet. Med. Assoc. 216(8):1273-1278.

Cohn, S.H.(ed) 1981. Non-invasive Measurements of Bone Mass and Their Clinical Application. CRC Press. Boca Raton, FL.

Cullinane, D.M., and T.A. Einhorn. 2002. Biomechanics of bone. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 17-32.

Curry, T.S., J.E. Dowdey, and R.C. Murrey, Jr (Eds). 1990. Christensen's introductin to the physics of diangnostic radiology. 4th ed.. Lea and Febiger. Philadelphia

Damien, E., J.S. Price, and L.E. Lanyon. 2000. Mechanical strain stimulates osteoblast proliferation through the estrogen receptor in males as well as females. J. Bone Min. Res. 15(11):2169-2177.

Davies, H., and R. McCarthy. 1994. Surface strain on the dorsomedial metacarpus of yearling Thoroughbreds at different speeds and gaits on a treadmill. Equine Vet. 17:25-28.

Dellmeier, G.R. 1989. Motivation in relation to the welfare of enclosed livestock. Appl. Anim.Behav. Sci. 22:129-138.

Delmas, P.D. 1990. Biochemical markers of bone turnover. Endo. Metab. Clinic. North Amer. 19:1-18.

Delmas, P.D. 1993. Biochemical markers of bone turnover. J. Bone Min. Res. 8(suppl. 2):S549-S555.

Donaldson, C.L., S.E. Hulley, J.M. Vogel, R.S. Hattner, J.H. Bayers, and D.E. McMillan. 1970. Effect of prolonged bedrest on bone mineral. Metab. 19:1071-1084

Egger, C.D., R.C. Mühlbauer, R. Felix, P.D. Delmas, S. C. Marks, and H. Fleisch. 1994. Evaluation of urinary pyridinium crosslink excretion as a marker of bone resorption in the rat. J. Bone Min. Res. 9(8):1211-1219.

Einhorn, T.A. 1992. Bone strength: The bottom line. Calc. Tissue Inter. 51:333-339.

El Shorafa, W.M., J.P. Feaster, and E.A. Ott. 1979. Horse metacarpal bone: Age, ash content, cortical area and failure stress interrelationships. J. Anim. Sci. 49(4):979-982.

Estberg, L., S.M. Stover, I.A. Gardner, C.M. Drake, B. Johnson, and A. Ardans. 1996. High-speed exercise history and catastrophic racing fracture in Thoroughbreds. Amer. J. Vet. Res. 57(11):1549-1555.

Evans, G.P., J.C. Behiri, L.C. Vaughan, and W. Bonfield. 1992. The response of equine cortical bone to loading at strain rates experienced in vivo by the galloping horse. Equine Vet. J. 24(2):125-128.

Everts, V., J.M. Delaisse, W. Korper, D.C. Jansen, W. Tigchelaar-Gutter, P. Saftig, and W. Beertsen. 2002. The bone lining cell: Its role in cleaning Howship's lacunae and initiating bone formation. J. Bone Min. Res. 17(1):77-90.

Eyre, D.R., T.J. Koob, and K.P. Van Ness. 1984. Quantitation of hydroxypyridinium cross-links in collagen by high-performance liquid chromatography. Analy. Biochem.. 137:380-388.

Eyre, D.R., I.R. Dickson, and K.P. Van Ness. 1988. Collagen cross-linking in human bone and cartilage: Age-related changes in the content of mature hydroxypyridinium residues. Biochem. J.. 252:495-500.

Eyre, D.R. 1995. The specificity of collagen cross-links as markers of bone and connective tissue degradation. Acta Orthopedic Scandinavia. 66(Suppl 266):166-170.

Fenton, J.I., M.W. Orth, K.A. Chlebek-Brown, B.D. Nielsen, C.D. Corn, K.S. Waite, and J.P. Caron. 1999. Effect of longeing and glucosamine supplementation on serum markers of bone and joint metabolism in yearling Quarter Horses. Can. J. Vet. Res. 63(4):288-291.

Field, R.A., R.A. Gebault, W.J. Means, D.L. Hixon, and W.C. Russel. 1999. Characteristics of metacarpal bones from cows of different ages and weights. 15:169-172.

Firth, E.C., P.R. van Weeren, D.U. Pfeiffer, J. Delahunt, and A. Barnevald. 1999. Effect of age, exercise and growth rate on bone mineral density (BMD) in third carpal bone and distal radius of Dutch Warmblood foals with osteochondrosis. Equine Vet. J. Suppl. 31:74-78.

Forwood, M.R., and C.H. Turner. 1994. The response of rat tibiae to incremental bouts of mechanical loading: A quantum concept for bone formation. Bone. 15(6):603-609.

Frost, H.M. 1990. Skeletal adaptations to mechanical usage (SATMU): 2. Redefining Wolff's Law: The remodeling problem. Anat. Rec. 226:414-422.

Fuchs, R.K., J.J. Bauer, and C.M. Snow. 2001. Jumping improves hip and lumbar spine bone mass in prepubescent children: A randomized controlled trial. J. Bone Min. Res. 16(1):148-156.

Fujimara, R., N. Ashizawa, M. Watanabe, N. Mukai, H. Amagal, T. Fukubayashi, K. Hayashi, K. Tokuyama, and M. Suzuki. 1997. Effect of resistance training on bone formation and resorption in young male subjects assessed by biomarkers of bone metabolism. J. Bone Min. Res. 12:656-662.

Garnero, P., E. Hausherr, M.C. Chapuy, C. Marcelli, H. Grandjean, C. Muller, C. Cormier, G. Breart, P.J. Meunier, and P.D. Delmas. 1996. Markers of bone resorption predict hip fracture in elderly women: The EPIDOS prospective study. J. Bone Min. Res. 11(10):1531-1538.

Garnero, P., and P.D. Delmas. 1996. Measurements of biochemical markers: Methods and limitations. In: J.P. Bilezikian, L.G. Raisz, and G.A. Rodan (Ed.) Principles of Bone Biology. pp 1277-1291. Academic Press, San Diego, CA.

Glade, M.J. 1991. Dietary yeast culture supplementation of mares during late gestation and early lactation. Effects on dietary nutrient digestibilities and fecal nitrogen partitioning. J. Equine Vet. Sci. 11:10-16.

Glade, M.J. 1993. Effects of gestation, lactation, and maternal calcium intake on mechanical strength of equine bone. J. Amer. Coll. Nutr. 12(4):372-377.

Goodship, A.E., L.E. Lanyon, and H. McFie. 1979. Functional adaptation of bone to increased stress. J. Bone Joint Surg. Amer. 61:539-546.

Goyal, H.O., F.J. MacCallum, M.P. Brown, and J.B. Delack. 1981.Growth rates at the extremities of limb bones in young horses. Can. Vet. J. 22(2):1-33.

Grafenau, P., B. Carstanjen, and O.M. Lepage. 2000. Osteocalcin: A biochemical marker of bone formation in equine medicine. Vet. Med. Czechoslovakia. 45(7):209-216.

Gross, T.S., K.J. McLeod, and C.T. Rubin. 1992. Characterizing bone strain distributions in vivo using three triple rosette strain gages. J. Biomech. 25(9):1081-1087.

Gross, T.S., S. Srinivasan, C.C. Liu, T.L. Clemens, and S. D. Bain. 2002. Noninvasive loading of the murine tibia: An in vivo model for the study of mechanotransduction. J. Bone Min. Res. 17(3):493-501.

Gundberg, C., and R.S. Weinstein. 1986. Multiple immunoreactive forms in uremic serum. J. Clin. Invest.77:1762-1767.

Gunness-Hey, M. and J.M. Hock, 1984. Increased trabecular bone mass in rats treated with human synthetic parathyroid hormone. Metabolic Disease Related Res. 5:177-181.

Hanson, P.D., and M.D. Markel. 1994. Radiographic geometric variation of equine long bones. Amer. J. Vet. Res. 55(9):1220-1227.

Hanson, P.D., M.D. Markel, and R. Vanderby. 1995. Diaphyseal structural properties of equine long bones. Amer. J. Vet. Res. 56(2):233-240.

Heaney, R.P., and T.G. Skillman. 1971. Calcium metabolism in normal human pregnancy. J. Clin. Endo. Metab. 33:661-670.

Heinonen, A., P. Oja, P. Kannus, H. Sievänen, A. Mänttäri and I. Vuori. 1993. Bone mineral density of female athletes in different sports. Bone Min. 23:1-14.

Hiney, K.M. 1998.The effects of forced exercise prior to race training on two-year-old racehorses. MS Thesis. Texas A&M University.

Hiney, K.M., G.D. Potter, P.G. Gibbs, and S.M. Bloomfield. 2000. Response of serum biochemical markers of bone metabolism to training in the juvenile racehorse. J. Equine Vet. Sci. 20(12):851-857.

Hintz, H.F., R.L. Hintz, and L.D. Van Vleck. 1979. Growth rate of Thoroughbreds. Effect of age of dam, year and month of birth, and sex of foal. J. Anim. Sci. 48(3):480-487.

Hodgson, D.R., and R.J.Rose (eds). 1994. The Athletic Horse. W.B. Sanders Co. Philadelphia, PA.

Hoekstra, K.E, B.D. Nielsen, M.W. Orth, D.S. Rosenstein, H.C. Schott and J.E. Shelle. 1999. Comparison of bone mineral content and bone metabolism in stall- versus pasture-reared horses. Equine Vet. J. Suppl. 30:601-604.

Hoffman, R.M., J.A. Wilson, L.A. Lawrence, D.S. Kronfeld, W.L. Cooper, and P.A. Harris. 2001. Supplemental calcium does not influence radiographic bone mineral content of growing foals fed pasture and a fat-and-fiber supplement. Proc. 17th Equine Nutrition Physiology Sympsium. Lexington, KY.pp122-123.

Hope, E., S.D. Johnston, R.L., Hagstad, R.J. Geor, and M.G. Murphy. 1993. Effects of sample collection and handling on concentrations of osteocalcin in equine serum. Amer. J. Vet. Res. 54(7):1017-1020.

Hou, J.C., G. Salem, R.F. Zernicke, and R.J. Barnard. 1990. Stuctural and mechanical adaptations of immature trabecular bone to strenuous exercise. J. Appl.Phys. 69(4):1309-1314.

Hoyt, S., and P.D. Siciliano. 1999. A comparison of ELISA and RIA techniques for the detection of serum osteocalcin in horses. Proc 16th Equine Nutrition Physiology Sympsium. pp 351-352.

Hung, C.T., S.R. Pollack, T.M. Reilly, and C.T. Brighton. 1995. Realtime calcium response of cultured bone cells to fluid flow. Clin. Orth. Related Res. 313:256-269.

Hyldstrup, L., C. Pyke, and I. Clemmensen. 1989. Determination of osteocalcin in serum by enzyme-linked immunoabsorbant assay (ELISA). J. Bone Min.Res. 4(Suppl. 1):980.

Inman, C. L., G.L. Warren, H.A. Hogan, and S.A. Bloomfield. 1999. Mechanical loading attenuates bone loss due to immobilization and calcium deficiency. J. Appl. Phys. 87(1):189-195.

Iwamota, J., J.K. Yeh and J.F. Aloia. 2000. Effect of deconditioning on cortical and cancellous bone growth in the exercise trained young rats. J. Bone Min Res. 15(9):1842-1848.

Jacobs, C.R., C.E. Yellowley, B.R. Davis, Z. Zhou, J.M. Cimbala, and H.J. Donahue. 1998. Differential effect of steady versus oscillating flow on bone cells. J. Biomech. 31:969-976.

Jackson, B., R. Eastell, J. Gray, P.E. Harris, S. Ricketts, L.E. Lanyon, and J.S. Price. 1997. Biochemical markers of bone turnover in growing horses: Interrelationships with seasonal patterns of growth. Bone. 20(4S):51S.

Jackson, B., R.E. Eastell, A.M. Wilson, L.E. Lanyon, A.E. Goodship, and J.S. Price. 1998. The effect of exercise on biochemical markers of bone metabolism and insulin-like growth factor-1 in two-year-old Thoroughbreds. J. Bone Min. Res. 13:521.

Jarvinen, T., P. Kannus, and H. Sievanen. 1999. Have the DXA-based exercise studies seriously underestimated the effects of mechanical loading on bone? J. Bone Min. Res. 14(9):1634-1635.

Jaworski, Z.F.G., and H.K. Uhtoff. 1986. Disuse osteoporosis: Current status and problems. In Current Concepts of Bone Fragility ed. H.K. Uhtoff and E.Stahl pp. 181-194. New York:Spriger-Verlag.

Jee, W.S.S. 1988. The skeletal tissues. In Cell and Tissue Biology: A Textbook of Histology. L.Weiss, M.D. ed. Urban and Schwarzenberg, Baltimore MD. 218-254.

Jeffcott, L.B., P.D. Rossdale, J. Freestone, C.J. Frank, and P.R. Towers-Clark. 1982. An assessment of wastage in Thoroughbred racing from conception to 4 years of age. Equine Vet J. 14:185-198.

Jeffcott, L.B., and R.N. McCartney. 1985. Ultrasound as a tool for assessment of bone quality in the horse. Vet. Rec. 116:337-342.

Jones, W. E. 1989. Racetrack breakdown epidemiology. Equine Vet. Data. 10(11):190-191.

Judex, S., and R.F. Zernicke. 2000a. Does the mechanical milieu associated with high-speed running lead to adaptive changes in diaphyseal growing bone? Bone. 26(2):153-159.

Judex, S., and R.F. Zernicke. 2000b. High-impact exercise and growing bone: relation between high strain rates and enhanced bone formation. J. Appl. Phys. 88:2183-2191.

Kannus, P., H. Sievanen, T.L.N. Jarvinen, M. Jarvinen, M. Kvist, P.Oja, I. Vuori, and L. Jozsa. 1994. Effects of free mobilization and low- to high-intensity treadmill running on the immobilization-induced bone loss in rats. J. Bone Min. Res. 9(10):1613-1619.

Kannus, P., H. Haapsalo, M. Sankelo, H. Sievanen, M. Pasanen, A. Heinonen, P. Oja and I. Vuori. 1995a. Effect on starting age of physical activity on bone mass in the dominant arm of tennis and squash players. Ann Intern Med 123:27-31.

Kannus, P., J. Parkkari, and S. Niemi. 1995b. Age adjusted incidence of hip fractures. Lancet. 346:50-51.

Kerr, D., T. Ackland, B. Maslen, A. Morton, and R. Prince. 2001. Resistance training over 2 years increases bone mass in calcium-replete postmenopausal women. J. Bone Min. Res. 16(1):175-181.

Khan, K., K. Bennell, J. Hopper, L. Flicker, C. Nowson, A. Sherwin, K. Crichton, P. Harcourt, and J. Wark.1998. Self-reported ballet classes undertaken at age 10-12 years and hip bone mineral density in later life. Osteopor. Inter.8:165-173.

Khan, K., H.A. McKay, H. Haapasalo, K.L. Bennell, M.R. Forwood, P. Kannus, and J.D. Wark. 2000. Does childhood and adolescence provide a unique opportunity for exercise to strengthen the skeleton? J. Sci. Med. Sport. 3(2):150-164.

Kiiskinen, A. 1977. Physical training and connective tissue in young mice-physical properties of achilles tendons and long bones. Growth. 41:123-137.

Kimmel, D.B. 1993. A paradigm for skeletal strength homeostasis. J. Bone Min. Res. 8(S2):S515-S522.

Kiuchi, A., Y. Arai, and S. Katsuta. 1998. Detraining effects on bone mass in young male rats. Inter. J. Sports Med. 19:245-249.

Kleerekoper, M., D.A. Nelson, E.L. Peterson, M.J. Flynn, A.S. Pawluszka, G. Jacobsen, and P. Wilson. 1994. Reference data for bone mass, calciotropic hormones, and biochemical markers of bone remodeling in older (55-75) postmenopausal white and black women. J. Bone Min. Res. 9:1267-1276.

Knight, D. A., and W.J. Tyznik. 1985. The effect of artificial rearing on the growth of foals. J. Anim. Sci. 60(1):1-5.

Knowles, T.G., and D.M. Broom. 1990. Limb bone strength and movement in laying hens from different housing systems. Vet Rec. 126:354-356.

Kodama, H., A. Yamaski, M. Nose, S. Niida, Y. Ohgame, M. Abe, M. Kumegawa, and T. Suda. 1991. Congenital osteoclast deficiency in osteopetrotic (op/op) mice is cured by injections of macrophage colony-stimulating factor. J. Exper. Med. 173:269-272.

Konieczynski, D.D., M.J. Truty, and A.A. Biewener. 1998. Evaluation of a bone's in vivo 24-hour loading history for physical exercise compared with background loading. J. .Ortho. Res. 16:29-37.

Kontulainen, S., P. Kannus, H. Haapasalo, A. Heinonen, H. Sievanen, P. Oja, and I. Vuori. 1999. Changes in bone mineral content with decreased training in competitive young adult tennis players and controls: A prospective 4-yr follow-up. Med. Sci. Sports Exer. 31:646-652.

Kontulainen, S., P. Kannus, H. Haapasalo, H. Seivanen, M. Pasanen, A. Heinonen, P. Oja, and I. Vuori. 2001. Good maintenance of exercise-induced bone gain with decreased training of female tennis and squash players: A prospective 5-year follow-up study of young and old starters and controls. J. Bone Min. Res. 16(2):195-201.

Kostenuik, P.J., J. Harris, B.P. Halloran, R.T. Turner, E.R. Morey-Holton, and D. D. Bilke. 1999. Skeletal unloading causes resistance of osteoprogenitor cells to parathyroid hormone and to insulin-like growth factor-1. J. Bone Min. Res. 14(1):21-31.

Kriska, A.M., R.B. Sandler, and J.A. Cauley. 1988. The assessment of historical physical activity and its relation to adult bone parameters. Amer. J. Epid. 127:1053-1061.

Lanyon, L.E. 1984. Functional strain as a determinant for bone remodeling. Calc. Tissue Inter.36(Suppl):S56-S61.

Lanyon, L.E. 1987. Functional strain in bone tissue as an objective, and controlling stimulus for adaptive bone remodelling. J. Biomech. 20(11/12):1083-1093.

Lanyon, L.E., A.E. Goodship, C.J. Pye, and H.J. MacFie. 1982. Mechanically adaptive bone remodeling. J. Biomech. 15:141-154.

Lanyon, L.E. 1993. The physiological basis of training the skeleton. Equine Vet. J. 8-13.

Lanyon, L.E. and C.T. Rubin. 1984. Static vs dynamic loads as an influence on bone remodeling. J. Biomech. 17(12):897-905.

Lanyon, L.E., C.T. Rubin, and G. Baust. 1986. Modulation of bone loss during calcium insufficiency by controlled dynamic loading. Calc. Tissue Inter. 38:209-216.

Larkin, N.C., and H.M.S. Davies. 1996. The application of a radiographic index to the prevention of dorsal metacarpal disease in Thoroughbred racehorses. Pferdeheilkunde. 12(4):595-598.

Lawrence, L.A., E.A. Ott, G.J. Miller, P.W. Poulos, G. Piotrowski, and R.L. Asquith. 1994. The mechanical properties of equine third metacarpals as affected by age. J. Anim.Sci. 72:2617-2623.

Lean, J.M., C.J. Jagger, T.J. Chambers, and J.W. M. Chow. 1995. Increased insulin-like growth factor I mRNA expression in rat osteocytes in response to mechanical stimulation. Amer. J. Phys. 268:E318-E327.

Leblanc, A.D., V.S. Scneider, H.J. Evans, D.A. Engelbretson, and J.M. Krebs. 1990. Bone mineral loss and recovery after 17 weeks of bed rest. J. Bone Min. Res. 5(8):843-850.

Lepage, O.M., M. Marcoux, and A.Tremblay. 1990. Serum osteocalcin or bone gla-protein, a biochemical marker for bone metabolism in horses: Differences in serum levels with age. Can. J. Vet. Res. 54:223-226.

Lepage, O.M., L. DesCoteaux, M. Marcoux, and A. Tremblay. 1991. Circadian rhythms of osteocalcin in equine serum: Correlation with alkaline phosphatase, calcium, phosphate and total protein levels. Can. J. Vet. Res. 55:5-10.

Lepage, O.M., D. Hartmann, R. Eicher, B. Uebelhart, P. Tschudi, and D. Uebelhart. 1998. Biochemical markers of bone metabolism in draught and warmblood horses. Vet. J. 156(3):169-175.

Lepage, O.M., B. Carstanjen, and D. Uebelhart. 2001. Non-invasive assessment of equine bone: An update. Vet. J. 161:10-23.

Les, C.M., J.H. Keyak, S.M. Stover, K.T. Taylor, and A.J. Kaneps. 1994. Estimation of material properties in the equine metacarpus with use of quantitative computed tomography. J. Orthop. Res. 12:822-833.

Li, K.C., R.F. Zernicke, R. J. Barnard, and A. F. Li. 1991. Differential response of rat limb bones to strenuous exercise. J. Appl. Phys. 70(2):554-560.

Loitz, B.J., and R.F. Zernicke. 1992. Strenuous exercise-induced remodeling of mature bone: Relationships between in vivo strains and bone mechanics. J. Exper. Biol. 170:1-18.

Lueken, A.S., S.B. Arnaud, A.K. Taylor, and D.J. Baylink. 1993. Changes in markers of bone formation and resorption in a bed rest model of weightlessness. J. Bone Min. Res. 8(12):1433-1437.

Ma, Y., W.S.S. Jee, Z. Yuan, W. Wei, H. Chen, S. Pun, H. Liang, and C. Lin. 1999. Parathyroid hormone and mechanical usage have a synergistic effect in rat tibial diaphyseal cortical bone. J. Bone Mineral Res. 14(3):439-448.

Marchant, J.N., and D.M. Broom. 1986. Effects of dry sow housing conditions on muscle weight and bone strength. J. Anim. Sci. 62:105.

Marks, S.C, and P.R. Odgren. 2002. Structure and development of the skeleton. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 3-15.

Markus, R. 2002. Mechanisms of exercise effects on bone. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 1477-1490.

Martin, R.B. 2000. Toward a unifying theory of bone remodeling. Bone. 26(1):1-6.

Martinez, D.A., M.W. Orth, K.E. Carr, R. Vanderby, Jr., and AC. Vailas. 1996. Cortical bone growth and maturational changes in dwarf rats induced by recombinant human growth hormone. Amer. J. Phys. 270:E51-E59.

Matsuda, J.J., R.F. Zernicke, A.C. Vailas, V.A. Pedrinin, A. Pedrinin-Mille, and J.A. Maynard. 1986. Structural and mechanical adaptation of immature bone to strenuous exercise. J. Appl. Phys. 60(6):2028-2033.

Maynard, J.A., A. Pedrini, and A.C. Vailas. 1995. Morphological and biochemical effects of strenuous exercise on immature long bones. Iowa Orthop. J. 15:162-167.

McCarthy, R.N., L.B. Jeffcott, and R.M. McCartney. 1990. Ultrasound speed in equine cortical bone: Effects of orientation, density, porosity, and temperature. J. Biomech. 23:1139-1143.

McCarthy, R.N., and L. B. Jeffcott. 1991. Treadmill exercise intensity and its effects on cortical bone in horses of various ages. Equine Exercise Physiology. 3:419-428

McCarthy, R.N., and L.B. Jeffcott. 1992. Effects of treadmill exercise on cortical bone in the third metacarpus of young horses. Res. Vet. Sci. 52:29-37.

McClure, S.R., L.T. Glickman, N.W. Glickman, and C.M. Weaver. 2001. Evaluation of dual energy x-ray absorptiometry for in situ measurement of bone mineral density of equine metacarpi. Amer. J. Vet. Res. 62(5):752-756.

McDonald, R., J. Hegenauer, and P. Saltman. 1986. Age-related differences in the bone mineralization pattern of rats following exercise. J. Geront. 41(4):445-452.

McKeever, M., G. Heusner, and D. Sperling. 1981. Quarter Horse foal growth – effect of month of birth, age of dam, and sex of foal. Proc. of 8th Equine Nutrition and Physiology Symposium. p.149-153.

McSheehy, P.M., and T.J. Chambers.1986. Osteoblast-like cells in the presence of parathyroid hormone release soluble factor that stimulates osteoclastic bone resorption. Endo. 119:1654-1659.

Meakim, D.W., E.A. Ott, R.L. Asquith, and J.P. Feaster. 1981. Estimation of mineral content of the equine third metacarpal by radiographic photometry. J. Anim. Sci. 53(4):1019-1026.

Meletti, Z., M. Shapiro, and C.S. Adams. 2000. Inorganic phosphate induces apoptosis of osteoblast-like cells in culture. Bone. 27(3):359-366.

Michael, E.M., G.D. Potter, K.J. Mathiason-Kochan, P.G. Gibbs, E.L. Morris, L.W. Greene, and D. Topliff. 2001. Biochemical markers of bone modeling and remodeling in juvenile racehorses fed differing levels of minerals. Proc. 17th Symposium of the Equine Nutrition and Physiology Society. Lexington, KY.

Minaire, P. 1993. Proceedings of the 1991 International Symposium on data on aging. Vital and Health Statistics. Series 5. Comparative International Vital and Health Statistics Reports 7:9-17.

Mori, S., and D.B. Burr. 1993. Increased intracortical remodeling following fatigue damage. Bone. 14:103-109.

Mosley, J.R., B.M. March, J. Lynch, and L.E. Lanyon. 1997. Strain magnitude related changes in whole bone architecture in growing rats. Bone. 20(3):191-198.

Mosley, J.R., and L.E. Lanyon. 1998. Strain rate as a controlling influence on adaptive modeling in response to dynamic loading of the ulna in growing male rats. Bone. 23(4):313-318.

Nelson, D.A., and M.L. Bouxsein. 2001. Exercise maintains bone mass, but do people maintain exercise? J. Bone Min. Res. 16(2):202-205.

Nielsen, B.D., G.D. Potter, E.L. Morris, T.W. Odom, D.M. Senor, J.A. Reynolds, W.B. Smith, and M.T. Martin. 1997. Changes in the third metacarpal bone and frequency of bone injuries in young Quarter Horses during race training – observations and theoretical considerations. J. Equine Vet. Sci. 17(10):541-549.

Nielsen, B.D., and G.D. Potter. 1997. Accounting for volumetric differences in estimates of bone mineral content from radiographic densitometry. Proc. 15th Equine Nutrition and Physiology Symposium. Ft. Worth TX. 367.

Nielsen, B.D., G.D. Potter, L.W. Greene, E.L. Morris, M. Murray-Gerzik, W.B. Smith, and M.T. Martin. 1998. Characterization of changes related to mineral balance in the young racing Quarter Horse. J. Equine Vet. Sci. 18:190-200.

Nijweide, P.J., E.H. Burger, and J. Klein-Nulend. 2002. The osteocyte. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 93-107.

Nishiyama, S. S.,Tomeda, T. Ohta, A. Higuchi, and I. Matsuda. 1988. Differences in basal and post-exercise osteocalcin levels in athletic and non-athletic humans. Calc. Tissue Inter. 43:150-154.

Noble, B.S., H. Stevens, J.R. Mosley, A.A. Pitsillides, J. Reeve, and L. Lanyon. 1997. Bone loading changes the number and distribution of apoptotic osteocytes in cortical bone. J. Bone Min. Res. 12(suppl. 1):S111.

Noble, B.S., and J. Reeve. 2000. Osteocyte function, osteocytes death and bone fracture resistance. Molec. Cell. Endo. 158:7-13.

Nolan, M.M., G.D. Potter, K.J. Mathiason, P.G. Gibbs, E.L. Morris, L.W. Greene and D.L. Topliff. 2001. Bone density in the juvenile racehorse fed differing levels of mineral. Proc. 17th Equine Nutrition and Physiology Symposium. 33-38.

Nomura, S., and T.Takano-Yamamoto. 2000. Molecular events caused by mechanical stress in bone. Matrix Biol. 19:91-96.

Notomi, T., N. Okimoto, Y. Okazaki, Y. Tanaka, T. Nakamura, and M. Suzuki. 2001. Effects of tower climbing exercise on bone mass, strength, and turnover in growing rats. J. Bone Min. Res. 16(1):166-174.

Nunamaker, D.M., D.M. Butterweck and M.T. Provost. 1989. Some geometric properties of the third metacarpal bone: a comparsion between the Thoroughbred and Standardbred racehorse. J. Biomech.. 22(2):129.

Nunamaker, D.M., D.M. Butterweck, and M. T. Provost. 1990. Fatigue fractures in Thoroughbred racehorses: relationships with age, peak bone strain, and training. J. Orthop. Res. 8(4): 604-611.

Ohashi, N., A.G. Robling, D.B. Burr, and C.H. Turner. 2002. The effects of dynamic axial loading on the rat growth plate. J. Bone Min. Res. 17(2):284-292.

Ott, S.M., E. W. Lipkin, and L. Newell-Morris. 1999. Bone physiology during pregnancy and lactation in young macaques. 14(10):1779-1788.

Ott, S, M. 2002. Histomorphometric analysis of bone remodeling. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 303-319.

Pagan, J.D., S.G. Jackson, and S. Caddel. 1996. A summary of growth rates of Thoroughbreds in Kentucky. World Equine Vet. Rev. 1:23-26.

Parfitt, A.M., and B. Chir. 1987. Bone remodeling and bone loss: Understanding the pathophysiology of osteoporosis. Clin. Obst. Gynec. 30(4): 789.

Pead, M., T.M. Skerry, and L.E. Lanyon. 1988a. Direct transformation from quiescence to formation in adult periosteum following a single brief period of loading. J. Bone Min.Res. 3:647-656.

Pead, M.J., R.S. Suswillo, T.M. Skerry, S. Vedi, and L.E. Lanyon. 1988b. Increased $^{3}$H-uridine levels in osteocytes following a single short period of dynamic bone loading in vivo. Calc. Tissue Inter. 43:92-96.

Pedersen, B.J., A. Schlemmer, C. Hassager, and C. Christiansen. 1995. Changes in the carboxyl-terminal propeptide of type I procollagen and other markers of bone formation upon five days of bed rest. Bone. 17(1):91-95.

Petersen, E.D., P.D. Siciliano, A.S. Turner, C.E. Kawcak, and C.W. McIlwraith. 2001. Effect of growth rate on serum-bone-specific alkaline phospatase and – osteocalcin in weanling horses. Proc. 17th Equine Nutrition Physiology Symposium. 186-187.

Piotrowski, G., M. Sullivan and P.T. Colahan. 1983. Geometric properties of equine metacarpi. J. Biomech. 16(2): 129-139.

Porr, C.A., D.S. Kronfeld, L.A. Lawrence, R.S. Pleasant, and P.A. Harris. 1998. Deconditioning reduces mineral content of the third metacarpal bone in horses. J. Anim. Sci. 76:1875-1879.

Porr, C.A., D.S. Kronfeld, L.A. Lawrence, R.S. Pleasant, and P.A. Harris. 2000. Diet and conditioning influence bone development. Equine Prac. 22(5):18-21.

Price, J.S., B. Jackson, R. Eastell, A.E. Goodship, A. Blumsohn, I. Wright, S. Stoneham, L.E. Lanyon, and R.G.G. Russell. 1995b. Age related changes in biochemical markers of bone metabolism in horses. Equine Vet. J. 27(3):201-207.

Price, J.S., B. Jackson, J. Gray, L.M. Wright, P. Harris, R.G.G. Russell, R.E. Eastell, C.W. McIlwraith, S.W. Ricketts, and L.E.Lanyon. 1997. Serum levels of molecular markers in growing bones: The effects of age, season, and orthopedic disease. Trans. Orthop.Res. Soc. 22:587.

Raab, D.M., T.D. Crenshaw, D.B. Kimmel, and E.L. Smith. 1991. A histomorphometric study of cortical bone activity during increased weight-bearing exercise. J. Bone Min. Res. 6:741-749.

Raab-Cullen, D.M., M.A. Thiede, D.N. Petersen, D.B. Kimmel, and R.R. Recker. 1994a. Mechanical loading stimulates rapid changes in periosteal gene expression. Calc. Tissue Inter. 55:473-478.

Raab-Cullen, D.M., M.P. Akhter, D.B. Kimmel, and R.R. Recker. 1994b. Periosteal bone formation stimulated by externally induced bending strains. J. Bone Min. Res. 9(5):1143-1152.

Raab-Cullen, D.M., M.P. Akhter, D.B. Kimmel, and R.R. Recker. 1994c. Bone response to alternate-day mechanical loading of the rat tibia. J. Bone Min. Res. 9(2):203-211.

Raub, R.H., S.G. Jackson, and J.P. Baker. 1989. The effect of exercise on bone growth and development in weanling horses. J. Anim. Sci. 67:2508-2514.

Reinholt, F.P., K. Hultenby, A. Oldberg, and D. Heingard. 1990. Osteopontin- a possible anchor of osteoclasts to bone. Proc. National Academy Science. 87:4473-4475.

Reller, E., J. Kivipelto, and E.A. Ott. 2001. Age related changes for serum bone metabolism markers in Thoroughbred and Quarter Horse foals. Proc. 17th Equine Nutrition Physiology Symposium. 472-473.

Robey, P.G. 1995. Biochemistry of bone. In Osteoporosis: Etiology, Diagnosis and Management. 2nd ed. Riggs, B.L. and Melton, L.J. III (eds) Lippincott-Raven Publishers. Philadelphia.41-59.

Robins, S.P., H.Woitge, R. Hesley, J.Ju, S. Seyedin, and M.J. Seibel. 1994. Direct, enzyme-linked immunoassay for urinary deoxpyridinoline as a specific marker for measuring bone resorption. J. Bone Min. Res. 9(10):1643-1649.

Robins, S.P., and J.D. Brady. 2002.Collagen cross-linking and metabolism. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 211-223.

Robling, A.G., D.B. Burr, and C.H. Turner. 2000. Partitioning a daily mechanical stimulus into discreet loading bouts improves the osteogenic response to loading. J. Bone Min. Res. 15(8):1596-1602.

Robling, A.G., D.B. Burr, and C.H. Turner. 2001. Recovery periods restore mechanosensitivity to dynamically to dynamically loaded bone. J. Exper. Biol. 204:3389-3399.

Rodan, G.A., and T.J. Martin. 1981. Role of osteoblasts in hormonal control of bone resorption: A hypothesis. Calc. Tissue Inter. 33:349-351.

Rodan, G.A., and S.B. Rodan. 1995. The cells of bone. In Osteoporosis: Etiology, Diagnosis and Management. 2nd ed. Riggs, B.L. and Melton, L.J. III (eds) Lippincott-Raven Publishers. Philadelphia.1-39.

Rossdale, P.D., R. Hopes, N.J. Wingfield Digby, and K. Offord. 1985. Epidemiological study of wastage among racehorse 1982 and 1983. Vet. Rec. 116:66-69.

Rossert, J., and B. de Crombrugghe. 2002. Biochemical markers of bone metabolism. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego.189-210.

Rubin, C.T. and L.E. Lanyan. 1982. Limb mechanics as a function of speed and gait: A study of functional strains in the radius and tibia of horse and dog. J. Exper. Biol. 101:187-211.

Rubin, C.T. 1984. Skeletal strain and the functional significance of bone architecture. Calc. Tissue Inter.36:S11-S18.

Rubin, C.T., and L.E. Lanyon. 1984. Regulation of bone formation by applied dynamic loads. J. Bone Joint Surg. 66:397-402.

Rubin, C.T., and L.E. Lanyon. 1985. Regulation of bone mass by mechanical strain magnitude. Calc. Tissue Inter. 37:411-417.

Rubin, C.T., S.D. Bain, and K.J. McLeod. 1992. Suppression of the osteogenic response in the aging skeleton. Calc. Tissue Inter. 50:306-313.

Rubin, J., X. Fan, D.M. Biskobing, W.R. Taylor, and C.T. Rubin. 1999. Osteoclastogenesis is repressed by mechanical strain in an in vitro model. J. Orthop. Res. 17:639-645.

Ryder, K.D., and R.L.Duncan. 2001. Parathyroid hormone enhances fluid shear-induced $[Ca^{2+}]_i$ signaling in osteoblastic cells through activation of mechanosensitive and voltage-sensitive $Ca^{2+}$ channels. J. Bone Min. Res. 16(2):240-248.

Saastamoinen, M.T., S. Hyyppa, and K. Houvinen. 1994. Effect of dietary-fat supplementation and energy-to-protein ratio on growth and blood metabolites of weanling foals. J. Anim. Phys. Anim. Nutr. 71:179-188.

Salem, G.J., R.F. Zernicke, A.C. Vilas, and D.A. Martinez. 1989. Biomechanical and biochemical changes in lumbar vertebrae of rapidly growing rats. Amer. J. Phys. 256:R259-R263.

Salem, G.J., R.F. Zernicke, D.A. Marinez, and A.C. Vailas. 1993. Adaptations of immature trabecular bone to moderate exercise: Geometrical, biochemical, and biomechanical correlates. Bone.14:647-654.

SAS. 2001. SAS User's Guide: Statistics. SAS Inst., Inc., Cary. NC

Seibel, M.J., R. Eastell, C.M. Gundberg, R. Hannon, and H.A.P. Pols. 2002. Biochemical markers of bone metabolism. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 1543-1571.

Sherman, K.M., G.J. Miller, T.J. Wronski, P.T. Colahan, M. Brown, and W. Wilson. 1995. The effect of training on equine metacarpal bone breaking strength. Equine Vet. J. 27(2): 135-139.

Skerry, T.M., and L.E. Lanyon. 1995. Interruption of disuse by short duration walking exercise does not prevent bone loss in the sheep calcaneus. Bone. 16(2):269-274.

Slemenda, C.W., M. Peacock, S. Hui, L. Zhou, and C.C. Johnston. 1997. Reduced rates of skeletal remodeling are associated with increased bone mineral density during the development of peak skeletal mass. J. Bone Min. Res. 12(4):676-682.

Snyder, A., J.R. Zierath, J.A. Hawley, M.D. Sleeper, and B.W. Craig. 1992. The effects of exercise mode, swimming vs. running, upon bone growth in the rapidly growing female rat. Mech. Ageing Devel. 66:59-69.

Steinberg, M.E., and J. Trueta. 1981. Effects of activity on bone growth and development in the rat. Clin. Orth. Rel. Res. 156:52-60.

Stover, S.M., R.R. Pool, R.B. Martin, and J.P. Morgan. 1992. Histological features of the dorsal cortex of the third metacarpal bone mid-diaphysis during postnatal growth in Thoroughbred horses. J. Anat. 181:455-469.

Takahashi, N., N. Udawaga, M. Takami, and T. Suday. 2002. Cells of Bone: Osteoclast generation. In Principles of Bone Biology. 2nd ed. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan (eds) Academic Press. San Diego. 109-126.

Thomson, K.L., G.D. Potter, K.J. Terrell, E.L. Morris, and K.J. Mathiason-Kochan. 2001. Cortical bone width in juvenile racehorses treated with exogenous somatotropin (eST). Proc. 17th Equine Nutrition Physiology Symposium. 108-113.

Tilton, F.E., J.J.C. and V.D. Schneider. 1980. Longterm follow-up of Skylab bone demineralization. Aviat. Space Environ. Med. 51:1209-1213.

Tipton, C.M., R.D. Mattles, and J.A. Maynard. 1972. Influence of chronic exercise on rat bones. Med. Sci. Sports. 4:55.

Turner, C.H. 1991. Homeostatic control of bone structure: An application of feedback theory. Bone. 12:203-217.

Turner, C.H., I. Owan, and Y. Takano. 1995. Mechanotransduction in bone: Role of strain rate. Amer. J. Phys. 269: E438-442.

Turner, C.H., and F.M. Pavalko. 1998. Mechanotransduction and functional response of the skeleton to physical stress: The mechanisms and mechanics of bone adaptation. J. Ortho. Sci. 3:346-355.

Tuukkanen, J., Z. Peng, and H.K. Vaananen. 1992. The effect of training on the recovery from immobilization-induced bone loss in rats. Acta Physiol Scand 145:407-411.

Uhthoff, H.K., and Z.F.G. Jaworski. 1978. Bone loss in response to long-term immobilization. J. Bone Joint Surg. 60B:420.

Uhthoff, H.K., G. Sekaly, and Z.F.G. Jaworski. 1985. Effect of long-term nontraumatic immobilization on metaphyseal spongiosa in young adult and old beagle dogs. Clin. Orthoped. Rel. Res.192:278-283

Umemura, Y., T. Ishiko, H. Tsujimoto, H. Miura, N. Mokushi, and H. Suzuki. 1995. Effects of jump training on bone hypertrophy in young and old rats. Inter. J. Sports Med. 16:364-367.

Umemera, Y., T. Ishiko, T. Yamauchi, M. Kurono, and S. Mashiko. 1997. Five jumps per day increase bone mass and breaking force in rats. J. Bone Min. Res. 12(9): 1480-1485.

Verborgt, O., G.J. Gibson, and M. B. Schaffler. 2000. Loss of osteocytes integrity in association with microdamage and bone remodeling after fatigue in vivo. J. Bone Min. Res. 15(1):60-67.

Vuori, I., A. Heinonen, P. Kannus, M. Pasanen, and P. Oja. 1994. Effects of unilateral strength training and detraining on bone mineral density and content in young women: A study of mechanical loading and deloading on human bones. Calc. Tissue Inter. 55:59-67.

Waite, K.L., B.D. Nielsen, and D.S. Rosenstein. 2000. Computed tomography as a method of estimating bone mineral content in horses. J. Equine Vet Sci. 20(1):49-52.

Warren, L.K., L.M. Lawrence, A.L. Parker, T. Barnes, and A.S. Griffin. 1998. The effect of weaning age on foal growth and radiographic bone density. J. Equine Vet. Sci. 18(5):335-342.

Wassen, M.H.M., J. Lammens, J.M. Tekoppele, R.J.B. Sakkers, Z. Liu, A.J. Verbout, and R.A. Bank. 2000. Collagen structure regulates fibril mineralization in osteogenesis as revealed by cross-link patterns in calcifying callus. J. Bone Min Res. 15(9):1776-1785.

Weinrab, M., G.A. Rodan, and D. D. Thompson. 1991. Immobilization-related bone loss in the rat is increased by calcium deficiency. Calc. Tissue Inter. 48:93-100.

Weitz, S.L., P. Benham, and S.S. Leung. 1999. Total deoxypyridinoline in serum and urine as measured by a novel adaptation of the Pyrilinks-D enzyme immunoassay. Amer. Soc. Bone Min. Res. 21st Annual Meeting. S371.

Whalen, R.T., and D.R. Carter. 1988. Influence of physical activity on the regulation of bone density. J. Biomech. 21(10):825-837.

Whalen, R.T., D.R. Carter, and C.R. Steele. 1993. The relationship between physical activity and bone density. Orthop. Trans. 12:56-65.

Whedon, G.D., L.Lutwak, P.Rambaut, M. Whittle, C. Leach, J.Reid, and M. Smith. 1977. Effect of weightlessness on mineral metabolic studies on Skylab orbital space flight. Calc. Tissue Res. (Suppl). 21:423-430.

Wojtowicz, A., A. Dziedzic Goclawska, A. Kaminski, W. Stachowicz, K. Wojtowicz, S.C. Marks Jr., and M. Yamauchi. 1997. Alteration of mineral crystallinity and collagen cross-linking of bones in osteopetrotic toothless (tl/tl) rats and their improvement after treatment with colony stimulating factor-1. Bone. 20:127-132.

Woo, S.L., S.C. Kuei, D.Amiel, M.A. Gomez, W.C. Hayes, F.C. White, and W.H. Akeson. 1981. The effect of prolonged physical training on the properties of long bone: A study of Wolff's Law. J. Bone Joint Surg. 63-A(5):780-787.

Yamauchi, M., D.R. Young, G.S. Chandler, and G.L. Mechanic. 1988. Cross-linking and bone collagen synthesis in immobilized and recovering primate osteoporosis. Bone 9:415-418.

Yamauchi, M., Y. Ogata, R.H. Kim, J.J. Li, L.P. Freedman, and J. Sodek. 1996. AP-1 regulation of the rat bone sialoprotein gene transcription is mediated through a TPA response element within a glucocorticoid response unit in the gene promoter. Matrix Biol. 15(2):119-130.

Yeh, J.K., and J.F. Aloia. 1990. Deconditioning increases bone resorption and decreases bone formation. Metab. 39:659-663.

Yeh, J.K., C.C. Liu, and J.F. Aloia. 1993. Effects of exercise and immobilization on bone formation and resorption in young rats. Amer. J. Phys. 264:E182-E189.

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