BIOCHEMICAL MARKERS OF BONE MODELING AND REMODELING IN JUVENILE RACEHORSES AT VARYING MINERAL INTAKES

A Thesis

by

ELENA MARIA ELLER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Animal Science

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Approved by:

Chair of Committee,	Pete G. Gibbs
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ABSTRACT

Biochemical Markers of Bone Modeling and Remodeling in Juvenile Racehorses at Varying Mineral Intakes. (May 2006)

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Chair of Advisory Committee: Dr. Pete Gibbs

Blood-borne biochemical markers were used to track comparative rates of bone turnover in horses fed differing amounts of Ca, P and Mg. Bone turnover was tracked by serum osteocalcin, bone resorption by the carboxyterminal telopeptide of type I collagen (ICTP), and bone formation by the carboxyterminal propeptide of type I procollagen (PICP). Twenty-four long-yearling Quarter Horses were blocked by gender and age, randomly assigned to one of four diets and subjected to 128 d of race training. Horses entered the study at approximately 20 months of age. The study was conducted in 32-d periods, each consisting of 28 d of race training followed by a 4-d fecal and urine collection, or a 4-d rest period. Blood samples were taken weekly during the training period. Serum and plasma samples were analyzed for concentrations of osteocalcin, the carboxyterminal telopeptide of type I collagen (ICTP) and the carboxyterminal propeptide of type I procollagen (PICP). Urine was collected for analysis of deoxypyridinoline (DPD) and creatinine.

Onset of training resulted in elevated concentrations of ICTP, PICP and osteocalcin. Horses consuming the highest levels of Ca, P and Mg exhibited higher concentrations of PICP and lower concentrations of ICTP indicating greater bone

formation coupled with lesser amounts of bone resorption. Further, ICTP, PICP and osteocalcin concentrations decreased dramatically following 4-d of confinement and relative inactivity. Therefore it appears that feeding minerals at levels greater than current NRC recommendations provided a protective effect on the developing skeleton of the young racehorse. Additionally, the biochemical markers used in this study were sensitive enough to track daily changes in bone activity resulting from daily changes in stress to the skeleton.

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CHAPTER I

INTRODUCTION

The incidence of skeletal injuries to young racehorses is a significant problem in the horse industry. These injuries are not only expensive in terms of direct veterinary costs, but in loss of opportunity for training and competition as well. Feeding and training mismanagement early in life are thought to contribute to the high frequency of injury in juvenile equine athletes. Juvenile racehorses entering training undergo a period of bone demineralization followed by a period of remineralization. While this process of remodeling is necessary for the skeleton to adapt to physical strain, the onset of speed work (50-60 d into training) often appears to coincide with the period of greatest bone demineralization (Nielsen et al., 1997). Thus, the bone is being subjected to excessive strain at a point when it is least prepared to handle that strain. A study by Nielsen et al. (1998) demonstrated that feeding Ca and other minerals at 30% above NRC (1989) recommendations to young Quarter Horses in race training reduced the extent of demineralization during the remodeling process. Further, Nielsen et al. (1997) found that horses that started with more bone were less likely to experience injury during training.

Proper bone development is dependant on both proper nutrition and the correct amount of physical activity. Research has repeatedly shown that a certain amount of exercise is necessary for the bone to remodel in order to withstand the strain that will be

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placed on it later in life. However, excessive loading at a time when the bone is not prepared to handle that strain can result in injury. Additionally, current literature indicates that the NRC (1989) recommendations for young horses in training are insufficient for maximizing bone growth and preventing skeletal injuries. Several studies have reported that feeding Ca and other minerals at levels greater than NRC (1989) recommendations during the time of training can be beneficial. Thus, a better understanding of how to feed and manage young horses in race training is needed in order to help better prepare the bone to withstand the intensity of training and racing.

The purpose of this study was 1) to evaluate the use of biochemical markers to track comparative rates of bone turnover, bone resorption, and bone formation in juvenile horses in race training and 2) to assess the impact of varying concentrations of Ca, P and Mg on bone activity.

CHAPTER II

REVIEW OF LITERATURE

The majority of career-ending injuries to racehorses involve the skeleton (Johnson, 1993). It has been documented that more than 50% of 2-year-old racehorses experience some form of injury during training (Jones et al., 1989; Rossdale et al., 1985). Several studies have reported that skeletal maturation and maximal bone density and mineral content are not reached until 4-6 years of age (Buckingham et al., 1992; Lawrence et al., 1994). Additionally, inertial properties of the third metacarpal change dramatically from 1 to 3 years of age, but changes are lesser past three years of age (Nunamaker et al., 1989). Starting long-yearling horses in training is often criticized because of the high occurrence of injury and the idea that immature bone is not prepared to withstand the stresses of racing; however, the skeleton of the young animal is more adaptable to training than the skeleton of the mature animal, thus this may be the optimal time to introduce training. Mansell et al. (1999) demonstrated that younger horses had a greater ability to respond to exercise following a period of idleness than did older horses. Additionally, a study of racing careers of horses in Australia, although not conclusive, demonstrated that horses that raced as 2-year-olds had a greater number of starts and raced in more seasons than horses that started racing as 3-year-olds (Bailey et al., 1999).

Bone is a unique tissue in that, although rather rigid, it responds fairly quickly to patterns of strain in the external environment. The ability of bone to alter its mass and shape according to the load that is placed upon it was first described in the nineteenth

century by J. Wolff (Duncan and Turner, 1995). Wolff's law has been used to describe the ability of exercise to enhance bone strength and has been widely demonstrated in numerous species. Cells within the bone detect variations in mechanical strain that fall outside the physiological window activating processes that alter the extra-cellular matrix of bone tissue in order to return strain to a desired level and prevent failure. When mechanical signals exceed the upper boundary of the physiological window, also known as the minimum effective strain, the bone undergoes changes in its structure in order to reduce strains back to a level below the upper boundary (Duncan and Turner, 1995). Conversely, disuse or decreased activity causes the unneeded bone mass to be removed through resorption (Iwamoto et al., 2000). Bone mineral content has been shown to decrease in weanlings and yearlings that are confined to stalls compared to those left out on pasture (Hoekstra et al., 1999; Bell et al., 2001).

Several studies in horses have demonstrated that exercise initiates skeletal adaptations. Schryver et al. (1978) found that bone from young exercised ponies tended to be stiffer and have a greater breaking strength than samples taken from age-matched controls. Sherman et al. (1995) found that cortical area and dorsopalmer bone diameter increased with duration of training in bones taken from 24 to 48 month-old Thoroughbred horses in race training. Increased dorsal cortical thickness in response to maximal exercise has been documented in two studies of yearling horses by McCarthy and Jeffcott (1991, 1992).

Bone modeling is site specific involving either addition of mineral to the site in order to increase bone density, or the removal and addition of bone to change the shape

of the bone (often referred to as remodeling). Growing and adult skeletons respond differently to mechanical stimuli (Bieweiner and Bertram, 1993). In the growing, exercising animal, adaptation of bone is most likely more of a change in bone geometry, as the primary factor influencing strength of long bones is moment of inertia or shape (Whalen et al., 1993). Thus, changing the shape of the bone results in a stronger structure than simply adding density. Research has shown that exercise training can result in increased cross sectional area of bone, increase in cortical thickness, or change in size of the medullary cavity (Woo et al., 1981). The drop in bone mineral density in horses over 6 months of age corresponds to replacement of primary bone through resorption (Stover et al., 1992). Further, Cornelissen et al. (1999) reported an increase in cross sectional area of the third metacarpal of horses between 5 and 11 months of age concurrent with a decrease in bone mineral density. A study by Sherman et al. (1995) found a positive correlation between duration of training and area moment of inertia, cortical area, and dorsopalmer bone diameter in bones taken from young racehorses. Additionally, most of the increase in the cross sectional area of bone was due to thickening in the dorsal cortex. Riggs and Boyde (1999) found that increased bone density in the distal region of the third metacarpal in long-yearlings in training was due to new bone formation.

While it has been shown that exercise can increase the strength of the skeleton, overloading the bone can result in increased risk of fracture. Fatigue due to repetitive loading causes micro-cracks, which can accumulate to dangerous levels if the skeleton is not provided enough time to repair itself. These micro-cracks, commonly referred to as

"bucked shins" or dorsal metacarpal disease, have been reported to occur in 70 to 80% of two-year-old racehorses (Norwood, 1978), and in the past, were thought to be a normal part of the training process. Accumulation of micro-damage can result in failure of the bone at strains that are within the physiological window (Burr et al., 1985). The presence of micro-cracks causes the bone to become less stiff, which magnifies the strain (Nunamaker et al., 1990) and initiates the repair mechanism of bone remodeling (Mori and Burr, 1993). During remodeling, the weakened bone is removed and replaced with new bone. The removal of the old bone causes a temporary increase in the porosity of the bone thus further increasing the susceptibility to failure.

Regardless of the stimuli, bone remodeling is an organized process that begins with bone resorption and ends with new bone formation. Resorption begins with recruitment of preosteoclasts, followed by differentiation into mature osteoclasts that then attach to the skeletal surface (Kleerekoper, 1996). During resorption (or demineralization) mineral is removed from the skeleton creating a cavity in the bone, which results in greater porosity and decreased bone strength. Thus there is a greater chance for micro-cracks to occur at the same strain. It is during this time that repetitive loading or an increase in the magnitude of loading can result in fatigue failure (Neilsen et al., 1997). While demineralization proceeds, preosteoblasts are recruited to the region where they mature into osteoblasts and attach to the base of the newly formed cavity (Kleerekoper, 1996). The osteoblasts synthesize and secrete the organic matrix that will become mineralized and form the new osteon (Parfitt, 1984). During the entire remodeling process, resorption and formation are tightly coupled, with resorption

proceeding rapidly followed by the much slower progression of the formation phase. In the event of skeletal repair, such as in response to micro-cracks, the amount of new bone formed is identical to the amount of bone removed resulting in zero net change. Conversely, during processes such as growth, bone formation exceeds resorption whereby a positive skeletal balance results from each remodeling cycle (Kleerekoper, 1996). Thus, an appropriate exercise program is important in order to achieve properly adapted bone while avoiding fatigue failure during the time when the bone is at its weakest.

The equine skeleton is composed of approximately 35% Ca, 15% P and 0.5% Mg. Interactions between these minerals and proper amounts in the diet are important in maximizing the potential strength of the skeleton. Ca is directly implicated to bone through incorporation into the hydroxyapetite (Porr et al., 2000) and Ca intake is important in the development of peak bone mass. Low Ca intake in humans has been associated with low bone mass (Weaver, 2000) and Ca supplementation has been shown to positively influence peak bone mass (Abrams and O'Brien, 2004). Ca deficiency in children is related to bone mineral insufficiency (Abrams and O'Brien, 2004) and in young horses has been linked with metabolic bone disease (NRC, 1989).

Like Ca, P is also a major constituent of bone. Inadequate dietary P may be linked with bone demineralization in horses (Lawrence et al., 1994). However, it is important that P levels in the diet not be allowed to exceed Ca levels as ratios of Ca:P less than 1:1 appear to inhibit Ca absorption (NRC, 1989). Although Mg does not comprise as great a percentage of bone as do both Ca and P, 60% of the Mg in the body

of the horse is found in bone (NRC. Mg deficiency has been shown to induce uncoupling of bone formation and resorption in the rat leading to loss of bone mass (Rude et al., 1999).

The mineral requirements for juvenile equine athletes are largely unknown. The most widely used publication for formulating horse rations is the National Research Council's Nutrient Requirements for Horses. The most recent NRC publication has significant limitations particularly in regard to young horses in training. In the absence of scientific data, recommendations for growing, exercising horses were extrapolated from data obtained from horses at rest. Recent research has demonstrated that these recommendations may be inadequate for maximizing bone development and preventing injuries during race training. Studies conducted by Schryver et al. (1978) and Young et al. (1989) have shown decreased urinary Ca and greater Ca retention when horses are exercised versus sedentary. Gray et al. (1988) found that horses in training had lower Ca excretion than horses not in training, despite receiving more Ca in the diet. In a study by Neilsen et al. (1998), horses in training receiving higher levels of Ca had greater Ca retention. Greater mineral content of the third metacarpal was seen in young race horses fed Ca at 151% to 169% of NRC (1989) recommendations (Nolen et al., 2001). Unlike Ca, an increased demand for P during exercise has not yet been clearly demonstrated. Young et al. (1989) reported increases in P retention in exercising horses. Conversely, Neilsen et al. (1998) found no change in P retention in young horses put into race training despite varying P concentrations in the diets. In the same study, Mg retention increased after three months of training along with mineral density of the third

metacarpal in horses provided with Ca supplementation. The authors hypothesized that increased Mg retention was caused by the increased bone formation resulting from increased Ca in the diet. The majority of the work done in Mg nutrition for exercising horses is confounded by varying concentrations of Ca and P. However, it seems that exercise and stage of training have some influence on Mg requirements (Stephens et al., 2001).

An important area of study in recent years has been the development of non-invasive techniques for studying the synthesis and degradation of bone. Significant progress in the development of biochemical markers for the assessment of bone metabolic activity has been driven by research in human osteoporosis. Several of these markers have proven useful in horses (Price et al., 1995a; Lepage et al., 2001). During bone formation, osteoblasts synthesize non-collagenous protein and type I collagen, which are partially secreted into the bloodstream (Lepage et al., 2001). Osteocalcin, also known as bone gla-protein (BGP), is non-collagenous, bone-specific protein that has been used extensively as a bone formation marker in past studies. Predominately synthesized by osteoblasts, osteocalcin is incorporated into the extracellular matrix, but a small fraction is released into circulation where it can be measured (Garnero and Delmas, 1996). However, since osteocalcin is part of the bone matrix, it is released into circulation during the resorptive phase as well (Kleerekoper, 1996). Additionally, it has been documented that BGP may also serve to regulate osteoclastic recruitment and regulation (Rodan and Rodan, 1995). Research has shown that when resorption and formation are uncoupled, osteocalcin is indeed a specific bone formation marker. Yet

when resorption and formation are coupled, serum levels at any one time contain components of both formation and resorption (Kleerekoper, 1996) therefore osteocalcin may better be interpreted as indicative of overall bone turnover (Garnero and Delmas, 1996). In addition to osteocalcin, there are several other markers of bone activity based on the metabolism of type I collagen which is the sole collagen in the organic matrix of bone. During the conversion of type I procollagen to collagen, there is a cleavage of the carboxyterminal propeptide (PICP) from the carboxy-terminal end of the protein during posttranslational modification, which is then released into circulation (Melkko et al., 1990). Concentrations of PICP exist in a stoichiometric relationship to the molecules of type I collagen formed thus, serum PICP is used as an indicator of bone formation.

As collagen is incorporated into collagen fibers, cross-links are formed between the terminal, non-helical propeptide of one collagen molecule to the helical region of another collagen molecule. During resorption, these cross-linked peptides, known as the carboxyterminal telopeptide of type I collagen (ICTP), are released into circulation. Thus, ICTP is used a marker for bone resorption (Eriksen et al., 1993). An additional marker released from the bone matrix during degradation by osteoclasts is deoxypyridinoline (DPD). DPD is one of the two nonreducible pyridinium crosslinks present in collagen that serves to stabilize the collagen molecule (Lepage et al., 2001). These crosslinks cannot be reutilized in collagen synthesis and are not metabolized *in vivo* therefore they are excreted in the urine (Garnero and Delmas, 1996).

Many studies have shown the potential use of these markers in horses for tracking skeletal growth and development, and for studying the effects of factors such as

training and nutrition on skeletal adaptation (Price et al., 1995b; Price, 1998; Hoekstra et al., 1999; Hiney et al., 2000). Although biochemical markers are not suited to predict bone mass, they have been correlated with changes in bone mass and it has been suggested that they could be useful in predicting fracture risk. In humans these markers are successfully used to monitor effectiveness of osteoporosis therapy in patients (Kleerekoper, 1996). More recently, studies in humans have indicated that these markers can predict patients at risk of osteoporotic fracture (Ebeling and Akesson, 2001). Jackson et al. (2005) conducted a study of 165 2-year-old racehorses to determine the relationship between serum concentrations of bone markers early in the training season and development of dorsal metacarpal disease (DMD). The authors found that ICTP concentrations early in the training season were significantly higher in horses that subsequently developed DMD. Although not proven, it is conceivable that with enough research, these markers could be independently used to predict fracture risk in elite equine athletes.

CHAPTER III

MATERIALS AND METHODS

Management of Animals

Twenty-four long yearling Quarter Horses were blocked by gender and age, and randomly assigned to one of four diets. Horses were staggered so as to enter the study at 20 months of age. All horses were gathered at the Texas A&M Horse Center where they were vaccinated and de-wormed prior to entering the study. Animals were then transported to Steephollow Farm where they were individually housed in 4X4 meter stalls. Routine hoof care and parasite control was performed as necessary throughout the trial. All procedures for animal care and use for this study were reviewed and approved by the Texas A&M University Agricultural Animal Care and Use Committee.

Experimental Diets

The four experimental diets were formulated to contain varying levels of Ca, P and Mg while meeting or slightly exceeding NRC (1989) recommendations for all other nutrients (Table 1).

Table 1. Calculated nutrient profiles of the concentrates (as fed basis)[a]

Nutrient	Diet A	Diet B	Diet C	Diet D[b]
Dry Matter, %	85.9	86.1	86.3	86.3
Protein, %	14.1	14	14.1	14.1
Crude Fat, %	5.1	5.1	5.0	5.0
Crude Fiber, %	2.1	3.2	3.4	5.4
DE, kcal/kg	3212	3115	3065	2924
Calcium, %	0.48	0.55	0.81	0.85
Phosphorus, %	0.25	0.30	0.44	0.52
Magnesium, %	0.12	0.15	0.22	0.23
Potassium, %	0.51	0.69	0.74	0.84
Salt, %	0.48	0.76	0.75	0.75
Sulfur, %	0.17	0.18	0.19	0.18
Total Copper, ppm	57	57	57	58
Total Iron, ppm	90	90	130	96
Total Manganese, ppm	76	84	91	127
Total Zinc, ppm	127	131	135	160
Added Cobalt, ppm	0.2	0.2	0.2	0.2
Added Copper, ppm	51	51	51	51
Added Iodine, ppm	0.1	0.1	0.1	0.1
Added Iron, ppm	29	29	29	29
Added Manganese, ppm	66	66	66	66
Added Selenium, ppm	0.3	0.3	0.3	0.3
Added Zinc, ppm	111	111	111	111
Vitamin A, IU/kg	84.18	84.18	84.18	84.18
Vitamin D, IU/kg	12.65	12.65	12.65	12.65
Vitamin E, IU/kg	144.8	144.8	144.8	144.8
Added Riboflavin, mg/kg	5.6	5.6	5.6	5.6
Added Thiamin, mg/kg	10.5	10.5	10.5	10.5
Lysine, %	0.76	0.76	0.76	0.76

[a]Calculated by Consolidated Nutrition, Ltd, Omaha, Nebraska

[b]Patriot 14-P, Consolidated Nutrition, Ltd., Omaha, Nebraska

An additional constraint for formulation of the diets was that the ratio of CA:P:Mg would be 3.5:2:1. It should be noted that diet D was a commercially available diet manufactured by Consolidated Nutrition, Ltd., Omaha, Nebraska. All concentrates were formulated, mixed and bagged by Consolidated Nutrition. Table 2 shows formulated nutrient profiles of the four diets as provided by Consolidated Nutrition. The total diet consisted of 40% coastal Bermudagrass hay and 60% of the assigned pelleted concentrate. All horses were backgrounded for a minimum of 1 week at the Horse Center were they were group housed in dirt-lot pens before being transported to Steephollow Farm. During this time, each horse received Diet D at approximately 2% of the average weight of the pen per day. Once they entered the study, the horses were individually fed their assigned diets at 12-hour intervals (0700 and 1900). The animals were given the entire 12 hours to consume the meal and any feed refusals were weighed and recorded. Adjustments in feed intake were made in order to maintain body condition between and score 5 to 6 and in order to allow for normal growth.

Table 2. Calculated mineral concentrations of diets (DM basis)[a]

	% Calcium	% Phosphorus	% Magnesium
Concentrations in total diet (60:40 concentrate:hay):			
Diet A	0.41	0.24	0.11
Diet B	0.48	0.29	0.13
Diet C	0.55	0.32	0.17
Diet D	0.63	0.40	0.18
Bermudagrass IFN# 1-09-209	0.30	0.19	0.11
Contribution from Bermudagrass (40% of total diet)	0.12	0.08	0.04
Contribution from concentrate portion (60% of total diet):			
Diet A	0.29	0.16	0.07
Diet B	0.36	0.21	0.09
Diet C	0.43	0.24	0.13
Diet D[b]	0.51	0.32	0.14

[a]Calculated by Consolidated Nutrition, Ltd, Omaha, Nebraska
[b]Patriot 14-P, Consolidated Nutrition, Ltd., Omaha, Nebraska

Training of Animals

The study was conducted in four, 32-day periods, each consisting of 28 days of race training followed by either a 4-day total fecal and urine collection or a 4-day rest period. The training protocol was designed to represent a typical commercial training regimen for racing Quarter Horses. During the first week, horses were ridden in a round pen and then moved to the track by the middle of the first week, depending on individual temperaments. For the first 3 days, horses were walked for 550 m, trotted for 110 m and galloped for 275 m. The distance galloped was increased 275 m per day for each day

after day 3 so that the horses were galloping a total of 1375 m by the end of week 1. During weeks 2 through 4 of the first period, horses were ridden 5 days per week at the same distances for the walk and the trot, but the gallop was increased to 8250 m. During the second period, the horses were ridden 4 days per week and the gallop was increased to 8440 m. In period 3, the horses were again ridden 4 days per week, with one of those days being designated as a sprint day. On sprint days, the horses were warmed up, sprinted for 230 m and then galloped. On non-sprint days, the horses were trotted 550 m and galloped 1925 m. During this time, the horses were galloped a total of 8210 m per week. Period 4 continued as in period 3 except the sprints were increased to 275 m and the gallop was decreased to a total of 8165 m per week. On rest days, the horses were walked for a minimum of 60 minutes on a mechanical walker.

Physical Measurements

Physical measurements of the animals were taken initially, and at 14-day intervals to monitor growth and analyze changes from pre-trial values. These measurements included body weight, body length, heartgirth circumference, height at the withers and hip, circumference of the forearm and gaskin and rump fat thickness. All measurements were taken in duplicate. Weight was determined using a certified livestock scale. Measurements of the heartgirth, forearm, gaskin and body length were taken using a plastic measuring tape. Placement markings were made by clipping the animal's hair, in order to ensure repeatability of the measurements. Heartgirth was determined using the top of the withers for placement of the measuring tape. Body

length was measured from the tuber ischii to the lateral tuberosity of the humerous. Height measurements were taken using a leveled height stick with the horse standing squarely on a flat, level surface. Rump fat thickness was determined using ultrasound at a point that was half way between the point of the hip and the point of the rump.

Sample Collection

Immediately prior to entering the study, and during the last 4 days of periods 2 and 4, horses were confined to tie stalls during which total urine and fecal output were collected. Feces were collected immediately upon defecation from a rubber mat placed beneath each horse. Every 3 hours, collections were thoroughly mixed, weighed and 10% aliquot was obtained and stored at - 20°C for later analysis. Urine was obtained via a standard urine collection harness. Urine output was quantified every 4 hours and a 10% sample was taken, strained through 4 layers of cheesecloth and stored at - 20°C for later analysis. During the total collection periods (beginning on d - 4, 60 and 124), horses were walked for 60 minutes daily on a mechanical walker in lieu of racetrack exercise. During this time urination was discouraged and any fecal matter was weighed and added to the daily totals, however none was added to the pooled sample. Hay and feed samples were taken daily during the collection periods. Fasting blood samples were taken prior to the morning meal at d - 4 (baseline), biweekly during periods 1 and 4 and weekly during periods 2 and 3. Additional blood samples were taken at the end of each 4-d rest period or total collection. All blood samples were collected at the same time

each day and were processed within 4 h of collection. Plasma and serum were harvested and stored at - 20°C for later analysis.

Analyses of Samples

Mineral analysis of feed, fecal and urine were conducted as part of a companion study (Stephens, 2002). Feed, hay and fecal samples were dried in a forced air oven at 62°C for 72 hours and ground in a Wiley mill with a 1 mm screen. Dried samples were digested using a perchloric acid digestion procedure and suspended in deionized, distilled water. Corn stalk reference material #8412 obtained from the National Institute of Standards and Technology was taken through each digestion and used as a control for mineral analysis. Ca and Mg concentrations in feed, fecal and urine samples were determined by atomic absorption spectrophotometry. Inorganic P was determined by colorimetry by the method of Fiske and Sabbarow (1925) using an Ultraspec III UV spectrophotometer. Mineral absorption and retention was determined by difference using mineral intake and excretion data.

Urine samples were analyzed for deoxypyridinoline by a solid-phase, chemiluminescent enzyme immunoassay using the Immulite® Pyrilinks-D kit from Diagnostic Products Corporation, Los Angeles, CA. In order to standardize deoxypyridinoline concentrations, urine was additionally analyzed for creatinine concentrations by colorimetry using the Ultraspec III UV spectrophotometer. Assay kits for creatinine were obtained from Sigma Diagnostics, Inc, St. Louis, MO. Plasma samples were analyzed for osteocalcin concentrations by radioimmunoassay (RIA) using

kits from DiaSorin, Stillwater, MN. Serum samples were analyzed for concentrations of the carboxyterminal telopeptide of type I collagen (ICTP) and the carboxyterminal propeptide of type I procollagen (PICP) by RIA using kits from DiaSorin. Amount of radioactive material was determined using a Packard Cobra II gamma counter. Inter- and Intra-assay coefficients of variations were determined using controls provided with the kits.

Statistical Analyses

Data were analyzed for group, day and group-by-day effects by analysis of variance using Stata Software (Stata Corp. 2001. College Station, TX). Where necessary, means were further separated using a Fisher-Hayter means comparison test. In order to visualize and analyze changes from baseline, data for each horse were normalized to baseline (day - 4) values. Data for physical measurements taken at d - 4 and d 128 were compared overall and by group using paired t-tests. In addition, linear regression with fixed time effects was used to identify the underlying relationship between mineral intake and biochemical markers. To facilitate interpretation, marginal effects in the form of elasticities were calculated for the model, setting all independent variables at the mean (Gujarati, 1999).

CHAPTER IV

RESULTS

Feed Intake

Total feed intake increased from d 0 to d 128 of the trial (Table 3). The increase in total feed intake can be explained by the increase in weight that was also seen in the horses as feed intake per body weight did not change throughout the trial. Feed intake corrected for body weight, and digested dry matter intake did not change significantly throughout the trial (Table 3).

Table 3. Feed intake and digestibility by day of trial (DM basis)

	D 0	D 64	D 128
Total Feed Intake (kg)	6.91[a]	7.48[b]	7.93[b]
SEM	0.25	0.36	0.23
Total Feed Intake (g/kg BW)	18.28	18.54	18.23
SEM	0.76	0.79	0.50
Total Digested Dry Matter Intake (kg)	4.89	5.03	5.27
SEM	0.19	0.23	0.14
Total Digested Dry Matter (g/kg BW)	12.94	12.47	12.14
SEM	0.54	0.49	0.31

[a,b]Rows means not sharing common superscripts differ (P<0.05)

There were significant differences in feed intake among the diet treatments (Table 4). Horses on diets A and C consumed less feed per day on average than did horses on diets B and D ($P<0.05$). However, when corrected for body weight, horses on diet C consumed significantly less ($P<0.05$) than the other groups (Table 4). Digested dry matter intake followed the same trends.

Table 4. Feed intake and digestibility by diet treatment (DM basis)

	Diet A	Diet B	Diet C	Diet D
Total Feed Intake (kg)	6.52[a]	8.26[b]	6.88[a]	7.97[b]
SEM	0.34	0.30	0.27	0.21
Total Feed Intake (g/kg BW)	17.59[a]	19.65[a]	16.66[b]	19.96[a]
SEM	0.94	0.59	0.73	0.52
Total Digested Dry Matter Intake (kg)	4.58[a]	5.93[b]	4.71[a]	5.18[a]
SEM	0.21	0.20	0.18	0.12
Total Digested Dry Matter (g/kg BW)	12.35[a,b]	13.70[a]	11.42[b]	13.01[a]
SEM	0.62	0.42	0.51	0.40

[a,b]Row means not sharing common superscripts differ ($P<0.05$)

Feed Analysis

Analyzed values for the Bermudagrass hay were similar to NRC (1989) values (Table 5). However, analyzed mineral concentrations in the concentrates were different from formulated values (Table 6). The analyzed Ca concentration in concentrates A and D (Patriot 14-P, Consolidated Nutrition) were less than formulated values while the concentration in concentrate B was greater than formulated values. Although designated as the high mineral diet, the concentrate used in diet D contained less Ca than did both

concentrates B and C. In the total diet, this resulted in a much narrower range in Ca concentrations among the treatments than was intended (Table 7). Phosphorus concentrations in all four concentrates were far less than formulated values (Table 7). This again resulted in a much narrower range in P concentrations among the diets. Analyzed Mg concentrations were similar to formulated values in the feeds and an acceptable range in Mg was achieved among the total diets (Table 7).

Table 5. Formulated versus analyzed mineral concentrations in Bermudagrass hay (DM basis).

	% Calcuim	% Phosphorus	%Magnesium
Bermudagrass IFN# 1-09-209	0.30	0.19	0.11
Analyzed Bermudagrass	0.32	0.15	0.11

(Stephens, 2002)

Table 6. Formulated (F) versus analyzed (A) mineral concentrations in the concentrate (DM basis).

	% Calcium		% Phosphorus		% Magnesium	
	F	A	F	A	F	A
Concentrate A	0.48	0.44	0.26	0.19	0.12	0.12
Concentrate B	0.60	0.64	0.35	0.33	0.15	0.14
Concentrate C	0.71	0.72	0.40	0.34	0.22	0.18
Concentrate D	0.85	0.55	0.54	0.36	0.23	0.19

(Stephens, 2002)

Table 7. Formulated (F) versus analyzed (A) mineral concentrations in the total diet (DM basis)

	% Calcium		% Phosphorus		% Magnesium	
	F	A	F	A	F	A
Diet A	0.41	0.39	0.24	0.17	0.11	0.11
Diet B	0.48	0.51	0.29	0.26	0.13	0.13
Diet C	0.55	0.56	0.32	0.26	0.17	0.15
Diet D	0.63	0.46	0.40	0.28	0.18	0.16

Groupings by Intake

The differences in analyzed mineral concentrations coupled with the differences in dry matter intake made reporting the data by use of assigned diets illogical. Therefore, data from all horses were arranged by Ca intake and horses were divided across diets into groups that corresponded to actual amounts of minerals consumed. The intake groups were designated as low (L), moderate (M), moderate-high (MH) and high (H) reflecting different mineral intakes (Table 8). The low group consumed an average

of 87.4 mg/kg BW·d^{-1} of Ca, 31.4 mg/kg BW·d^{-1} of P and 19.6 mg/kg BW·d^{-1} of Mg. The moderate group consumed on average 122 mg/kg BW·d^{-1} of Ca, 49 mg/kg BW·d^{-1} of P and 28.8 mg/kg BW·d^{-1} of Mg. The moderate high group consumed on average 136.3 mg/kg BW·d^{-1} of Ca, 65 mg/kg BW·d^{-1} of P and 36.8 mg/kg BW·d^{-1} of Mg. The high intake group consumed 153 mg/kg BW·d^{-1} of Ca, 66.3 mg/kg BW·d^{-1} of P and 37.8 mg/kg BW·d^{-1} of Mg (Table 8). Calcium intakes for the L, M, MH and H groups were 97%, 138%, 155% and 170% of NRC (1989) recommendations, respectively. Intakes of P and Mg ranged from 81% to 100% and 89% to 167% of NRC (1989) recommendations, respectively.

Table 8. Mineral consumption for horses grouped by calcium intake (mg/kg BW/d)

	Calcium	Phosphorus	Magnesium
Low	87.4	31.4	19.6
SEM	13.6	8.3	4.2
Moderate	122.0	49.0	28.8
SEM	2.9	7.4	5.0
Moderate High	136.3	65.0	36.8
SEM	4.8	4.1	2.5
High	153.0	66.3	37.8
SEM	8.8	5.2	4.0

Physical Measurements

Physical measurements were similar between the groups at d 0 and at d 128 (Table 9). The horses in the low mineral intake group gained significantly less ($P<0.05$) weight with an average gain of 37.50 kg when compared to horses in the high group that had an average gain of 65.22 kg. There were no differences between the groups in gain of wither height, hip height, body length, heartgirth circumference or rump fat thickness (Table 9).

Table 9. Influence of diet and training on parameters of growth

	Low	SEM	Moderate	SEM	Mod-High	SEM	High	SEM
Weight, kg								
Initial	390.80	23.81	386.90	18.69	382.99	15.39	368.80	8.96
Final	428.30	22.49	438.66	14.40	443.65	15.63	434.02	7.66
Gain	37.50[a]	7.27	51.76[a,b]	5.50	60.57[a,b]	6.88	65.22[b]	7.27
Wither Height, cm								
Initial	144.91	2.40	148.08	0.58	148.33	1.59	148.70	1.25
Final	148.97	2.17	152.02	0.95	153.93	1.37	152.09	1.39
Gain	4.06	0.95	3.94	0.61	5.59	0.97	3.39	0.71
Hip Height, cm								
Initial	150.62	2.37	152.90	0.95	153.42	1.37	151.77	0.89
Final	153.20	2.50	156.05	0.91	155.58	1.80	154.63	1.08
Gain	2.70	0.79	2.38	0.65	2.16	0.97	2.16	0.32
Body Length, cm								
Initial	162.69	3.65	161.86	2.64	166.56	3.17	161.66	1.59
Final	169.22	2.70	168.09	2.56	169.55	3.37	165.21	1.55
Gain	6.54	2.00	6.22	1.07	2.98	1.67	3.55	1.85
Heartgirth, cm								
Initial	169.29	2.96	170.50	1.33	170.34	2.69	170.13	1.74
Final	172.34	2.21	174.75	1.40	175.51	2.32	176.90	1.21
Gain	3.05	1.52	4.26	1.16	5.18	1.36	6.78	1.39
Rump Fat, mm								
Initial	4.40	0.92	3.90	0.87	3.60	0.64	3.58	0.68
Final	3.90	0.33	4.60	0.67	3.85	0.79	4.80	0.88
Gain	-0.50	1.20	0.70	1.11	0.25	1.08	1.25	0.67

[a,b]Row means not sharing common superscripts differ ($P<0.05$)

Urinary Deoxypyridinoline Crosslinks

Deoxypyridinoline (DPD) concentrations were expressed relative to creatinine concentrations in order to standardize for urine output. DPD concentrations were not different due to treatment, but did differ by day of trial ($P<0.05$) with increased mean concentrations at d 128 (Table 10).

Table 10. Mean urinary deoxypyridinoline crosslinks (DPD) in juvenile rachorses at varying mineral intakes

	DPD (nmol/mmol creatinine)			Δ DPD (nmol/mmol creatinine)		
	D0	D64	D128	D0	D64	D128
Low	37.77	151.18	135.10	0	128.77	112.69
SEM	15.45	63.62	12.74		64.52	12.14
Moderate	61.06	94.26	178.22	0	33.20	117.16
SEM	35.08	26.78	27.15		30.79	44.37
Moderate-High	97.36	103.74	204.42	0	6.39	87.54
SEM	46.47	35.67	38.02		62.19	60.11
High	113.47	88.61	235.31	0	-24.86	104.85
SEM	41.39	28.85	14.37		38.42	55.11
All Groups	79.13[a]	106.32[a]	187.67[b]	0	28.19	106.24
SEM	18.44	18.06	14.63		25.67	21.30

[a,b]Row means not sharing superscripts differ ($P<0.001$)

Due to the large variation among the groups at d0, the data were normalized to d 0 values. The normalized data again showed no effect of treatment (Table 10). At d 64, mean DPD concentrations in the low group appear to be higher than in the other three groups, yet due to the large variance in the data, the means were not statistically different (Figure 1). Although other studies have successfully used DPD concentrations in horses to describe bone resorption, the extremely high individual variance precludes statistical confidence at traditional levels in this study. DePriest et al. (1995) also reported high

day-to-day intra-individual variation in human subjects (between 10.7 and 15.5%) using an ELISA assay and over 25% using HPLC methods.

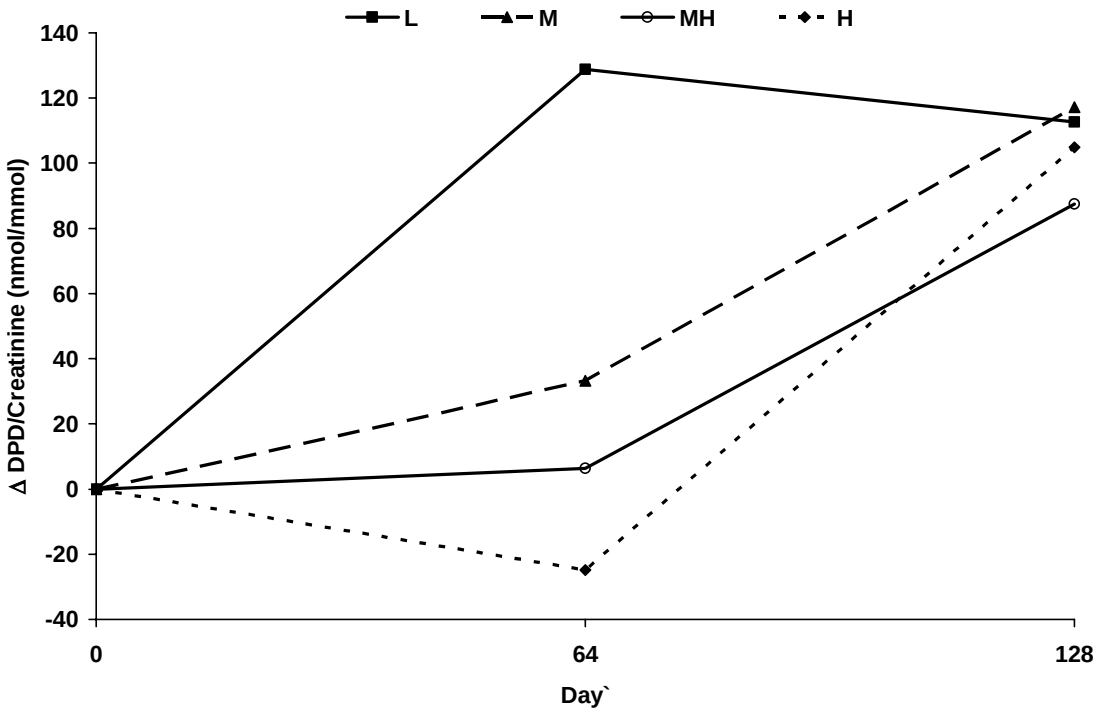

Figure 1. Mean change in urine DPD/creatinine concentrations in juvenile racehorses at varying mineral intakes.

Serum ICTP

Serum ICTP concentrations were not different between treatment groups, but did differ significantly ($P<0.001$) by day of trial. ICTP increased with the introduction of race training, from d 0 to d 14 ($P<0.01$), and remained elevated through d 60 (Figure 2;

Appendix 8). Concentrations during the second half of the training period (d 71 through d 124) were lower than those seen during the first half, however, concentrations were still higher than d 0 values with the exception of two days (d 78, d 85). At d 128, 4 days following the completion of training and the end of a 4-day total collection, ICTP concentrations were similar to pretrial values. Particularly noteworthy was a significant drop ($P<0.0001$) in ICTP concentrations that was seen at d 64 following 4 days of confinement concurrent with total urine and fecal collections. During the collection periods, horses were completely inactive for 96 hours with the exception of 1 hour per day when they were walked on a mechanical walker. ICTP concentrations increased again by d 71 ($P<0.05$) following the return to training.

When data were normalized, there was a significant effect of treatment as the low group had higher ICTP concentrations ($P<0.05$) on average than the moderate-high and high groups (Figure 3). This was particularly pronounced at d 60 (although there was not a significant treatment by day interaction) and toward the end of the training period when ICTP concentrations in the moderate-high and high groups had returned to d 0 values. Further, regression analysis revealed a significant negative relationship (Figure 4) between Ca intake and ICTP ($P<0.005$). Calculated elasticities for the relationship between Ca and ICTP from d 32 to d 128 indicated that each 1 % increase in Ca in the diet (g/day) led to an 18.9% decrease in serum ICTP. Serum ICTP intra-assay coefficient of variation (CV) was less than 5% and inter-assay CV was 5.7%.

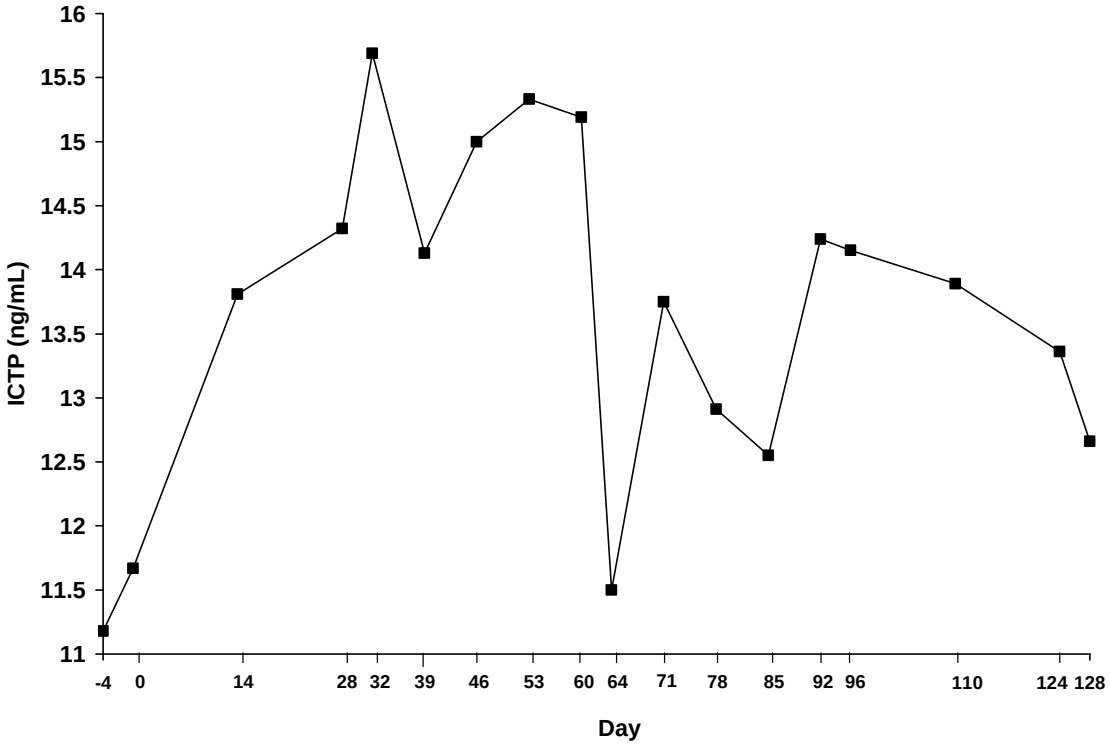

Figure 2. Mean serum ICTP concentration in juvenile racehorses by day of trial (all groups).

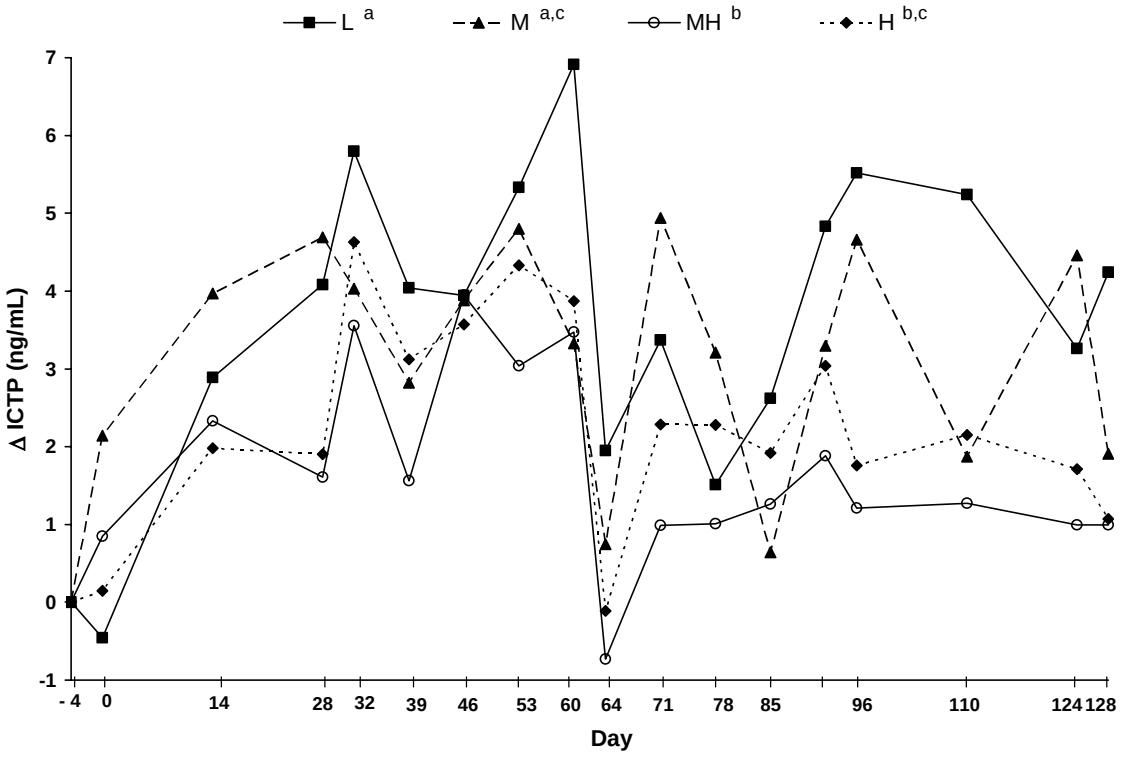

Figure 3. Mean change in serum ICTP concentrations in juvenile racehorses at varying mineral intakes. [a,b,c]Groups not sharing common superscripts differ ($P<0.05$).

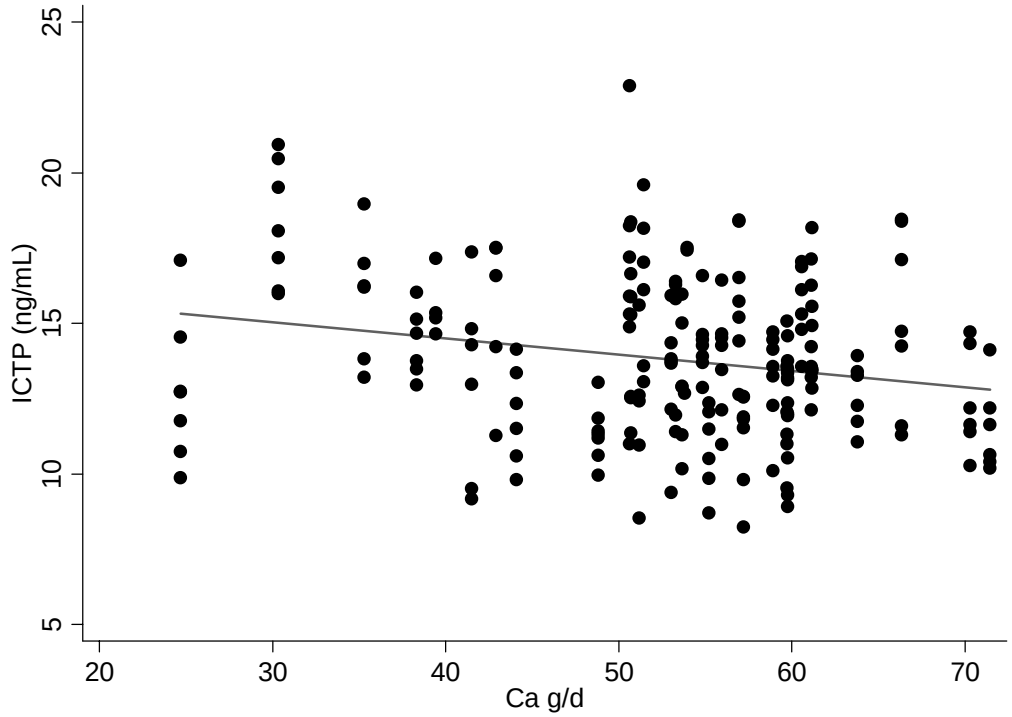

Figure 4. Relationship between ICTP and Ca intake in juvenile racehorses in training.

Serum PICP

Serum PICP was different by day of trial (P<0.001) with concentrations increasing significantly by 14 days into training and remaining elevated through d 110 (Figure 5, Appendix 10). By d 128 of training, concentrations were no longer different from d 0 values. Similar to the ICTP data, precipitous decreases in PICP were seen following 4 days of confinement at d 64 (P=0.08) and d 128 (P<0.05).

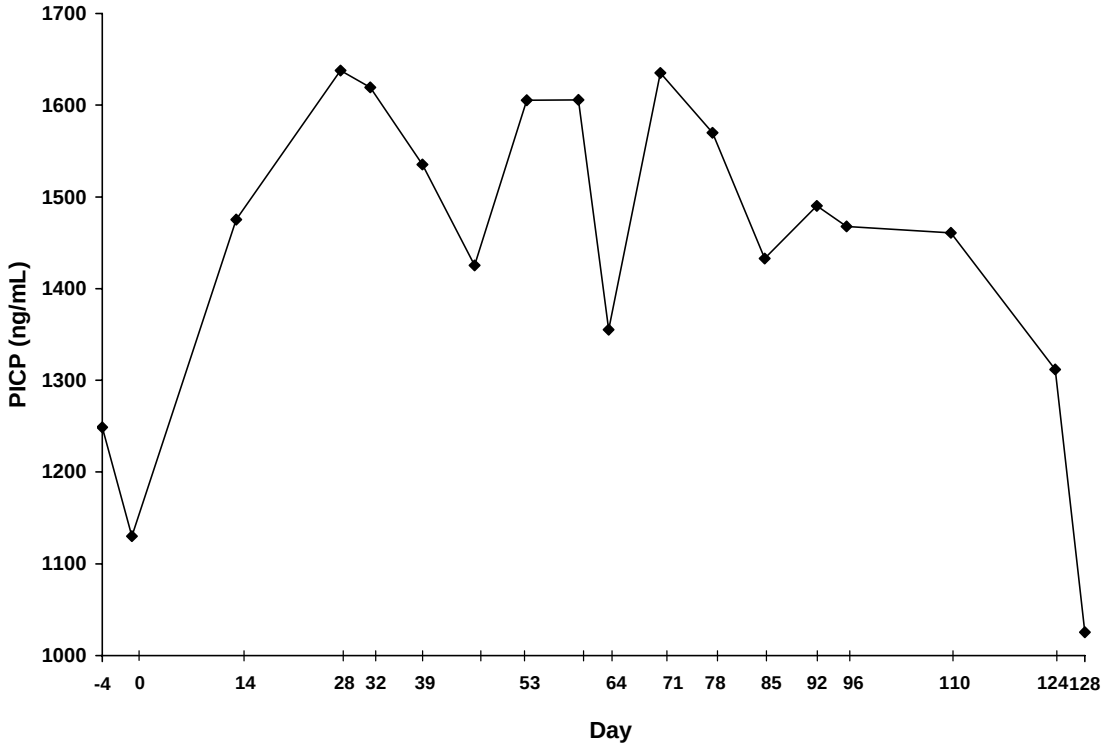

Figure 5. Mean serum PICP concentrations in juvenile racehorses by day of trial.

Additionally, there was a significant effect of treatment on PICP concentrations ($P<0.001$) with horses in the moderate-high and high groups exhibiting significantly higher concentrations than the two lower mineral intake groups. Horses in the low intake group exhibited the lowest concentrations with a trial average of 1,180.48 ng/mL, followed by the moderate group with a trial average of 1,382.08 ng/mL. Average concentrations for the moderate-high and high groups were similar at 1,619.26 ng/mL and 1,589.99 ng/mL, respectively (Appendix 8B). Regression analysis revealed a significant positive relationship (Figure 6) between Ca intake and PICP ($P<0.001$).

Calculated elasticities for the relationship between Ca and PICP from d 32 to d 128 indicated that each 1 % increase in Ca in the diet (g/day) led to a 32% increase in serum PICP.

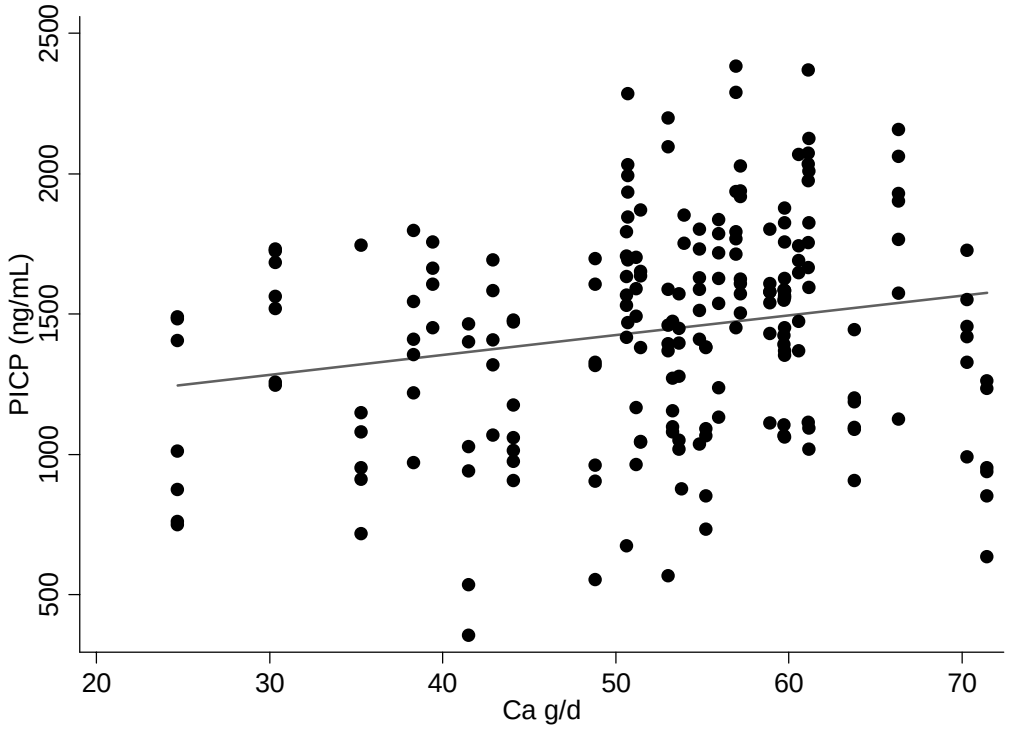

Figure 6. Relationship between PICP and Ca intake in juvenile racehorses in training.

When data were normalized, there was a similar effect of treatment ($P<0.001$) with the high intake group exhibiting significantly higher serum PICP than the low and moderate groups ($P<0.05$). This was particularly pronounced from d 56 to d 64,

although there was not a significant treatment by day interaction (Figure 7). Intra-assay CV for serum PICP was less than 5% and inter-assay CV was 3.9%.

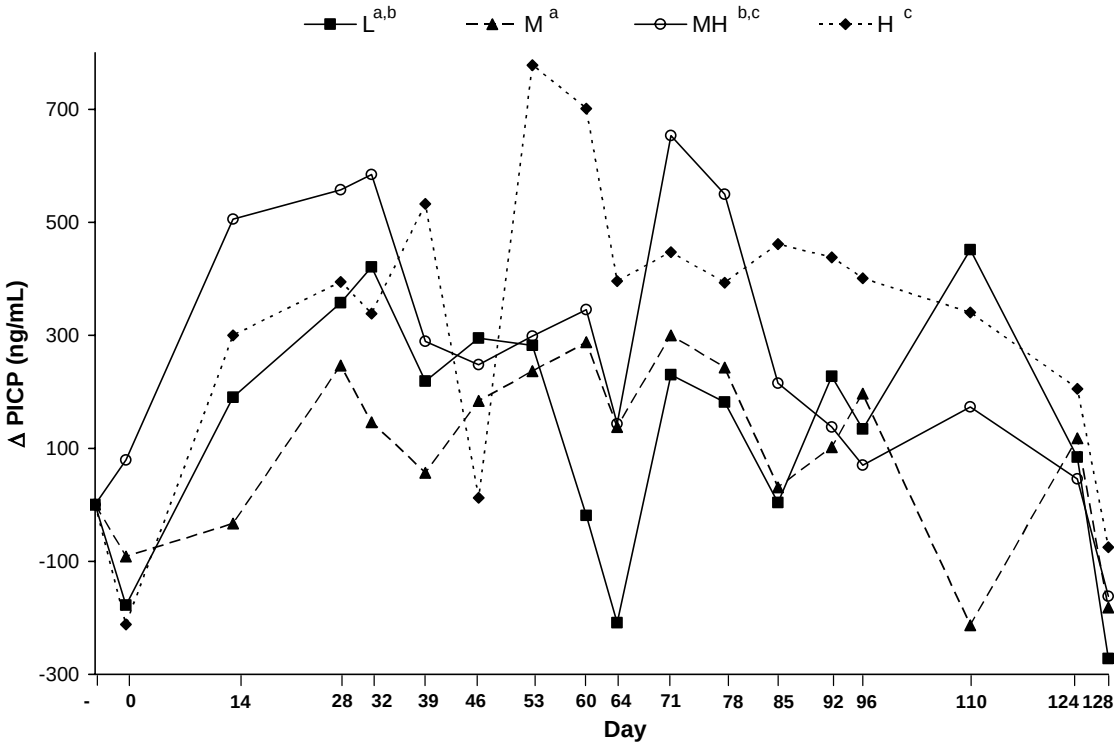

Figure 7. Mean change in serum PICP concentrations in juvenile racehorses at varying mineral intakes. [a,b,c]Groups not sharing common superscripts differ (P<0.05).

Plasma Osteocalcin

Osteocalcin concentrations differed by day of trial (Figure 8) with the same trend for decreased concentrations following total collections at d 0 (P<0.05) and d 64 (P=0.1). Although osteocalcin appeared to be somewhat elevated during training, values were not significantly different from baseline (d -4) at any time. Additionally, osteocalcin was

different by treatment ($P<0.05$), although the only difference was seen between the low and high intake group (Table 11). When data were normalized in order to take out variation at baseline, there was no effect of treatment (Table 11). Plasma osteocalcin intra-assay CV was less than 5% and inter-assay CV was 7.4%.

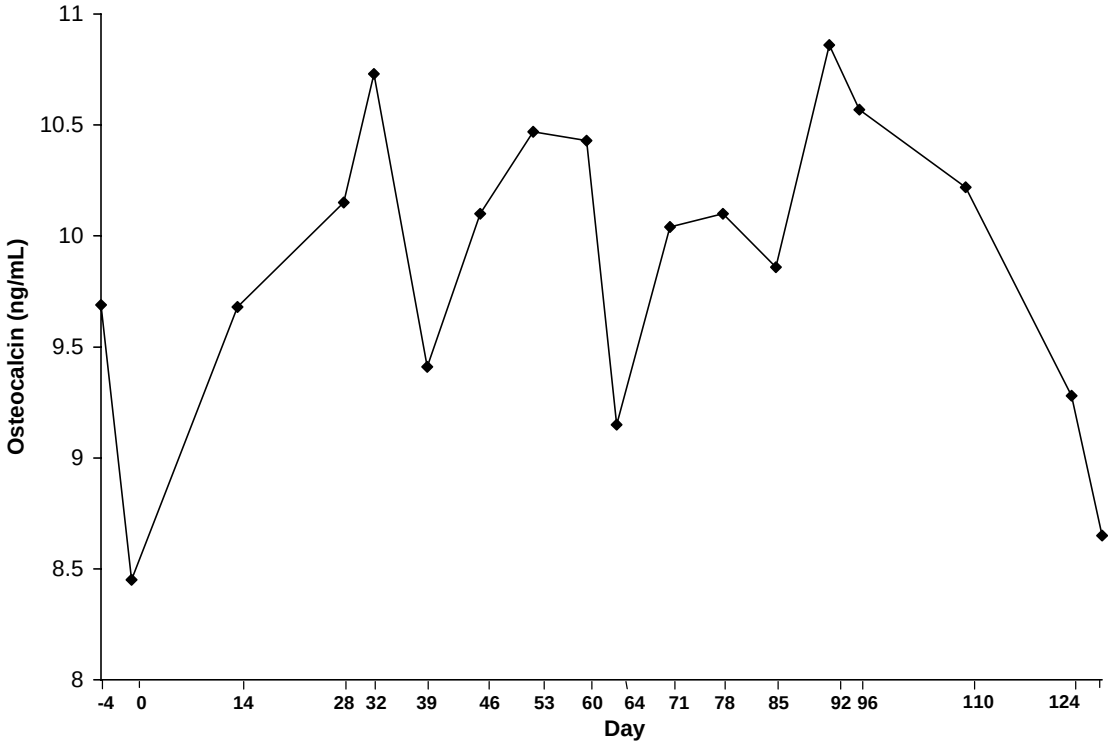

Figure 8. Mean plasma osteocalcin concentrations in juvenile racehorses by day of trial (all groups).

Table 11. Mean osteocalcin concentrations in juvenile racehorses at varying mineral intakes

	Low	Moderate	Mod-High	High
Osteocalcin, ng/ml	10.45[a]	9.87[a,b]	9.79[a,b]	9.45[b]
SEM	0.33	0.19	0.21	0.19
ΔOsteocalcin, ng/ml	0.65	-0.16	0.43	0.50
SEM	0.29	0.23	0.16	0.19

[a,b]Column means not sharing common superscripts differ (P<0.05)

CHAPTER V

DISCUSSION

Two complications with the study lead to reorganization of the animals from the predetermined diet treatment groups into groupings of animals based on their actual Ca intake. The first problem occurred with a suspected manufacturing inconsistency, in which the concentrates did not contain the formulated amounts of Ca, P and Mg specified in the research protocol. Second, feeding the horses to maintain a constant body condition during training instead of feeding to a percentage of body weight created an additional complication. Horses on the designated "low" feed were, in some cases, actually taking in more mineral than horses consuming a feed with higher mineral concentration. Reorganization of the horses into groups reflecting actual mineral intake per kg of body weight provided the opportunity to better evaluate the effects of varying mineral concentrations in the diet.

Exercise Effects

Exercising untrained horses at high speeds, such as in race training, increases both strain magnitude and strain rate thereby initiating skeletal adaptations.
In the present study, concentrations of ICTP and PICP increased significantly from baseline values with the onset of race training and remained elevated through the majority of the training period. The fact that both markers followed similar patterns indicates that resorption and formation in theses horses were coupled. Julen-Day et al.

(1997) also reported an increase in bone activity with exercise. Within just 14 days of training under saddle, osteocalcin concentrations increased significantly in 2-year-old horses. Other studies have not reported the same dramatic increase in bone markers with the introduction of exercise. In a study by Price et al. (1995b) of 2-year-old horses, PICP and ICTP decreased throughout the trial in both a control group and a group exercised 3 days per week on a high-speed treadmill. Although the authors attributed this to the normal age related decrease seen in bone markers, ICTP and PICP concentrations were significantly higher in the exercised horses. Age of the horses and intensity of the exercise program were two major differences between the study conducted by Price et al. (1995b) and the present study, and most likely contributed to the differences in the pattern of bone markers in the exercised animals. This age related decrease seen in biochemical markers has been shown in numerous studies. Lepage et al. (1990) reported a significant negative correlation between osteocalcin and age when comparing horses between birth and 20 years of age, indicating a slowing of the rate of bone formation in adults. Further, in a study by Price et al. (1995a), concentrations of bone markers such as bone specific alkaline phosphatase (BALP), ICTP and PICP decreased as horses aged, with the greatest changes occurring during the first 2 years.

As compared to the present study, a study by Hiney et al. (2000) of racehorses of a similar age put into a similar training protocol did not show increases in biochemical markers as quickly during the onset of race training, although ICTP did gradually increase throughout the trial. The reason for the discrepancy between these two studies is unclear since the age of the horses and the intensity of training was similar. However,

the horses in the study conducted by Hiney et al. were preconditioned for 14 weeks on a high-speed treadmill prior to initiation of training on the track. Additionally, even in a similar training protocol it is difficult to standardize speed and effort between horses; an issue compounded by different exercise riders. Design of the exercise program can result in differences in the way the bone remodels. In a study by Sherman et al. (1995) longer duration of training resulted in greater cortical area and dorsopalmer third metacarpal bone diameter in bone taken from 2, 3 and 4-year-old Thoroughbreds. Additionally, peak principle strain as well as peak strain rate increases linearly with increasing speed in the horse (Davies et al., 1993). Therefore, it is likely that the differences in exercise intensity between the present study and the study conducted by Hiney et al. (2000) accounted for the differences seen in the pattern of change in bone markers.

Higher concentrations of ICTP (bone resorption marker) during the first half of the training protocol in this study seem to agree with the decrease in bone density seen in other studies. In a study of long-yearlings in race training, Neilsen et al. (1997) reported decreased bone density at d 62 of training followed by a subsequent increase out to d 244. Decreased density of the lateral cortex was also seen at d 42 and d 56 of race training in 2-year-old horses (Julen-Day et al., 1997).

It is unclear why osteocalcin concentrations did not increase significantly due to introduction of exercise as did ICTP and PICP. Other studies have successfully used osteocalcin concentrations in horses to describe changes in bone activity due to age, and in response to exercise. The radioimmunoassay technique used in this study has been

used in other studies involving horses (Julen-Day et al., 1997; Black et al., 1999; Lepage et al., 1998; Hiney et al., 2000) and coefficients of variation in this study were low. However, degradation of osteocalcin molecules occurs within a few hours if serum is left at room temperature before being frozen. The assay used in this study employs polyclonal antibodies raised against bovine osteocalcin. The bovine RIA does not recognize the N-terminal midpeptide fragment of osteocalcin which may affect the sensitivity of the assay to detect significant changes (Garnero and Delmas, 1996). Blood samples in this study were placed on ice following collection, serum was separated by refrigerated centrifugation within 4 hours, and samples were immediately frozen. Although care was taken in handling of the samples it is possible that fragmentation of the intact osteocalcin molecule occurred causing lowered sensitivity of the assay.

As was mentioned earlier, DPD was disappointing in its effectiveness to monitor changes in bone resorption due to the high variance in the data. The method used for determination of DPD concentrations in this study was different than methods used in previous studies. The chemiluminescent enzyme immunoassay employed here was developed for use with the IMMULITE Automated Analyzer. This assay uses the same monoclonal antibody as an enzyme-linked immunosorbent assay from MetraBiosystems, Inc. While data from the company indicates that the two assays are equally valid, this may not be true. Similar problems with this particular assay have been reported in another study (Hiney, 1999). In addition, data from other studies involving horses have failed to show an effect of exercise on DPD. In a study by Hoekstra et al. (1999) initiation of exercise did not produce significant changes in DPD concentrations in

horses that were either confined or left on pasture. Black et al. (1997) also saw no change in DPD concentrations in Standardbreds subjected to a moderate exercise program. It must also be taken into consideration that DPD concentrations were analyzed in urine taken during confinement periods only. Given the data obtained from the other bone markers, it is possible that DPD concentrations might somehow be effected by inactivity of the animal thereby further complicating interpretation.

Effects of Confinement

Several studies have shown that disuse or reduction in physical activity causes a reduction in bone mass in many species (Merchant and Broom, 1986; Iwamoto et al., 2000; Bell et al., 2001). Stall confinement caused a decrease in bone density in yearling horses that remained low even after 56 d of conditioning (Hoekstra et al., 1999). Of particular interest in this study were the dramatic decreases seen in osteocalcin, PICP and ICTP following just 4 days of confinement and relative inactivity. Although not as dramatic, a similar decrease in bone markers was observed by Hiney et al. (2000). Osteocalcin, ICTP and PICP decreased in long-yearlings after 2 days of stall confinement following 14 weeks of treadmill training. Similarly, Nielsen et al. posited this effect after a pair of studies where decreased confinement periods of young horses resulted in less dramatic increases in bone remodeling (Neilson et al 1998, 1997). As the change in all serum markers in these studies were similar following confinement, it seems as though the juvenile equine skeleton responds to inactivity with an overall decrease in total bone activity. Human studies have documented significant increases in

bone resorption markers after just a few days of bed rest (Lueken et al., 1993). A study by Pedersen et al. (1995) reported a decrease in PICP concentrations and an increase in osteocalcin after just 3 days of bed rest in humans. These studies indicate that bone is sensitive to daily changes in physical activity and responds quickly to differences in strain. Further, blood-borne biochemical markers are sensitive enough to track daily changes in bone activity.

Diet Effects

In this study, Ca intake ranged from 97 to 170% of NRC (1989) recommendations. Horses in the low mineral intake group experienced greater bone resorption, as measured by ICTP, than horses in the two highest intake groups. Additionally, greater bone formation, as measured by PICP, was seen in horses in the high mineral intake group when compared to horses in the two lowest intake groups. Osteocalcin showed no differences due to mineral intake most likely for the reasons mentioned earlier. Given that bone formation and resorption were coupled, osteocalcin is probably best interpreted as indicative of total bone activity. Therefore greater amounts of bone formation coupled with lesser amounts of bone resorption in the high group would result in the same amount of total bone activity as horses experiencing the opposite. In agreement with these findings, Neilsen et al. (1998) evaluated Ca retention and bone mineral content in a group of young horses in race training fed a diet containing either 0.38% or 0.31% Ca. Greater Ca retention along with greater mineral content of the third metacarpus was reported in horses fed the high Ca diet. In a study of

mature horses introduced to exercise on a treadmill, increase in bone mineral content in response to training only occurred in horses fed diets containing 0.69% Ca, and greater increases in osteocalcin were observed in horses fed 0.69% Ca than in horses fed 0.35% Ca (Porr et al., 2000). In contrast, Schryver et al. (1978) reported no benefit in feeding a diet containing 0.6% Ca versus a diet containing 0.4% Ca to young Standardbreds put into training.

When evaluating the results of mineral intake, it is important to consider whether the requirements were based on a percentage basis in the diet, or as grams needed per day. It may often be assumed that as feed intake increases to meet the demands for energy associated with increased work, any additional mineral requirements will also be met. This would be expected when mineral concentrations are sufficient relative to energy and horses actually receive enough energy to support work. However, in young, exercising horses this is not necessarily the case. In the study conducted by Neilsen et al. (1998), when total Ca intake was considered, the control diet provided only 80 to 85% of NRC (1989) recommendations and the high mineral diet provided approximately 100% of recommendations. When examined from this perspective, it may not be appropriate to infer suitability of NRC recommendations based on studies where total daily intake is not evaluated. In the present study, treatment groups were based on absolute mineral intake. These data suggest that feeding Ca and other minerals at intakes greater than current NRC recommendations may have a protective effect on the bone. Calcium is directly associated with bone through its incorporation into

hydroxyapatite and removal of Ca from bone to meet metabolic demands during dietary Ca deficiency can result in a weakened skeleton.

Increased ICTP concentrations early in the training season have been correlated to development of dorsal metacarpal disease (DMD) (Jackson et al., 2005). Further, young horses with greater cortical mass in the lateral, medial and dorsal aspects of the third metacarpal in relation to the palmer aspect experienced fewer bone injuries during race training (Neilsen et al., 1997). Greater amounts of bone formation coupled with lesser bone resorption, as was seen in the high and moderate-high intake groups in the present study, would likely result in greater mineral density and therefore greater strength.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Of all the factors influencing the skeletal architecture in growing horses, physical activity is probably the most important. Stimulating the skeleton to remodel early on can better prepare these animals to withstand the intensity of racing. However, at some point, the beneficial effects of exercise are lost and bone failure can occur. Therefore the goal of any training program should be to adequately strengthen the skeleton while avoiding overtraining that can result in injury. Certainly the challenge of the racing industry is to reduce the number of injuries to young horses through the development of better training and management techniques, and possibly by monitoring the state of bone, particularly through the initial stages of training. Fatigue strain of immature bone is the primary cause of dorsal metacarpal disease, which leads to delays in training and loss of opportunity for competition. It has also been suggested that an additional cause of dorsal metacarpal disease is the repair process itself. The remodeling process causes an increase in the porosity of the bone during the resorption phase resulting in weakened bone. It has been proposed that the introduction of speed work in the majority of typical race training programs occurs at the point when the bone is at its weakest.

An additional challenge in management of skeletal development in these athletes is proper nutrition. As bone formation increases in response to exercise, so does the need for additional Ca and other minerals, but to what extent, researchers are still unsure. Even in humans there are incomplete data on how Ca requirements are modified due to different amounts of physical activity (Weaver, 2000).

The data presented here indicate that bone activity increases due to the introduction of race training in long-yearling horses. Further, since formation and resorption were coupled, it is likely that these horses were undergoing bone remodeling specifically rather than just bone modeling. Additionally, concentrations of the bone resorption marker were highest during the first 60 days of training which agrees with other studies that have reported decreased bone mineral density from around day 42 to day 60 of training. The traditional method of training young racehorses may actually be the most direct cause of injury as the bone is not being properly prepared. In most training programs, horses are conditioned at slow speeds for extended periods. Thus the bone has remodeled for the strains associated with long, slow, distance work and is unprepared for the loads associated with speed work. In traditional training programs, it is typically when speed work is introduced that problems arise. A suggested alternative is the introduction of short sprints early in training in order to induce changes that would prepare the bone for racing speeds. Hence, the bone adapts early on to the stress it will ultimately undergo without introducing the high strain, cyclic fatigue associated with long bouts of speed.

Of particular interest was the precipitous drop that occurred in ICTP, PICP and osteocalcin after just 4 days of the horses being placed in confinement. These data are in agreement with studies in humans that have documented significant responses of bone markers within days of introduction of bed rest. This reveals not only that bone responds rather quickly to daily changes in the amount of load upon it, but that some of these biochemical markers are sensitive enough to pick up daily changes in the resulting

bone activity. This dramatic response to inactivity helps to further support the idea that long periods of stall confinement can result in a negative outcome on the bone. In this study, skeletal disuse due to confinement triggered at least as much response as increased loading. Management practices of yearlings prior to training are often not considered by trainers to have any impact on the animal's career. Yearlings are typically confined to stalls in preparation for sales and at the conclusion of the fall sales, the horses will usually be placed immediately into a training program. The data presented here and in other studies indicates that the response of the skeleton to confinement is both rapid and dramatic. Thus many of these animals are beginning training with already weakened bone.

In addition to changes that were noted due to exercise, differences in bone marker concentrations were observed among the groups. Horses consuming lower amounts of Ca, P and Mg had significantly higher concentrations of the bone resorption marker which has been linked to greater risk for development of DMD. Higher mineral intakes resulted in greater amounts of bone formation coupled with lesser amounts of bone resorption suggesting a protective effect of these minerals on bone. Reducing the amount of bone resorption taking place should decrease porosity of the bone, thereby increasing its resistance to damage. Therefore, feeding Ca, P and Mg at levels above NRC (1989) recommendations appears to provide a protective effect on the bone during race training. Consequently current recommendations may not be sufficient to maximize bone strength. However, it is not possible to make any kind of specific recommendations based on these data alone. The effect of any one mineral was

confounded by varying levels of the other two minerals, particularly in regards to P and Mg. It would certainly be beneficial to study the individual effects each of these minerals may have on bone activity in young race horses.

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APPENDICES

1. INGREDIENT COMPOSITION OF THE CONCENTRATES[a]

Ingredient	Diet A	Diet B	Diet C	Diet Db
Corn	79.27	64.57	59.63	31.61
Oats	-	10.1	10	10
Soybean Meal	1	13.5	15	7.5
Wheat Middlings	-	-	3.8	39.79
Corn Gluten Meal	10.85	1.3	-	-
Yeast Culture	0.5	0.5	0.5	0.5
Calcium Carbonate	0.91	0.95	1.38	1.67
Monocalcium Phosphate	-	-	0.55	-
Salt	0.77	0.77	0.77	0.77
Magnesium Oxide	-	-	0.11	-
Potassium Chloride	0.21	-	-	-
Selenium 0.06%	0.05	0.05	0.05	0.05
Lysine	0.49	0.11	0.06	0.16
Flavoring	0.15	0.15	0.15	0.15
Trace Mineral Premix	0.1	0.1	0.1	0.1
Vitamin Premix	0.05	0.05	0.05	0.05
Fat	2	2.2	2.2	2
Molasses	3	5	5	5
Calcium Propionate	0.15	0.15	0.15	0.15
Binder	0.5	0.5	0.5	0.5

[a]Formulation provided by Consolidated Nutrition, Inc., Omaha, Nebraska
[b]Patriot 14-P, Consolidated Nutrition, Inc.

2. CALCIUM, PHOSPHORUS AND MAGNESIUM INTAKE

Horse	Day	Diet	Group	Total Ca Int (mg/kg BW/d)	Total P Int (mg/kg BW/d)	Total Mg Int (mg/kg BW/d)
1A	0	A	Low	111.54	43.21	30.04
2A	0	A	Low	144.06	55.34	38.04
6A	0	A	Low	111.07	42.22	28.06
1C	0	C	Low	103.16	39.19	26.53
2C	0	C	Low	72.02	27.86	19.34
5A	0	A	Moderate	180.17	68.32	46.15
1B	0	B	Moderate	128.80	49.56	34.15
3B	0	B	Moderate	144.79	55.03	37.29
5C	0	C	Moderate	107.57	40.8	27.56
1D	0	D	Moderate	145.40	55.59	37.98
3C	0	C	Moderate-High	143.73	54.68	37.09
2D	0	D	Moderate-High	165.74	63	42.7
4D	0	D	Moderate-High	162.28	61.7	41.82
6D	0	D	Moderate-High	138.49	52.42	35.32
4B	0	B	High	144.11	54.79	37.14
6B	0	B	High	139.34	52.85	35.71
4C	0	C	High	169.68	64.54	43.78
6C	0	C	High	115.99	44.2	30.05
5D	0	D	High	138.11	52.33	35.31
1A	64	A	Low	66.33	21.97	15.44
2A	64	A	Low	89.99	27.4	17.89
6A	64	A	Low	80.46	24.43	15.9
1C	64	C	Low	106.37	37.22	25.96
2C	64	C	Low	94.75	33.31	23.38
5A	64	A	Moderate	117.45	36.19	23.93
1B	64	B	Moderate	125.28	40.66	26.13
3B	64	B	Moderate	123.55	40.11	25.8
5C	64	C	Moderate	124.32	43.41	30.21
1D	64	D	Moderate	121.32	51.12	35.4
3C	64	C	Moderate-High	139.34	48.72	33.96
2D	64	D	Moderate-High	139.60	58.91	40.25
4D	64	D	Moderate-High	128.18	54.07	37.06
6D	64	D	Moderate-High	137.80	58.13	39.88
4B	64	B	High	159.49	52.82	34.9
6B	64	B	High	152.49	49.35	31.6
4C	64	C	High	166.22	58.13	40.53
6C	64	C	High	141.15	49.34	34.38
5D	64	D	High	146.53	61.84	42.23

Horse	Day	Diet	Group	Total Ca Int (mg/kg BW/d)	Total P Int (mg/kg BW/d)	Total Mg Int (mg/kg BW/d)
1A	128	A	Low	101.64	31.08	20.82
2A	128	A	Low	107.28	31.45	21.12
6A	128	A	Low	86.88	25.33	17.02
1C	128	C	Low	145.25	47.5	35.41
2C	128	C	Low	112.50	36.53	27.29
5A	128	A	Moderate	124.77	35.79	24.06
1B	128	B	Moderate	127.66	55.54	27.03
3B	128	B	Moderate	128.23	55.58	26.85
5C	128	C	Moderate	117.96	38.15	28.54
1D	128	D	Moderate	135.34	55.72	39.22
3C	128	C	Moderate-High	133.90	43.77	32.64
2D	128	D	Moderate-High	146.82	60.24	42.45
4D	128	D	Moderate-High	82.74	35.66	24.74
6D	128	D	Moderate-High	120.40	49.65	34.93
4B	128	B	High	138.30	59.86	28.84
6B	128	B	High	134.17	57.83	27.63
4C	128	C	High	139.32	45.73	34.05
6C	128	C	High	133.24	43.28	32.33
5D	128	D	High	125.40	51.38	36.22

3. PHYSICAL MEASUREMENTS OF HORSES

Horse	Diet	Group	Day	Weight (kg)	Hip Height (cm)	Whither Height (cm)	Body Length (cm)	Heartgirth (cm)	Rump Fat (mm^2)
1A	A	L	0	365.47	149.23	146.05	156.85	164.78	8
1C	C	L	0	467.62	159.39	153.04	173.67	176.53	4
2A	A	L	0	343.68	147.96	140.34	156.21	164.78	3
2C	C	L	0	424.49	151.13	145.42	169.23	176.53	4
6A	A	L	0	352.76	145.42	139.70	157.48	163.83	3
1B	B	M	0	421.77	154.94	147.96	170.82	170.82	6
1D	D	M	0	344.13	149.86	146.05	155.58	168.91	3
3B	B	M	0	422.22	154.94	149.23	162.88	175.26	4.5
5A	A	M	0	339.14	152.40	147.96	157.48	167.32	1
5C	C	M	0	407.24	152.40	149.23	162.56	170.18	5
2D	D	MH	0	360.48	151.13	145.42	165.74	163.20	3.5
3C	C	MH	0	340.50	149.23	144.15	160.34	164.78	5.5
4D	D	MH	0	429.03	155.58	148.59	178.44	173.99	4.5
5B	B	MH	0	384.54	156.21	152.40	161.93	172.72	2.5
6D	D	MH	0	400.43	154.94	151.13	166.37	147.00	2
3D	D	H	0	347.31	151.13	146.69	159.39	165.10	5
4B	B	H	0	410.42	158.75	153.04	167.96	177.80	6
4C	C	H	0	356.39	154.94	150.50	165.10	168.59	4
5D	D	H	0	370.92	149.86	145.42	160.02	168.28	2.5
6B	B	H	0	365.92	152.40	150.50	158.75	170.82	2
6C	C	H	0	361.84	150.50	146.05	158.75	170.18	2
1A	A	L	64	371.83	152.40	149.86	161.29	168.59	4.5
1C	C	L	64	458.54	161.29	156.85	173.99	179.07	5
2A	A	L	64	354.12	149.86	145.42	157.48	162.24	2.5
2C	C	L	64	437.66	151.77	147.32	168.91	173.99	5
6A	A	L	64	376.82	147.96	142.24	159.39	166.37	4
1B	B	M	64	456.27	155.58	153.67	171.45	176.53	4.5
1D	D	M	64	363.20	152.40	148.59	157.48	168.91	3
3B	B	M	64	443.56	157.48	151.77	169.55	177.80	4

Horse	Diet	Group	Day	Weight (kg)	Hip Height (cm)	Whither Height (cm)	Body Length (cm)	Heartgirth (cm)	Rump Fat (mm^2)
5A	A	M	64	365.02	153.04	150.50	155.26	168.91	1.5
5C	C	M	64	424.94	153.67	151.13	159.39	172.72	4
2D	D	MH	64	379.54	-	-	-	-	-
3C	C	MH	64	363.65	175.90	146.69	163.20	167.96	2.5
4D	D	MH	64	444.01	159.39	153.04	177.80	175.90	4.5
5B	B	MH	64	417.23	158.12	156.21	163.51	176.21	3
6D	D	MH	64	427.21	154.94	153.04	165.10	176.53	3
3D	D	H	64	384.54	152.40	149.23	163.83	170.18	3
4B	B	H	64	440.38	156.21	152.40	167.64	179.07	8
4C	C	H	64	398.61	156.85	153.67	154.94	173.99	4
5D	D	H	64	381.36	151.13	147.32	153.67	169.55	3
6B	B	H	64	400.43	154.31	153.04	163.20	176.53	4
6C	C	H	64	381.81	152.40	149.86	154.31	174.63	2.5
1A	A	L	128	376.82	153.67	151.13	167.64	167.64	3
1C	C	L	128	491.23	160.02	156.21	175.26	179.07	3.5
2A	A	L	128	395.43	150.50	147.32	160.02	167.64	4
2C	C	L	128	472.16	150.50	146.69	173.99	175.26	4
6A	A	L	128	405.88	148.59	143.51	169.23	172.09	5
1B	B	M	128	467.62	155.58	153.67	174.63	176.53	4
1D	D	M	128	397.25	151.77	149.23	161.29	170.18	4
3B	B	M	128	465.35	158.75	154.31	173.04	178.44	3
5A	A	M	128	411.78	154.94	150.50	163.83	175.26	5
5C	C	M	128	451.28	154.94	152.40	167.64	173.36	7
2D	D	MH	128	412.23	154.31	153.67	165.74	168.91	7
3C	C	MH	128	400.43	150.50	149.23	167.01	171.45	2.75
4D	D	MH	128	476.25	159.39	155.58	182.88	177.80	3
5B	B	MH	128	471.25	160.02	157.48	167.64	181.61	3.5
6D	D	MH	128	458.09	153.67	153.67	164.47	177.80	3
3D	D	H	128	419.50	153.04	147.96	168.91	175.90	3.5

Horse	Diet	Group	Day	Weight (kg)	Hip Height (cm)	Whither Height (cm)	Body Length (cm)	Heartgirth (cm)	Rump Fat (mm^2)
4B	B	H	128	460.81	158.12	154.94	167.01	179.71	9
4C	C	H	128	438.56	156.85	154.94	166.37	176.85	5
5D	D	H	128	407.69	151.13	147.96	162.56	172.09	3
6B	B	H	128	444.92	155.58	154.94	167.64	180.34	4.5
6C	C	H	128	432.66	153.04	151.77	158.75	176.53	4

4. DPD CONCENTRATIONS FOR ALL HORSES

Horse	Diet	Group	Day	DPD (nmol/day)	Creatinine (µmol/day)	DPD/Creatinine (nmol/mmol)
1A	A	L	0	720.791	33536.53	21.493
1A	A	L	64	1021.52	3657.63	279.285
1A	A	L	128	944.748	5944.39	158.931
2A	A	L	0	709.562	24517.5	28.941
2A	A	L	64	890.078	22277.2	39.955
2A	A	L	128	591.874	4539.81	130.374
5A	A	M	0	1204.158	50863.41	23.674
5A	A	M	64	1068.547	21743.94	49.142
5A	A	M	128	1284.088	5014.93	256.053
6A	A	L	0	927.725	9352.59	99.194
6A	A	L	64	-	-	-
6A	A	L	128	-	-	-
1B	B	M	0	1016.544	19863.46	51.177
1B	B	M	64	1184.24	15284.29	77.481
1B	B	M	128	1316.869	5911.85	222.751
3B	B	M	0	1037.151	78780.61	13.165
3B	B	M	64	982.768	6198.79	158.542
3B	B	M	128	1064.385	7738.91	137.537
4B	B	H	0	821.063	46485.37	17.663
4B	B	H	64	828.734	40987.85	20.219
4B	B	H	128	1334.438	6189.93	215.582
5B	B	MH	0	1287.12	8932.75	144.09
5B	B	MH	64	1422.988	76197.63	18.675
5B	B	MH	128	993.6	7972.03	124.636
6B	B	H	0	817.976	6019.3	135.892
6B	B	H	64	1305.175	20342.8	64.159
6B	B	H	128	-	-	-
1C	C	L	0	801.158	39487.93	20.289
1C	C	L	64	1268.881	5246.05	241.874
1C	C	L	128	1284.894	12683.14	101.307
2C	C	L	0	879.7	46510.94	18.914
2C	C	L	64	1494.728	34281.17	43.602
2C	C	L	128	766.688	5118.86	149.777
3C	C	MH	0	767.97	18585.25	41.321
3C	C	MH	64	1304.741	10773.68	121.105
3C	C	MH	128	1431.795	5143.25	278.383
4C	C	H	0	1012.42	43893.76	23.065

Horse	Diet	Group	Day	DPD (nmol/day)	Creatinine (µmol/day)	DPD/Creatinine (nmol/mmol)
4C	C	H	64	1338.343	11432.65	117.063
4C	C	H	128	-	-	-
5C	C	M	0	1126.569	5662.83	198.941
5C	C	M	64	1178.76	7559.96	155.921
5C	C	M	128	1366.25	8198.16	166.653
6C	C	H	0	917.993	4327.23	212.144
6C	C	H	64	1164.41	33085.61	35.194
6C	C	H	128	1321.172	6301.1	209.673
1D	D	M	0	888.633	48416.8	18.354
1D	D	M	64	612.203	20261.47	30.215
1D	D	M	128	550.938	5096.81	108.095
2D	D	MH	0	939.004	48749.29	19.262
2D	D	MH	64	1281.044	5950.03	215.3
2D	D	MH	128	-	-	-
3D	D	H	0	1020.662	24354.56	41.908
3D	D	H	64	805.838	10062.51	80.083
3D	D	H	128	1290.066	5291.89	243.782
4D	D	MH	0	1242.45	54383.11	22.846
4D	D	MH	64	1411.235	41064.36	34.366
4D	D	MH	128	-	-	154.903
5D	D	H	0	798.244	3191.43	250.121
5D	D	H	64	921.384	4286.61	214.945
5D	D	H	128	1370.925	5036.54	272.196
6D	D	MH	0	1341.251	5173.31	259.263
6D	D	MH	64	1080.095	8355.54	129.267
6D	D	MH	128	1406.681	5415.46	259.753

5. ANOVA TABLE FOR DEOXYPYRIDINOLINE

Source	df	SS	MS	F-value	P-value
DPD					
Model	11	167.977.144	15270.649	2.57	0.0126
Group	3	14656.179	4885.393	0.82	0.4892
Day	2	118456.915	59228.457	9.95	0.0003
Group *Day	6	37523.553	6253.925	1.05	0.4058
Error	46	273851.373	5953.291		
Total	57	441828.516	7751.377		
Normalized DPD					
Model	11	172955.628	15723.239	2.32	0.0231
Group	3	24301.6714	8100.557	1.20	0.3222
Day	2	105488.982	52744.491	7.78	0.0012
Group *Day	6	38451.027	6408.504	0.95	0.4723
Error	46	311812.740	6778.538		
Total	57	484768.368	8504.708		

6. ICTP, PICP AND OSTEOCALCIN CONCENTRATIONS

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
1A	A	L	-4	948.1	8.4	9.3624
1A	A	L	0	749.7	8.63	9.15
1A	A	L	14	1181.25	12.15	8.8538
1A	A	L	28	1222.35	12.7	10.562
1A	A	L	32	1417.7	14.64	12.416
1A	A	L	39	1483.05	11.76	9.9598
1A	A	L	46	1404.7	14.542	14.178
1A	A	L	53	1489.15	12.7	12.192
1A	A	L	60	1010.45	17.082	12.275
1A	A	L	64	760.9	10.73	8.0281
1A	A	L	71	748.55	12.73	8.3986
1A	A	L	78	875.55	9.87	9.527
1A	A	L	85	1218.35	12.94	12.206
1A	A	L	92	1410.45	16.03	11.464
1A	A	L	96	1355.25	15.14	-
1A	A	L	110	1796.95	13.48	15.072
1A	A	L	124	1543.2	14.66	13.373
1A	A	L	128	970.5	13.76	12.098
2A	A	L	-4	1059.4	9.1	9.8575
2A	A	L	0	1522.75	9.48	10.799
2A	A	L	14	1503.65	11.94	12.247
2A	A	L	28	1665.55	11.92	11.809
2A	A	L	32	1645.75	14.65	12.208
2A	A	L	39	668.95	10.63	6.751
2A	A	L	46	1256.7	12.914	11.065
2A	A	L	53	1165.9	13.601	-
2A	A	L	60	-	-	-
2A	A	L	64	-	-	-
2A	A	L	71	-	-	-
2A	A	L	78	-	-	-
2A	A	L	85	-	-	-
2A	A	L	92	-	-	-
2A	A	L	96	-	-	-
2A	A	L	110	-	-	-
2A	A	L	124	-	-	-
2A	A	L	128	-	-	-
5A	A	M	-4	1166.9	13.84	10.433
5A	A	M	0	1140.5	15.79	6.0325
5A	A	M	14	858.4	19.251	5.5364
5A	A	M	28	1053.95	16.58	10.974
5A	A	M	32	818.65	14.45	9.009
5A	A	M	39	1407.15	17.49	11.228
5A	A	M	46	1692.15	17.51	11.823
5A	A	M	53	1317.55	16.572	-

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
5A	A	M	60	1582.35	14.22	9.9799
5A	A	M	64	1068.05	11.26	9.9282
5A	A	M	71	-	-	-
5A	A	M	78	-	-	-
5A	A	M	85	1635.1	13.06	11.294
5A	A	M	92	1381.25	18.15	15.352
5A	A	M	96	1870.6	19.59	12.223
5A	A	M	110	1044.35	16.1	11.863
5A	A	M	124	1652.3	17.018	10.55
5A	A	M	128	1043.55	13.59	8.8229
6A	A	L	-4	1134.05	11.2	13.116
6A	A	L	0	766.8	9.98	10.978
6A	A	L	14	1491.7	14.47	-
6A	A	L	28	1585.55	19.06	14.91
6A	A	L	32	1623.95	19.31	14.413
6A	A	L	39	1682.6	20.93	14.728
6A	A	L	46	1257.4	15.97	12.908
6A	A	L	53	1563	20.47	14.117
6A	A	L	60	1518.4	19.506	14.429
6A	A	L	64	1246.8	17.17	15.074
6A	A	L	71	1731.25	18.06	15.525
6A	A	L	78	1723.7	16.07	14.611
6A	A	L	85	1080.15	13.19	12.45
6A	A	L	92	1148.8	16.97	15.175
6A	A	L	96	951.95	16.19	10.957
6A	A	L	110	1745.05	18.963	10.984
6A	A	L	124	910.35	13.81	9.8432
6A	A	L	128	716.8	16.228	9.4267
1B	B	M	-4	1935.75	9.46	8.6404
1B	B	M	0	1251.35	10.997	8.7702
1B	B	M	14	1488.45	11.51	7.7513
1B	B	M	28	1570.05	13.82	8.4064
1B	B	M	32	1419.55	11.57	9.0132
1B	B	M	39	1503.45	11.51	7.9875
1B	B	M	46	1937.6	11.89	8.8647
1B	B	M	53	2026.05	12.562	7.1348
1B	B	M	60	1608.15	12.55	9.5338
1B	B	M	64	1571.75	8.22	7.4246
1B	B	M	71	1623.6	11.81	9.603
1B	B	M	78	1917.8	9.8	7.8305
1B	B	M	85	1061.9	9.28	8.2201
1B	B	M	92	1557.4	10.524	8.3317
1B	B	M	96	1368.55	12.34	9.1503
1B	B	M	110	1582.85	11.93	8.6191
1B	B	M	124	1565.1	13.106	8.2371

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
1B	B	M	128	1353.35	13.76	9.2157
3B	B	M	-4	1268.55	10.1	
3B	B	M	0	1390.8	12.359	8.4577
3B	B	M	14	1261.05	14.34	10.383
3B	B	M	28	1951.75	13.18	11.422
3B	B	M	32	1870	15.365	11.33
3B	B	M	39	1513.45	14.46	10.119
3B	B	M	46	1036.05	14.62	10.856
3B	B	M	53	1586.4	16.58	10.105
3B	B	M	60	1731.7	13.69	11.757
3B	B	M	64	1628.35	12.86	10.474
3B	B	M	71	1801.9	13.9	11.67
3B	B	M	78	1409.9	14.267	12.708
3B	B	M	85	1392.5	9.52	11.186
3B	B	M	92	1585.05	11.3	13.32
3B	B	M	96	1548.2	13.57	11.165
3B	B	M	110	1105.4	10.98	11.025
3B	B	M	124	1423.55	15.07	10.494
3B	B	M	128	1065.6	12.05	8.9471
4B	B	H	-4	1055.95	12.385	8.0253
4B	B	H	0	923.65	10.029	7.9605
4B	B	H	14	1063.8	11.77	8.8415
4B	B	H	28	1611.3	12.79	10.543
4B	B	H	32	1684.75	12.72	8.8269
4B	B	H	39	1550.75	11.39	8.9416
4B	B	H	46	991.75	12.18	8.3944
4B	B	H	53	-	-	-
4B	B	H	60	1725.55	14.33	7.2255
4B	B	H	64	1418.35	10.26	8.8828
4B	B	H	71	1455.15	11.63	6.803
4B	B	H	78	1328.35	14.712	8.2876
4B	B	H	85	1089.6	11.74	7.0539
4B	B	H	92	1094.45	13.27	6.6187
4B	B	H	96	1200.75	11.05	7.9896
4B	B	H	110	1187.45	13.383	7.3531
4B	B	H	124	1443.5	12.27	7.4726
4B	B	H	128	906.85	13.91	5.977
5B	B	MH	-4	1322.05	14	9.6779
5B	B	MH	0	-	-	-
5B	B	MH	14	-	-	-
5B	B	MH	28	1535.2	10.74	8.2672
5B	B	MH	32	1664.2	14.85	9.5537
5B	B	MH	39	1946.05	18.68	-
5B	B	MH	46	1484.5	20.827	7.6671
5B	B	MH	53	-	-	-

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
5B	B	MH	60	-	-	-
5B	B	MH	64	-	-	-
5B	B	MH	71	-	-	-
5B	B	MH	78	-	-	-
5B	B	MH	85	-	-	-
5B	B	MH	92	-	-	-
5B	B	MH	96	-	-	-
5B	B	MH	110	1820.1	14.97	8.7615
5B	B	MH	124	-	-	-
5B	B	MH	128	-	-	-
6B	B	H	-4	1108.1	9.41	6.9841
6B	B	H	0	689.4	12.143	6.0363
6B	B	H	14	1407.45	10.92	8.4965
6B	B	H	28	1649.2	14.31	8.7801
6B	B	H	32	880.5	16.646	7.4572
6B	B	H	39	1753.05	17.12	9.5313
6B	B	H	46	1114.6	13.206	6.6256
6B	B	H	53	2368.75	14.21	9.8199
6B	B	H	60	2034	13.45	-
6B	B	H	64	1975.75	12.11	9.4038
6B	B	H	71	1665.45	16.26	8.5143
6B	B	H	78	2072.2	13.57	8.993
6B	B	H	85	1876.8	8.9	8.1219
6B	B	H	92	1627.2	14.57	8.5033
6B	B	H	96	1582.6	13.48	9.1132
6B	B	H	110	1823.9	13.35	9.5385
6B	B	H	124	1756.65	13.179	8.2401
6B	B	H	128	1451.55	11.98	7.8697
1C	C	L	-4	1057.8	9.29	-
1C	C	L	0	720.6	10.44	9.4446
1C	C	L	14	803.5	10.51	9.6348
1C	C	L	28	1446.6	12.459	10.862
1C	C	L	32	1466.65	14.8	9.7866
1C	C	L	39	960.45	11.413	8.8596
1C	C	L	46	1315.7	11.3	10.002
1C	C	L	53	1328.6	11.843	9.4277
1C	C	L	60	553.6	13.03	8.271
1C	C	L	64	904.7	10.611	9.1627
1C	C	L	71	1605.8	9.95	9.5543
1C	C	L	78	1696.7	11.176	10.351
1C	C	L	85	851.85	10.63	7.8038
1C	C	L	92	1261.6	10.39	10.564
1C	C	L	96	1234.85	14.11	10.918
1C	C	L	110	952.05	12.17	9.4466
1C	C	L	124	938.7	10.19	8.5406

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
1C	C	L	128	636.3	11.62	7.9868
2C	C	L	-4	964.45	11.34	6.9002
2C	C	L	0	516.75	8.5006	4.7701
2C	C	L	14	1136.3	14.71	7.591
2C	C	L	28	1031.45	13.61	6.4705
2C	C	L	32	1114.1	14.92	8.1903
2C	C	L	39	1463.6	14.81	8.0389
2C	C	L	46	1401.75	14.29	8.1202
2C	C	L	53	1027.85	17.36	7.7855
2C	C	L	60	-	-	-
2C	C	L	64	355.35	9.5	4.0621
2C	C	L	71	940.1	12.97	6.7602
2C	C	L	78	534.4	9.1529	4.4928
2C	C	L	85	-	-	-
2C	C	L	92	-	-	-
2C	C	L	96	-	-	-
2C	C	L	110	-	-	-
2C	C	L	124	-	-	-
2C	C	L	128	-	-	-
3C	C	MH	-4	1635.8	11.11	7.5751
3C	C	MH	0	1325.95	12.77	7.3911
3C	C	MH	14	1751	14.59	9.1284
3C	C	MH	28	1998.65	16.37	8.6177
3C	C	MH	32	2091	15.75	9.4081
3C	C	MH	39	2285.45	15.27	9.205
3C	C	MH	46	1992.2	18.352	9.4816
3C	C	MH	53	1692.05	15.87	9.3942
3C	C	MH	60	1932.65	16.649	8.4942
3C	C	MH	64	1469.25	12.51	8.1045
3C	C	MH	71	2032	11.36	7.9046
3C	C	MH	78	1843.9	12.56	10.147
3C	C	MH	85	1395.55	15.95	-
3C	C	MH	92	1449.05	15.007	7.6175
3C	C	MH	96	1571.15	11.29	-
3C	C	MH	110	1017	12.89	7.419
3C	C	MH	124	1278.55	12.91	7.4489
3C	C	MH	128	1048.95	10.17	6.2501
4C	C	H	-4	1420.95	11.96	9.3826
4C	C	H	0	1367.55	12.16	10.617
4C	C	H	14	1734.65	17.76	10.919
4C	C	H	28	2256.15	14.666	12.068
4C	C	H	32	2167.15	16.14	12.063
4C	C	H	39	2156.9	14.73	10.648
4C	C	H	46	1125.05	18.37	11.127
4C	C	H	53	2061.7	18.44	12.294

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
4C	C	H	60	1929.6	17.1	11.645
4C	C	H	64	1765.85	11.58	11.564
4C	C	H	71	1902.8	11.29	12.204
4C	C	H	78	1574.25	14.23	10.948
4C	C	H	85	2124.25	18.17	11.501
4C	C	H	92	1824.35	14.91	12.435
4C	C	H	96	2009	15.55	13.121
4C	C	H	110	1593.95	13.47	9.8792
4C	C	H	124	1093.75	13.41	9.9958
4C	C	H	128	1018.25	12.84	8.1353
5C	C	M	-4	898.75	9.76	9.5474
5C	C	M	0	1197.3	13.89	10.307
5C	C	M	14	1372.2	14.05	9.9038
5C	C	M	28	1683.55	17.91	11.336
5C	C	M	32	1616.75	18.5	13.843
5C	C	M	39	674.15	10.989	7.2387
5C	C	M	46	1565.9	17.182	-
5C	C	M	53	1634.05	18.23	11.625
5C	C	M	60	1791.7	15.3	12.641
5C	C	M	64	1530.5	14.88	11.625
5C	C	M	71	1417	22.89	10.776
5C	C	M	78	1706.85	15.9	10.581
5C	C	M	85	1474.65	11.95	10.038
5C	C	M	92	1154.1	16.38	11.882
5C	C	M	96	1270.5	16.28	10.359
5C	C	M	110	-	-	-
5C	C	M	124	1098.4	15.804	9.9595
5C	C	M	128	1080.5	11.4	8.7991
6C	C	H	-4	1845.7	12.65	13.383
6C	C	H	0	1002.3	14.5	8.6886
6C	C	H	14	1765.7	17.09	13.076
6C	C	H	28	2054.75	16.48	12.086
6C	C	H	32	1626.3	17.78	14.289
6C	C	H	39	1750.9	17.43	-
6C	C	H	46	1851	17.517	-
6C	C	H	53	-	-	-
6C	C	H	60	-	-	-
6C	C	H	64	-	-	-
6C	C	H	71	-	-	-
6C	C	H	78	-	-	-
6C	C	H	85	-	-	-
6C	C	H	92	-	-	-
6C	C	H	96	-	-	-
6C	C	H	110	-	-	-
6C	C	H	124	-	-	-

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
6C	C	H	128	-	-	-
1D	D	M	-4	1017.25	10.11	10.761
1D	D	M	0	848.9	10.92	8.1576
1D	D	M	14	1141.95	13.98	8.9346
1D	D	M	28	1261.75	15.24	8.7854
1D	D	M	32	1292.6	13.53	8.663
1D	D	M	39	1472.3	-	8.7974
1D	D	M	46	974	11.49	9.6067
1D	D	M	53	907.15	13.35	8.4199
1D	D	M	60	1013.25	14.14	8.7137
1D	D	M	64	1175.8	9.79	7.7525
1D	D	M	71	1477.8	10.59	9.6715
1D	D	M	78	1058.35	12.32	9.3262
1D	D	M	85	876.7	12.675	8.7704
1D	D	M	92	-	-	-
1D	D	M	96	-	-	-
1D	D	M	110	-	-	-
1D	D	M	124	-	-	-
1D	D	M	128	-	-	-
2D	D	MH	-4	805.55	13.45	10.611
2D	D	MH	0	1559.55	14.706	8.298
2D	D	MH	14	2016.15	16.904	10.073
2D	D	MH	28	1911.3	17.9	10.936
2D	D	MH	32	2349.05	21.628	12.476
2D	D	MH	39	567.1	9.3749	6.0429
2D	D	MH	46	1460.65	13.66	10.412
2D	D	MH	53	1587.95	13.72	11.94
2D	D	MH	60	1369.5	15.909	10.863
2D	D	MH	64	1393.65	12.136	-
2D	D	MH	71	2095.3	14.35	12.097
2D	D	MH	78	2197	13.81	14.028
2D	D	MH	85	2067.35	13.554	-
2D	D	MH	92	1689.55	15.3	12.181
2D	D	MH	96	1473.35	17.032	11.38
2D	D	MH	110	1741.45	16.11	11.777
2D	D	MH	124	1645.95	14.79	-
2D	D	MH	128	1368.55	16.882	11.111
3D	D	H	-4	1529	12.23	-
3D	D	H	0	1443.45	10.97	8.4989
3D	D	H	14	1947.8	10.268	9.7806
3D	D	H	28	1977.8	10.51	9.3027
3D	D	H	32	2311.4	16.617	10.289
3D	D	H	39	2280.9	11.41	8.8321
3D	D	H	46	1382.2	13.35	8.8051
3D	D	H	53	2007	13.21	-

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
3D	D	H	60	2149.7	16.217	9.2407
3D	D	H	64	1854.75	9.73	7.4349
3D	D	H	71	2142.45	11.008	8.0811
3D	D	H	78	1619.25	11.94	9.0788
3D	D	H	85	1681.7	13.59	-
3D	D	H	92	2221.35	12.04	8.6774
3D	D	H	96	1887.65	11.313	-
3D	D	H	110	-	-	-
3D	D	H	124	-	-	-
3D	D	H	128	-	-	-
4D	D	MH	-4	1796.45	15.75	10.447
4D	D	MH	0	1693.1	15.6	-
4D	D	MH	14	1911.65	16.63	11.224
4D	D	MH	28	2492.8	15.73	-
4D	D	MH	32	2134.1	14.85	-
4D	D	MH	39	2289.9	15.2	12.095
4D	D	MH	46	1451	16.51	10.431
4D	D	MH	53	1768.35	18.43	11.156
4D	D	MH	60	1936.75	18.383	-
4D	D	MH	64	1713.65	12.63	-
4D	D	MH	71	2381.85	14.416	12.139
4D	D	MH	78	1791.8	15.72	-
4D	D	MH	85	1450.65	17.15	10.775
4D	D	MH	92	1755.35	15.18	-
4D	D	MH	96	1605.2	15.34	10.872
4D	D	MH	110	1662.95	14.65	11.782
4D	D	MH	124	-	-	-
4D	D	MH	128	-	-	-
5D	D	H	-4	1054.2	9.21	10.527
5D	D	H	0	1316.55	8.93	7.7547
5D	D	H	14	1892.35	11.91	11.416
5D	D	H	28	831.4	10.5	7.2272
5D	D	H	32	1374.05	15.724	10.884
5D	D	H	39	1717.5	14.51	10.175
5D	D	H	46	1625.25	14.65	11.213
5D	D	H	53	1785.65	14.26	11.191
5D	D	H	60	1836.4	13.46	10.555
5D	D	H	64	1131.5	10.966	8.5025
5D	D	H	71	1236.1	16.424	10.537
5D	D	H	78	1538.3	12.12	10.533
5D	D	H	85	1701.65	12.403	9.4215
5D	D	H	92	1590.3	15.59	-
5D	D	H	96	1491.8	12.6	10.697
5D	D	H	110	-	-	-
5D	D	H	124	1165.4	10.94	8.7537

Horse	Diet	Group	Day	PICP (ng/ml)	ICTP (ng/mL)	Osteocalcin (ng/mL)
5D	D	H	128	962.6	8.52	7.153
6D	D	MH	-4	1196.85	10	9.2562
6D	D	MH	0	1174.3	10.62	-
6D	D	MH	14	1776.7	11.51	10.117
6D	D	MH	28	1604.95	-	9.6897
6D	D	MH	32	1441.95	15.01	10.381
6D	D	MH	39	1112.45	13.57	9.595
6D	D	MH	46	1607.9	14.71	10.394
6D	D	MH	53	1579.55	14.45	-
6D	D	MH	60	1578.35	13.25	10.854
6D	D	MH	64	1430.7	10.1	8.9926
6D	D	MH	71	1539.25	14.13	10.384
6D	D	MH	78	1800.65	12.26	-
6D	D	MH	85	1382.05	8.69	9.1642
6D	D	MH	92	1090.8	12.35	9.9582
6D	D	MH	96	1065.35	11.48	9.4676
6D	D	MH	110	1380.65	12.05	9.5305
6D	D	MH	124	850.7	9.84	7.7835
6D	D	MH	128	734	10.49	9.3437

7A. ANOVA TABLE FOR ICTP

Source	df	SS	MS	F-value	P-value
ICTP					
Model	71	816.912	11.506	1.83	0.0004
Group	3	383.060	12.695	2.01	0.1124
Day	17	570.054	33.533	5.32	0.0000
Group *Day	51	192.452	3.774	0.60	0.9851
Error	249	1568.808	6.300		
Total	320	2385.720	7.455		
Normalized ICTP					
Model	71	913.776	12.870	2.29	0.0000
Group	3	173.282	57.761	10.26	0.0000
Day	17	559.385	32.905	5.85	0.0000
Group *Day	51	199.081	3.904	0.69	0.9412
Error	249	1401.577	5.629		
Total	320	2315.354	7.235		

7B. MEANS TABLE FOR ICTP BY GROUPINGS

	Low	Moderate	Mod-High	High
ICTP, ng/ml	13.30	13.69	14.31	13.33
SEM	0.35	0.31	0.30	0.25
ΔICTP, ng/ml	3.47^{a}	$3.06^{a,c}$	1.67^{b}	$2.2^{b,c}$
SEM	0.32	0.28	0.27	0.27

^{a,b,c}Column means not sharing common superscripts differ (P<0.05)

7C. FIXED EFFECTS MODEL OF DIETARY CA ON SERUM ICTP

Variable	Coefficient	SE	t	P
cagd	-0.05	0.02	-3.12	0.002
int1	16.80	1.01	16.61	0.000
int2	17.44	1.00	17.49	0.000
int3	18.05	1.00	18.06	0.000
int4	17.70	1.02	17.31	0.000
int5	14.15	1.00	14.09	0.000
int6	16.49	1.02	16.14	0.000
int7	15.54	1.02	15.21	0.000
int8	15.19	1.06	14.38	0.000
int9	17.10	1.07	16.01	0.000
int10	17.04	1.07	15.96	0.000
int11	16.53	1.10	15.03	0.000
int12	16.11	1.09	14.72	0.000
int13	15.41	1.09	14.08	0.000

F(14, 189)	477.89
Prob > F	0
Adj R-squared	0.9705
Number of obs	203

8A. ANOVA TABLE FOR PICP

Source	df	SS	MS	F-value	P-value
PICP					
Model	71	23892084.60	336508.234	2.98	0.0000
Group	3	9261961.08	3087320.360	27.30	0.0000
Day	17	8870328.74	521784.043	4.61	0.0000
Group *Day	51	5208570.65	102128.836	0.90	0.6612
Error	251	28389393.00	113105.152		
Total	322	52281477.60	162364.837		
Normalized PICP					
Model	71	16646861.100	234462.832	1.63	0.0034
Group	3	2795055.590	931685.196	6.46	0.0003
Day	17	8280761.970	487103.645	3.38	0.0000
Group *Day	51	5500992.670	107862.601	0.75	0.8926
Error	251	36173071.500	144115.823		
Total	322	52819932.600	164037.058		

8B. MEANS TABLE FOR PICP

	Low	Moderate	Mod-High	High
PICP, ng/ml	1180.48a	1382.08b	1619.26c	1589.99c
SEM	41.17	34.21	44.65	41.71
∆PICP, ng/ml	144.82a,b	103.41a	275.01b,c	315.53c
SEM	40.20	42.87	53.95	39.30

[a,b,c]Column means not sharing common superscripts differ (P<0.05)

8C. FIXED EFFECTS MODEL OF DIETARY CA ON SERUM PICP

Variable	Coefficient	SE	t	P
cagd	8.78	2.34	3.75	0.000
int1	1069.37	146.19	7.31	0.000
int2	983.84	146.19	6.73	0.000
int3	1169.92	146.59	7.98	0.000
int4	1118.42	149.97	7.46	0.000
int5	877.58	147.28	5.96	0.000
int6	1150.53	149.81	7.68	0.000
int7	1114.01	149.81	7.44	0.000
int8	941.36	154.85	6.08	0.000
int9	964.99	156.58	6.16	0.000
int10	963.61	156.58	6.15	0.000
int11	954.31	161.23	5.92	0.000
int12	826.24	160.48	5.15	0.000
int13	539.90	160.48	3.36	0.001

F(14, 190)	245.32
Prob > F	0
Adj R-squared	0.9476
Number of obs	204

9. ANOVA TABLE FOR OSTEOCALCIN

Source	df	SS	MS	F-value	P-value
Osteocalcin					
Model	71	268.038	3.775	0.89	0.7224
Group	3	44.919	14.973	3.51	0.0161
Day	17	127.064	7.474	1.75	0.0356
Group *Day	51	103.058	2.021	0.47	0.9990
Error	220	938.109	4.264		
Total	291	1206.148	14.145		
Normalized osteocalcin					
Model	71	223.507	3.148	1.10	0.3018
Group	3	17.488	5.829	2.04	0.1099
Day	17	103.420	6.084	2.13	0.0077
Group *Day	51	91.417	1.792	0.63	0.9734
Error	175	499.808	2.856		
Total	246	723.315	2.940		

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