THE EFFECTS OF PHYTASE ON CALCIUM & PHOSPHOROUS DIGESTIBILITY AND BONE COMPOSITION IN WEANLING HORSES

by

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(Under the Direction of Gary Heusner)

ABSTRACT

The addition of phytase at 1500 FTU/kg to low-P and adequate-P diets was examined for changes in bone and plasma minerals, as well as nutrient digestibility. There was no change in growth data due to the addition of phytase. Additionally, phytase did not affect bone calcium in either the splint or third metacarpal. Phosphorous in the splint bone was decreased by the addition of phytase ($P < .05$). The mineral levels between the two bones types were different ($P < .0001$), with the third metacarpal containing nearly double the percentage of calcium and phosphorous. Plasma minerals were not affected by phytase or the different diets. In addition, there was no effect on energy or calcium digestibility. Phosphorous digestibility was affected at day 120 by the diets. The low-P diet had higher digestibility percentages. Phosphorous digestibility was also improved by the addition of phytase to the adequate-P diet.

INDEX WORDS: Bone, Calcium, Horses, Phosphorous, Phytase, Phytate

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CHAPTER 1

INTRODUCTION

The use of horses for sport has a long history. However, over time and with an increase in the cost of equine ownership, the horse has become used in competition at earlier ages. Many competitive disciplines have added or expanded their young horse competitions and have added large purses for the winners. Due to the high cost of ownership, people attempt to make a return on their investment in as little time as possible; otherwise the investment quickly becomes a loss. In doing this, horses become very vulnerable to early break downs. The equine skeletal system is not done growing by ages two and three (Lawrence at al, 1994), so it is definitely not suitable or ready for competition. In addition, while the Jockey Club cites that the number of two-year old races has declined over the past few years, the purses have increased. This creates even more motivation to train and compete animals at a younger age. Seventy percent of young racehorses in training are noted to be subject to fatigue fractures of the third metacarpal bone (Nunamaker et al., 1999). Many other disciplines have similar such incentives, such as the Quarter Horse Futurities, and Young Jumper Championships, which encourage the use of young horses at high levels of competition.

This push towards early use leads to tragic events, such as the break down of Ruffian, Barbaro and many racehorses. However, change is not likely to occur anytime soon, especially with the increase in group ownerships of horses. This collective ownership makes horses more affordable to the middle class enthusiast, yet also does not allow for the huge wealth of the more traditional owner to be put into the sport. Therefore, it seems that we must make these horses

stronger. By potentially increasing the bone strength of young horses, fractures may become less prevalent.

The use of phytase in other species of livestock has shown an increase in bone strength, as well as nutritional efficiency (Tsai et al., 2007; Cromwell et al., 1995; Kim et al., 2005). Therefore, this project was conducted in order to see whether the use of phytase in young horses can increase bone mineral content, and perhaps prevent competitive horses from breaking down early.

CHAPTER 2

REVIEW OF LITERATURE

Phytic Acid

Much of the phosphorous that is found in cereal grains is in the form of phytic acid (Oatway et al., 2001). Phytic acid, PA, also known as myo-inositol hexakisphosphate, is a phosphorylated inositol composed of a six carbon ring with a phosphate group attached to each carbon atom (Figure 1). Phytic Acid was first discovered in 1855 and has largely been viewed as an antinutritional component of grains since then, due to its ability to bind key nutrients (Oatway et al, 2001).

The majority of phosphorous found in cereals and legumes is in the form of phytic acid, with it representing up to 85% of the total P (Tsao et al, 1997). Plants use phytic acid as a storage form of phosphorous for the seed. In addition, it also forms phytic acid when it receives more P than is needed. The highest concentration of PA exists in the seed, at the time of ripeness (Barrier-Guillot et al., 1996). The unique structure of PA makes it unavailable to most monogastric animals, due to their lack of intrinsic phytase (Oatway et al, 2001).

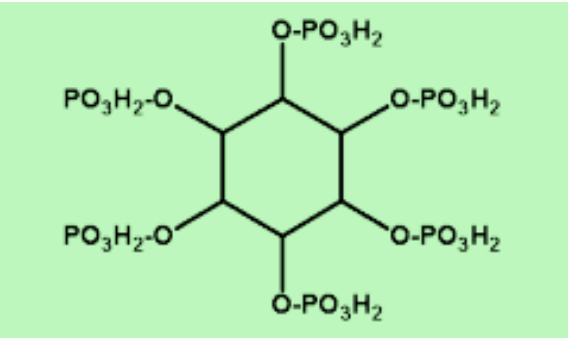

Figure 1: Basic Structure of Phytic Acid (Taken from phytochemicals.info)

Since the PA cannot be degraded, it is excreted in the feces and causes an overload of phosphorous in the soil and water runoff. Excess phosphorous in the environment is the major cause of eutrophication of water sources (Correll, 1999). Eutrophication can cause toxic algae blooms and fish kills, which are greatly detrimental to the environment (Sharpley, 1999). Excess phosphorous has become such an environmental concern that it has allowed for more research into PA and the use of phytase.

In addition to the excess phosphorous excretion, PA also binds to many key nutrients in an animal's diet. PA has a strong ability to chelate with many minerals, with a strong affinity for divalent cations (Maga, 1982). The mineral most affected by PA is zinc, as it forms the most stable of the PA-mineral complexes (Oatway et al, 2001). However, many other minerals are affected by PA, such as calcium, sodium, magnesium, chlorine, manganese, iron, and copper (Oatway et al, 2001). These minerals bind to the phosphate groups of the molecule, and vary in the number bound (Rickard et al, 1997). When calcium is bound to phytic acid it creates a compound called phytate. Similarly, the calcium-magnesium salt of phytic acid is known as phytin (Maga, 1982). These complexes are not easily broken due to the high stability of the bonds between the negatively charged phosphates of the phytic acid and the positive metal ions and require the work of the enzyme phytase (Oatway et al, 2001). Due to these strong bonds, many dietary minerals become bound to the phytic acid and are thus unavailable to animals without phytase. The addition of PA has shown to decrease the bioavailability of Calcium in dogs, and has also been used to induce rickets in these animals (Hoff-Jorgensen, 1946). This corresponds to the stable complex that forms between calcium and PA and its detriment to the animal (Graf, 1983). According to work done by Hoff-Jorgensen in 1946, the addition of PA salt to the diets of infants greatly decreases the availability of the calcium in the diet to the child. In

addition, this seems to be greater in older infants, and puppies, than the younger subjects (Hoff-Jorgensen, 1946).

In addition to binding with minerals, PA also has the ability to bind to proteins and starches (Oatway et al, 2001). It binds to proteins through electrostatic charges, while binding to starch through hydrogen bond formation at the hydroxyl group of the starch compound to the phosphate of the phytic acid (Rickard et al, 1997). The negative charges of phytate interact with basic amino acids such as lysine, histidine and arginine to form phytate-protein complexes at pH levels lower than the isoelectric point of proteins (Cosgrove, 1966).

Phytase

Phytases are the enzymes that break down phytic acid into its component parts of myo-inositol and inositol phosphates through dephosphorylation (Odani et al., 1997). There are many different types of phytases that are found in the environment, with many different cleavage sequences and start points. Phytases work through a series of stepwise reactions that cleave the individual phosphates from the molecule (Odani et al., 1997). The order in which the phosphates are cleaved are variable to the phytase source (Greiner et al, 2000). In addition, phytases are classified as acidic phosphatases as they have an optimum pH of 4.5 (Lim et al, 2000). Some phytases also seem to be mineral dependent enzymes and perform at a higher rate with the addition of Mg (Odani et al., 1997).

When feeding animals phytase, the enzyme is primarily derived from one of three sources, either *Aspergillus niger*, *Peniophora lycii*, or *Escherichia coli* (Augspurger et al, 2003). The majority of early research was performed using the *Aspergillus* form, but recently evidence has shown that the *E.coli* derived phytase may actually be more effective (Augspurger et al,

2003). The E.coli phytase has the highest specific activity of all known phytases, having nearly eight times greater specific activity to that of the *Aspergillus niger* phytase (Lim et al, 2000). In addition, this phytase cleaves in the sequence of 6/1/3/4/5, which is unique to this enzyme (Greiner et al, 2000).

E.coli phytase is composed of an α and an α/β subunit. A large central cavity divides the two subunits and is the location of the active site of the protein (Lim et al, 2000). The phytase's preferred substrate is phytic acid, which is also thought to enhance catalysis through conformational changes that increase the acidity of the general environment (Lim et al, 2000).

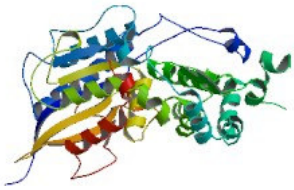

Figure 2: The three-dimensional structure of *E.coli* derived phytase (Lim et al., 2000)

The differences in the α subunits between the phosphatases is thought to be the reason for the greater catalytic ability of the E.coli phytase, due to a β-hairpin structure that lines the rim of the binding pocket opposite of the binding site (Lim et al, 2000). When the enzyme binds with the phytic acid, it preferentially hydrolyzes the 6-phosphate, thus classifying it as a 6-phytase. This is the same start point as the *Paramecium* phytase, yet the following cleavage sequence varies (Greiner et al, 2000). Therefore, the cleavage order of the *E.coli* phytase is thought to be unique to the enzyme (Greiner et al, 2000). The order in which they are cleaved is also thought to be important (Greiner et al., 2000). The E.coli phytase prefers to bind phytic acid due to its six

phosphates, yet will bind to other phosphorylated inositols that contain fewer phosphate groups. However, it has a strong preference for the six phosphate groups of PA (Lim et al, 2000).

Phytase in Animal Diets

In traditional monogastric diets, producers have had to add inorganic phosphorous to rations in order to meet the nutritional phosphorous requirements of the animals. However, in recent years, environmental concerns regarding the high fecal levels of phosphorous have become increasingly important (Selle et al., 2007). Phytase has become a good way to decrease phosphorous output by making the phosphorous that was once unavailable to the animal in the form of phytic acid available as inorganic phosphates (Odani et al., 1997). Therefore, the producer should not need to add the inorganic supplement (Murry et al., 1997).

Several studies have shown that when swine diets are supplemented with phytase, there is an increase P utilization, serum mineral content and also increases the breaking strength of pig bones (Cromwell et al., 1993; Haper et al., 1997; Lei et al., 1994). In addition, studies have shown that phytase will increase Ca and other mineral digestibility (Tsai et al., 2007). In a study conducted by Murry et al. in 1997, pigs on a low phosphorous diet that were supplemented with phytase were able to perform as well as pigs on adequately formulated rations. In general, phytase was shown to bring the pigs that were fed a phosphorous deficient diet back up to the basal serum mineral rates of the pigs that were fed an adequate- P level diet. However, the pigs on the adequate diet that were fed phytase were less efficient, but still performed better than the pigs on the low-P diet that were not supplemented with phytase (Murry et al., 1997).

Table 4. Serum P, Ca, Mg, Zn, and Cu concentrations at d 35 of pigs fed microbial phytase in pearl millet-soybean mean diets[a]

		Low-P			Adequate-P				*P*-value		
Item	Phy[b], units/kg:	0	700	1,000	0	700	1,000	SEM	P	Phy	P × Phy
P, mg/dL[c]		7.92	9.66	9.34	9.64	9.52	9.79	.218	.002	.004	.003
Ca, mg/dL		10.68	10.82	10.23	11.03	10.53	11.23	.951	.37	.81	.68
Mg, mg/dL		21.07	21.23	19.60	20.47	19.83	21.89	1.340	.92	.98	.37
Zn, mg/dL		.86	1.04	.98	.85	.87	1.07	.079	.52	.09	.35
Cu, mg/dL		1.83	1.80	1.73	2.00	1.88	1.79	.165	.20	.32	.83

[a]Data are means of three pigs per treatment.
[b]Phy = microbial phytase.
[c]Linear phytase effect for low-P diets, $P < .01$. Quadratic phytase effect for low-P diets, $P < .02$.

Figure 3: Table showing serum mineral concentration of pigs on a phytase trial (Murry et al., 1997)

In addition, Augspurger conducted research to compare the effectiveness of E.coli phytase in contrast with that of commercial fungal phytase (Auspurger et al., 2003). In his work, which was conducted using chickens and pigs, the *E.coli* phytase was more effective in all species as related to P release. The most dramatic change occurred in young chicks, in which the P-release value was .125% for the *E.coli* derived phytase as compared to .032 and .028% for the commercial phytases (not *E.coli* derived) (Augspurger et al, 2003). The theory as to why the *E.coli* phytase is superior to the fungal phytase is related to its pH sensitivities and its ability to withstand the proteolytic enzymes of the GI tract. *E.coli* phytase is better conformed to maintain its effectiveness in the stomach of an animal due to its acidic nature, which is the main site of phytase activity in young pigs (Yi et al., 1996). In addition, it is not harmed by the enzyme pepsin, which is one of the primary enzymes of the stomach (Augspurger et al., 2003).

While no research has yet been published regarding the use of *E.coli* phytase in equine rations, work has been done with *A.niger* phytase. While the P in forage is thought to be 100% available, horses that consume diets high in concentrate, receive a large amount of phytate phosphorous. In a study conducted by Van Doorn et al (2004), eight mature horses were fed one of four diets containing different phosphorous sources and one that included phytase. The diets were formulated to have a control diet that had a standard phosphorous level, and two high phosphorous level diets. Of the two high phosphorous diets, one was formulated with the

addition of a supplemental inorganic phosphorous, while the other diet contained no inorganic phosphate and had a higher phytate content. An additional group of horses were fed the high phytate diet with the addition of 1000 FTU/kg of phytase. These animals were set up for total collection and apparent digestibilities of phosphorous, calcium and magnesium were determined. While there was no improvement in the apparent digestibility of phosphorous or magnesium, there did seem to be an increase in apparent digestibility of calcium when on the added phytase diet as compared to the high phytate diet with no phytase (Van Doorn et al., 2004). Therefore, it seems that phytase does aid in the digestion of calcium by releasing it from the phytate molecule and making it available to the horse.

However, a similar study conducted at Oklahoma State University showed no differences in the digestibility of dry mater, phosphorous, calcium or magnesium (Patterson et al., 2002). Only four mature horses were used. In this study one basal diet was used with no added phytase, 300 FTU/kg phytase, 600 FTU/kg phytase, or 900 FTU/kg phytase. Therefore, the amount of phytase that was used in this study was lower than what van Doorn had used, which may have been a factor. In addition, Hainze et al. (2004) performed a digestibility study containing eight yearlings on four diets with or without phytase. This study also did not see a difference in phosphorous digestibility, and did not calculate calcium digestibility. However, fecal phosphorous levels were decreased on one of the diets when phytase was added. This diet was high in grain content, which is also high in phytate. Therefore, the addition of the phytase may be effective in reducing P pollution in high phytate diets.

Since phosphorous pollution due to horses is not as great of a concern as in the poultry and swine industry, the research that has been conducted in the equine field has been scant. Studies in horses have focused also primarily on horses at least a year old and have only used

phytases derived by A. niger. Additionally, none of these studies have looked at the effect that phytase supplementation has on bone strength or bone minerals. The focus has been on digestibility results and primarily on phosphorous. However, according to Matsui et al. (1999), phytate is readily absorbed by the horse in the large intestine perhaps due to phytase activity of the hind gut. Therefore, it is not surprising that research does not show an increase in phosphorous digestibility. In addition, the repeatability of the equine phytase trials is questionable given the small numbers of horses used on these trials.

Calcium

Calcium is an integral component of the equine skeleton. 99% of the calcium in the equine body is found in the bones and the teeth, with calcium constituting about 1-2% of total body weight (Cashman, 2002). In addition, calcium accounts for 35% of bone ash (El Shorafa et al., 1979). Calcium also has many other functions in the equine body relating to muscle contraction and other processes vital to survival. While the majority of Ca is in the bones of the animal, it can readily be mobilized in the event of deficiency, in order to maintain homeostasis in the blood. Therefore a negative calcium balance is not necessarily apparent in serum Ca values (Krook and Lowe, 1964). A proper amount of calcium is absolutely necessary for the health of the animal. Animals with a calcium deficiency will often show signs of weakened bones and decreased weight gain. In addition, recent studies have shown that overly high level of dietary calcium can cause gastric ulcers due to gastrin secretion (Dufner et al, 2005). However, other symptoms of toxicity are rarely seen in the presence of adequate phosphorous levels, due to its competitive absorption in the small intestine (Jordan et al., 1975). Therefore, proper nutritional balance is vital to a healthy horse.

In the horse, calcium is absorbed actively in the small intestine (Staderman et al., 1992). Dietary calcium absorption is directly related to the calcium demand of the horse (Jones and Rasmusson, 1980). In general, the absorption efficiency of calcium in horses is thought to be about 50% (NRC, 2007). However, true absorption efficiency in young horses can be up to 70%, due to their high calcium demand, and tapers down as the horse ages (NRC, 2007). In the horse, the upper section of the small intestine has been shown to be the major site of calcium digestion. However, the lower small intestine also has significant potential for calcium absorption (Schryver et al., 1970). In order for calcium to be absorbed there must be an adequate amount of vitamin D present in the body. Vitamin D is crucial for calcium absorption since it influences calcium-binding protein transcription, alkaline phosphatase and calcium activated ATPases. These compounds all facilitate the absorption and transport of Ca in the animal. Therefore, the maintenance of calcium homeostasis in the horse is dependent upon many factors (Krook and Lowe, 1964).

Generally, when fed an adequate diet, horses do not exhibit many calcium deficiencies. However, with the movement of the horse into more of a confined setting and a diet that consists of high concentrate levels, calcium supplementation is common (NRC, 2007). In addition, the composition of the diet with respect to other nutrient amounts can have a great effect on calcium absorption. Increasing dietary magnesium has been shown to increase Ca absorption (Hintz and Schryver, 1973), while increasing phosphorous in the diet decreases calcium absorption efficiency due to the two minerals competitive nature in the small intestine. Dietary oxalate also decreases calcium absorption (NRC, 2007). With as little as a 1% inclusion of oxalate in an equine diet, calcium digestibility can decrease by 66 percent (Swartzmann et al., 1978). Additionally, diets high in fat and fiber can also affect calcium digestibility. Diets high in fats

and fibers have been reported to decrease bone mineral content of the third metacarpus (Hoffman et al., 2000).

In addition to dietary considerations, calcium absorption is influenced by age, exercise and production state (NRC, 2007). As a foal ages from 6 to 24 months, calcium digestibility substantially decreases. However, other factors such as calcium source change and varying requirements due to training may influence the decrease (Cymbaluk et al., 1989). Mineral deposition due to bone growth also affects calcium absorption and increases the animal's calcium requirement (NRC, 2007). Due to the reservoir nature of bone, as bone is being formed, more dietary calcium is required, but during times of inactivity, calcium is mobilized from the bones, thus not needed in as large amounts in the diet (Bronner, 1993). Dietary calcium requirements also increase during late gestation, in order to support the growth of the developing fetus. The greatest need for calcium being during the tenth month of gestation, due to dramatic fetal foal growth (NRC, 2007). Lactation also is a period of great dietary calcium need, as the mare mobilizes calcium in order to provide adequate nutritional values to the newborn foal (NRC, 2007). Training state additionally requires an increase in calcium, as bone formation is impacted by exercise (NRC, 2007). Young horses that are exercised can use excess dietary calcium to maximized bone strength (Nielsen, 2005). According to Nolan et al. (2001), diets that contained 151 and 169 percent of the 1989 NRC recommendations for calcium increased mineralization of the third metacarpus, as compared to diets that only contained 97 or 136 percent of the requirement. Therefore the addition of calcium to young horses' diets is crucial for adequate bone strength, especially for animals in training.

Phosphorous

In addition to calcium, phosphorous is a major constituent of bones. Phosphorous makes up between 14 and 17 percent of the equine skeleton (El Shorafa et al., 1979). Phosphorous is also crucial to energy transfer reactions, as it is found in adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Phosphorous is an important component of nucleic acids, the phospholipids that make up cell membranes and phosphoproteins (NRC, 2007).

Phosphorous is generally found in the form of phytate in the equine diet, as they consume a large quantity of plant matter (NRC, 2007). Phytate is poorly absorbed by the horse, even though phytase exists in the hindgut of the animal (Hintz et al., 1973). Therefore, horses are commonly supplemented with inorganic phosphates in order to meet their nutritional requirements (NRC, 2007). Phosphorous is primarily absorbed in the dorsal large colon and the small colon of the horse (Schryver et al., 1972). Matsui et al. (1999), confirmed the site of absorption, and also showed that phytate phosphorous is easily absorbed in the lower large intestine, as it is degraded in the upper small intestine and the lower large intestine.

Phosphorous absorption typically ranges from 30-55 percent (NRC, 2007). However many factors influence the absorption of phosphorous, including amount and type fed, as well as age and calcium levels. High phosphorous concentrations increase phosphorous retention as well as plasma phosphorous concentrations (Schryver et al., 1971). However, high dietary calcium levls decrease phosphorous absorption, as they compete for uptake in the intestinal tract of the animal (NRC, 2007). In addition, age appears to affect the efficiency of phosphorous digestibility. Younger horses consuming milk have higher absorption than mature horses (Grace et al., 1999). While horses that are 8 months old also have a higher phosphorous efficiency than 12 month old horses (Cymbaluk, 1990). A probable reason for the greater efficiency is likely due

to the mineral source. Mature horses generally consume more phosphorous of plant origin, while lactating and growing horses are often supplemented with inorganic phosphates (NRC, 2007). Recent evidence has also proposed that P absorption can vary with the demand of the animal (Stephens et al., 2004).

Calcium:Phosphorous Ratio

Upon meeting the calcium and phosphorous requirements of the horse, the ratio at which they are given is extremely important (NRC, 2007). Due to the competitive nature of the minerals in the digestive tract of the horse, the ratio of calcium to phosphorous should be at least 1:1. If calcium is lower, absorption will be impaired (NRC, 2007). Diets that are adequately supplemented with calcium, yet are too high in phosphorous may also cause skeletal abnormalities (Schryver et al., 1971). Excess phosphorous can result in nutritional secondary hyperparathyroidism (NSH). While not common, it can be a concern in horses that are fed diets high in phosphorous, low in calcium or high in oxalates (Mason et al., 1988). Horses that are afflicted with this condition acquire a shifting lameness and in severe cases an enlargement of the maxilla and mandible (Krook and Lowe, 1964). Many phosphorous excess concerns come from the high phosphorous content in grain, and a potentially low calcium level in forages (NRC, 2007). Therefore, it is greatly important that horses in this situation are supplemented with calcium.

Excess calcium in the diet, however, tends not to be a concern, as long as there is adequate phosphorous available. Ratios as high as 6:1, in growing horses, have shown to be acceptable, as long as the horse's diet meets its basal phosphorous needs (Jordan et al., 1975).

Therefore, calcium becomes the true concern in formulating a horse's diet in order to maintain good health and provide for growth.

Bone Development and Metabolism

With 70% of young racehorses suffering stress fractures of the third metacarpal during training (Nunamaker et al., 1990), the importance of strong bones is crucial to a young horse. Bone tissue is dynamic, in that it is constantly undergoing resorption and remodeling. As a bone grows longer, the bone is resorbed along the inside cavity in order to widen the marrow cavity (Pike and Brown, 1967). There are two main cell types that facilitate bone regulation, osteoblasts and osteoclasts. The osteoblasts are the cells that are responsible for bone growth, while the osteoclasts are the bone-resorption cells. In the bone, resorption always occurs prior to new bone deposition (Parfitt and Chir, 1987). As the animal matures, and bone growth in size is no longer needed, balance between bone destruction and formation still occurs at an equal rate (Pike and Brown, 1967).

Mineral composition in bone is believed to be primarily in a hydroxyapatite, with a basic formula of $Ca_{10}(PO_4)_6(OH)_2$. Additionally, there are three zones within the crystal lattice: the hydration shell, the crystal surface and the crystal interior. Of the three zones, there is rapid turnover of minerals in the crystal surface, and the slowest turnover is in the interior. There are also mineral substitutions within the hydroxyapatite (Pike and Brown, 1967). Fluoride can be substituted for the hydroxyl group and actually increases the stability of the molecule (Posner, 1967), while magnesium can be substituted for Ca.

The regulation of serum calcium is very important in bone maintenance. The bone is used not only for skeletal support, but also as a reservoir of minerals. Calcium balance is determined

by the amount of calcium absorbed through the small intestine and resorbed through bone against the amount that is deposited in the bone and also lost in the urines and feces (Pike and Brown, 1967). Therefore, in order to balance the calcium in the body, a tightly regulated endocrine system exists. Parathyroid hormone and thyrocalcitonin are the primary regulators of calcium balance. Parathyroid hormone (PTH) works on the kidney and bone in order to increase plasma calcium. The primary effect of PTH is to activate adenyl cyclase, which works in a diverse number of way in order to change blood calcium. Thyrocalcitonin works as an antagonist to PTH and reduces the rate of calcium resorption in the bone, thus decreasing blood calcium. Vitamin D also directly works on bone tissue by aiding in the resorption of bone and the maintenance of adequate plasma calcium levels (Pike and Brown, 1967).

In horses, the majority of bone growth is in utero, and bone growth is complete at roughly age five (Lawrence et al., 1994). The most rapid increase in mechanical properties of cortical bone occurs directly after parturition, due to weight bearing (Bigot et al., 1996). After birth, the bone mineral density of the third metacarpal in pasture raised foals increases from day 15 to day 135 by 52% (Firth et al., 2000). In horses ranging from two months of age to 4 years, the modulus of elasticity and the bending strength of bone was not different. Therefore, equine bone may be mechanically comparable across this age range (Bigot et al., 1996). However, bone mineral content increases linearly with age until it reaches a maximum around six years of age. At age one, there is approximately 76% of maximum bone mineral content. The trend is similar for breaking load, the amount of force that is required to break the bone. Therefore, skeletal maturity is not achevied until age 4-6 (Lawrence et al., 1994). As horses are used for heavy work and competition during these growing years, it is important to keep in mind the that the animal is

not fully developed, thus there tends to be higher instances of injury (Nunamaker et al., 1990; El Shorafa et al., 1979).

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CHAPTER 3

THE EFFECTS OF ADDED PHYTASE ON BONE MINERALS, PLASMA MINERALS, AND NUTRIENT DIGESTIBILITIES IN WEANLING HORSES

Abstract

The purpose of this study was to determine whether the addition of phytase to equine weanling diets would affect bone minerals, nutrient digestibility and plasma minerals and growth. In a 2 X 2 factorial design, two diets of different phosphorous levels (adequate-P with inorganic P and low-P without inorganic P) were tested against two phytase levels (no added phytase or 1500 FTU/kg added). The horses received the diets for 120 days. Bone samples were taken at days 0 and 120. Fecal samples were taken at day 60 and 120. Additionally plasma was collected on days 0, 60 and 120. Final weights were higher in the horses on the adequate phosphorous diet ($P< 0.05$). However, average daily gain was not affected. Phytase addition did not significantly influence bone calcium in the splint bone of the horse, and neither did the difference in diet. Phytase did increase bone calcium of the third metacarpal (MCIII) in the horses on the adequate-P diet from 14.48 to 18.76% of wet weight, yet decreased bone calcium on the low-P diet from 19.17 to 16.23%. Phytase decreased the percentage of phosphorous in the splint bone of the weanlings, yet had no effect on MCIII. Plasma calcium and phosphorous concentrations were not affected by any of the diet-phytase combinations. Additionally, there were no differences in calcium or energy digestibility among all horses, regardless of diet or

phytase level. Phosphorous digestibility was significantly higher in the horses on the low phosphorous diet. There was also a diet by phytase interaction within the adequate-P diet, with the addition of phytase causing P digestibility to increase from 9.48% to 26.04%. These results do not provide any support to the claim that phytase improves bone strength in horses. Additionally, the inability to lower diets to a phosphorous deficient level probably is why there were few differences between the animals on the two diets.

Introduction

In recent years, the use of horses at earlier ages has become increasingly popular. With the use of younger horses comes an increase in injuries and breakdowns of these animals. Seventy percent of young thoroughbred racehorses in training suffer stress fractures of the third metacarpal (Nunamaker et al, 1990). The careers of most racehorses are usually over before maximum bone strength is reached (Lawrence et al., 1994). Due to this shockingly high number of injuries, it becomes increasingly more important to find ways to improve the strength of equine bone. In many studies conducted on pigs and chickens, the addition of phytase has been shown to increase bone strength (Tsai et al., 2007, Cromwell et al., 1995, Kim et al., 2005). The addition of phytase to the equine diet has not shown any increase in phosphorous digestibility, but has shown a potential for an increase in calcium digestibility (Van Doorn et al., 2004). Additionally, the use of E.coli derived phytase has shown a greater improvement in growth and nutrient digestibility than the more common fungal derivative (Augspurger et al., 2003). However, E.coli derived phytase use in the horse has not been studied. Therefore, the objective of this study was to determine whether the addition of E.coli derived phytase would increase bone mineralization and mineral digestibility in young horses, as it does in pigs and chickens.

Materials & Methods

Experimental Design

Sixteen stock horse type weanlings at an average age of 176 ± 31 days were used in a 2x2 Factorial design experiment in order to study the effects of phytase (Phyzyme(R) XP 5000G, donated courtesy of Danisco Animal Nutrition St.Louis, MO) and differing levels of dietary phosphorous on the bone strength, growth and nutrient digestibility in young horses. The weanlings consisted of 12 colts and 4 fillies. Horses were blocked based on starting weight and sex. Horses were weighed at day 0 of the trial and weighed an average of 214.17 ± 40.51 kg. Total feed intake was set at 2.5 % of individual body weight with a concentrate to forage ratio of 70:30 and the horses were reweighed every 14 days in order to adjust intake to growth. Prior to being placed on an experimental diet, the weanlings underwent surgery in order to collect bone samples. The day following surgery, the horses were placed on one of four experimental diets for 120 days: low phosphorous (90% of NRC (1989) requirements), without added phytase; low phosphorous, added phytase; adequate phosphorous (110% of NRC (1989) requirements), without added phytase; adequate phosphorous, added phytase. Each horse was housed in 1.524m x 3.658m stall and left inside 24 hrs during the initial 14 day recovery period. From day 14 through day 120, the horses were turned out for 8 hours on dry lot with 2-3 others for exercise and kept in stalls the remaining 16 hours. The horses were bandaged to protect the surgical site from day 0 to day 13, with the bandages changed every other day. Following day 120, the horses again underwent another surgery on the opposite front limb in order to collect final bone samples. All care and procedures were approved by the University of Georgia Institutional Animal Care and Use Committee, AUP #A2007-10037-0.

Experimental Diet

The weanlings were fed a common mixed grain ration that consisted primarily of corn, oats, and soybean meal (Table 1). Total feed intake provided to the weanlings was 2.5% of their body weight, with 70% of the ration consisting of concentrate and the remaining 30% consisting of Russell Bermudagrass hay. The diets contained contained an average available phosphorous percentage of .0990% and .0842% in the adequate-P and low-P diets, respectively. Horses were reweighed every two weeks and diet amounts were reformulated to account for increases in weight gain. Weanlings were matched by weight and gender and then randomly placed on one of two diets with or without added phytase.

Table 1. Composition of Experimental Diets[a]

Ingredient	Control (%)	Low P (%)
Cracked corn	26.76	27.58
Whole Oats	23.81	23.69
Soybean Meal	11.04	10.93
Fat	3.35	3.07
Molasses	3.00	3.00
Calcium Carbonate	.88	.95
Salt	.51	.51
Trace Mineral Mix[b]	.08	.08
Vitamin A, D, E[c]	.05	.05
Vitamin E[d]	.14	.14
Dicalcium Phosphate	.38	0.0
Bermuda grass Hay	30	30
Nutrient Composition, calculated		
Crude Protein, %	13.1	13.1
Lysine, %	.64	.64
DE, Mcal/kg	2.87	2.87
Calcium, %	.59	.54
Phosphorous, %	.34	.27
Copper, ppm	12	12
Zinc, ppm	123	123

[a]Percentage on an as-fed basis
[b]Provided per kg of mix: Zn, 12%; Mg, 14%; Cu, 13228 ppm; Se, 1323 ppm.
[c]Provided per kg of mix: vitamin A, 4.41 million IU; vitamin D, 2.2 million IU; vitamin E, 4409 IU.
[d] Contains 44092 IUs vitamin E/kg.

All other nutrients were formulated to meet NRC (1989), for 6 month old weanlings with rapid growth. Each weanling was administered a cube that consisted of 37.5% dehydrated alfalfa, 50% molasses and 12.5% cottonseed hulls. The horses that were designated to receive added phytase

received cubes supplemented with 1.2 g of the phytase at the start, and eventually up to 1.8 g due to the increase in animal size and feed consumption. The cubes were formulated to provide 1500 FTU/kg diet of phytase per animal per day. The cubes weighed an average of 127 g and one cube was fed per horse per day. The horses were fed the grain and half of a cube twice daily, and fed all of their hay once in the morning. Uneaten feed was weighed back each morning and recorded. Fresh clean water was available ad libitum.

Sample collection

At day zero, the weanlings were taken into the UGA College of Vet Med Teaching Hospital for bone samples to be removed. Bone samples were collected so that they could later be analyzed for calcium and phosphorous content. The horses received Naxcel (2.2mg/kg, IV), and Phenylbutazone (4.4 mg/kg, IV) pre and postoperatively. They were premedicated with Xylazine (1.1mg/kg IV) and Butorphenol (0.3 mg/kg IV) and induced with Diazepam (0.05 mg/kg IV) and Ketamine (1.1 mg/kg IV) and anesthesia was maintained with GKX [(Guaifenesin, Ketamine, and Xylazine) 2 ml/kg/hr]. Once anesthetized a 2 inch incision was made over the lateral center of a front forelimb, with exception of two animals which had incisions medially. The two exceptions were due to scars on the proposed lateral incision site, which made them unusable as incision sites. The horses then had a small portion of the splint bone, approximately two centimeters long, removed as well as a core sample taken from third metacarpal. The core sample was obtained by using a 1/8" Michele Trephine to bore a hole into the bone to the point of the medullary cavity. The horse then had staples used to close the incision and the legs were wrapped for support. In addition to the bone samples, the horses also had two vials of blood collected from the jugular vein in heparinized Vacutainers, and a radiograph taken of the limb that the samples were taken from immediately post-op. The bone

samples were stored in Vacutainer tubes and frozen at -20°C until further analysis was completed. Additionally, the blood was spun down and plasma extracted and frozen at -20° C until further analysis was completed.

The animals also had radiographs taken of the sampled limbs every subsequent 15 days. The radiographs were taken with a portable x-ray.

Fecal grab samples were taken twice per animal during the 120 day period in order to evaluate calcium, phosphorous and energy digestibility. They were collected on day 60 and 120. Samples were obtained by collecting grab samples rectally three times daily, at 7am, 3pm, and 11 pm, for 3 consecutive days. The samples were then stored in large Ziploc bags and stored at -20° C for later analysis. Blood samples were also taken on days 0, 60 and 120 in heparinized Vacutainer tubes from the jugular vein, for plasma analysis of calcium and phosphorous content. The samples were then spun down in a centrifuge for 20 minutes at 750 RCG. The plasma was pipetted off and then frozen at -20° C until analyzed.

Chemical Analysis

The bone samples were thawed to 25°C at time of analysis and weighed. The samples were placed individually in a 100ml kjeldahl flask. Six mL of nitric acid and three glass boiling beads were added to the flasks. The samples were boiled on individual burners under the fume hood. The samples were cooked until approximately half of the nitric acid had boiled off. The flasks were then cooled to room temperature and 5 ml of perchloric acid were added. The flasks were then returned to the burners and were cooked until a noticeable change in the fume color, from yellowish to a white cloud, was observed. The flasks were then kept on the heat for an additional thirty minutes. Once removed from the heat, the samples were cooled to room temperature and then transferred to 100 ml volumetric flasks. Once transferred, the flasks were

brought up to 100ml volume and capped for later analysis. Before being analyzed on the atomic absorbance, the solutions were diluted with a 0.1% Lanthanum Oxide solution. 20μl of sample was diluted with 9980μl the lanthanum solution. The samples were then vortexed and analyzed for Ca using a Perkin Elmer AAnalyst Atomic Absorption Spectrophotometer.

The bone samples were also analyzed for phosphorous content. This analysis was completed colormetically on a Beckman Coulter spectrophotometer using the molybdenum blue method (Quinlan and DeSesa, 1955).

Calcium , phosphorous and energy digestibility were determined through the fecal samples collected. The digestibility was determined using indigestible acid detergent fiber as an internal marker (Cochran et al., 1986). Prior to analysis, the samples were thawed and subsequently weighed. Once weighed, they were mixed with enough water to make a slurry, the water volume recorded, and blended in a laboratory blender in order to create a homogenous sample. Once blended, samples were divided into an aluminum pan and a plastic storage container; the sample weight was recorded. The pan was placed in a drying oven at 37.78^{o}C for 72 hours in order to calculate dry matter. The plastic storage container was freeze dried for 144 hours, or until all moisture had been removed. The samples prepared were processed for analysis by being ground through a 1mm screen. Duplicate 1 gram aliquots of each sample were then weighed out and prepared for analysis through the same procedure as the bone samples. Once cooked and in the 100 mL volumetric flasks, the 10 μL of sample was diluted with 4990 μL of 0.10% Lanthanum oxide and analyzed for calcium using a Perkin Elmer AAnalyst 400 atomic absorption spectrophotometer. Phosphorous content was calculated colormetrically on a Beckman Coulter spectrophotometer using the molybdenum blue method (Quinlan and DeSesa,

1955). Additionally, gross energy was determined by a bomb calorimeter (Parr 1261, Parr Instrument co., Moline, IL)

Indigestible acid detergent fiber was calculated by incubating 5 g of the ground sample with 30 mL of inoculum, which consisted of a 2:1 ratio of McDougall's buffer to rumen fluid, in sample tubes with one-way stoppers. The samples were incubated in a water bath at 39^{o}C for 6 days. After each ten samples were prepared, a blank that consisted of only inoculum was also prepared. Once incubated, the samples were spun down at 2500 rpm for 15 minutes. The supernatant was poured off and the remaining sample in the tube was placed in a drying oven at 60^{o} C for 72 hours. The samples were then analyzed for ADF using the Van Soest method (Van Soest et al, 1991).

Feed samples were also collected during the fecal collection periods. The feed samples were analyzed for calcium, phosphorous and energy content in the same manner as the fecal samples.

The blood was spun down for twenty minutes at 750 RCG and the plasma, subsequently, extracted. The blood plasma was then precipitated with 15% TCA solution in order to remove the plasma proteins, and the supernatant was pipetted off. The samples were then stored at 5^{o} C for approximately 48 hours before they were analyzed for calcium and phosphorous content. For calcium analysis, 4900 μL of 0.10% Lanthanum Oxide was used to dilute 100 μL of sample. The diluted samples were then analyzed for calcium content using a Perkin Elmer AAnalyst 400 atomic absorption spectrophotometer. The phosphorous was analyzed colorimetrically on a Beckman Coulter spectrophotometer using the molybdenum blue method (Quinlan and DeSesa, 1955).

Statistical Analysis

Data were analyzed using a SAS (SAS Institute Inc, Cory, NC) program for the factorial design and the general linear model. The SAS PROC MIXED model was used to determine least squares means and the treatment differences (significance at $P < .05$). Results were presented as least squares means for diet, phytase and diet by phytase interaction.

Results

Growth Performance

The growth performance data is shown in Table 2. All animals started at roughly the same weight, with there being no significant difference among the treatments (P>.05). However, there was a diet effect on end weight (P<.05), with the horses on the adequate phosphorous diet weighing more than those on the low phosphorous diet. However, the horses on the adequate phosphorous diet had numerically larger starting weights than the horses on the low-P diet. Average daily gain was not affected by the diets (P>.20) or the phytase (P>.15). Numerically, the horses that did not receive phytase had a slightly higher gain rate, but it was not statistically significant. In addition, the standard error values for each item are fairly high, which also shows a lack of difference between the four treatment groups.

Table 2. Growth Performance of weanling horses fed phytase in varying phosphorous level diets.[a]

		Low-P		Adequate-P					
Item:	Phy[b], (FTU/kg):	0	1500	0	1500	SEM	P[c]	Phy	P x Phy
Start weight (kg)[d]		207.79	206.51	222.22	228.25	10.1929	.0747	NS	NS
End weight (kg)		303.94	291.56	321.69	323.14	9.8819	.0216	NS	NS
ADG (kg/day)		.8012	.7087	.8289	.7908	.06066	NS	.1725	NS

[a] Data are means of four horses per treatment, except the Adequate-P, 0 Phytase which contained three.
[b] Phy= Phytase
[c] P= Diets containing varying levels of phosphorous
[d] NS= not significant, P >.20

Bone composition results

The starting calcium levels of the splint bone varied between diet and phytase (Table 3). The horses on the adequate phosphorous diet had higher starting calcium levels than those on the

Table 3. Bone minerals[a] of weanling horses fed variable levels of phosphorous with or without phytase.[b]

		Low-P		Adequate-P					
Item:	Phy[c] , FTU/kg	0	1500	0	1500	SEM	P[d]	Phy	P x Phy
Calcium (%):									
Day 0:									
Splint[e]		6.32	5.38	9.39	7.43	.60	.0014	.0109	NS
MCIII[f]		14.32	12.43	18.52	13.71	1.23	.0345	.0065	.1697
Day 120:									
Splint		5.86	6.57	7.43	6.93	.78	.1896	NS	NS
MCIII		19.17	16.23	14.48	18.76	1.66	NS	NS	.0263
Phosphorous (%):									
Day 0:									
Splint		8.37	4.75	4.63	4.12	1.29	.0853	.0579	.1627
MCIII		7.02	5.57	8.22	7.11	1.86	NS	NS	NS
Day 120:									
Splint		5.73	4.87	5.08	4.08	.55	.1641	.0489	NS
MCIII		9.85	7.92	5.05	8.70	1.73	NS	NS	.0757

[a] As a percentage of wet weight

[b] Data are means of four horses per treatment, except the Adequate-P, 0 Phytase which contained three.

[c] Phy= Phytase

[d] P= Diets containing varying levels of phosphorous

[e] NS= not significant, P >.20

[f] MCIII = Metacarpal III

low phosphorous diet. In addition, the horses that did not receive phytase started with higher bone calcium percentage than the horses that did receive phytase (P<.05). There was no significance of age or blocking group on splint bone calcium level, or a diet by phytase interaction (P>.20). The same higher starting calcium levels held true for the third metacarpal bone, with both the horses on the adequate phosphorous diet and the horses not receiving phytase having higher bone calcium percentages(P<.05). Again, there was no significance of age, blocking group or diet by phytase interaction. Splint bone calcium content at Day 120 did not differ significantly between diets (P>.15), phytase addition or diet by phytase interaction (P>.20). Numerically, the horses on the adequate phosphorous level diet had higher calcium

percentages in the splint than those on the low phosphorous diet. However, the horses on adequate phosphorous started with higher bone calcium levels than those on the low-P diet. The third metacarpal also did not have any significant differences between diets and phytase addition ($P>.20$). However there was a diet by phytase interaction ($P<.05$), but none of the individual groups significantly varied from the others. Numerically, the horses on the low phosphorous with no added phytase had the highest bone calcium percentage. Phytase did increase MCIII bone calcium of the horses on the adequate-P diet from 14.48 to 18.76%. Age and blocking group did not cause any significant variability.

The starting phosphorous levels did not vary as much as the starting calcium levels, in the splint bone. However, the low phosphorous without phytase group had a much higher phsophorous level than the other groups. The third metacarpal starting phosphorous did not vary significantly. In both bones, age, blocking group and diet by phytase interaction did not play a significant role. Final bone phosphorous percentage in the splint bone was numerically higher in the weanlings that were on the low phosphorous diet with the low-P horses having 5.73 and 4.87% P as compared with the adequate-P horses having 5.08 and 4.08% P. It was also significantly higher in the horses that were not given supplemental phytase ($P<.05$). There was no importance to age or blocking group variability or diet by phytase interaction. There were also no significant differences between diets or phytase levels in the phosphorous percentages of the third metacarpal on day 120. There was a trend of a diet by phytase interaction, but it was not significant, with phytase decreasing bone P in the low-P horses from 9.85 to 7.92%, but increased bone P from 5.05 to 8.70% in the adequate-P horses.

Plasma mineral concentrations

The initial plasma calcium concentration was the similar for all diet-phytase groups at an average of 9.62 mg/dL (Table 4). There was no significance of diet, phytase, diet by phytase interaction, age or blocking group. Calcium concentration did increase on day 60 to 11.82 mg/dL. Again, there was no significance of diet, phytase, diet by phytase interaction, age or blocking group. However, the no added phytase was numerically slightly higher in calcium concentration. The final plasma calcium also did not have any significant differences due to diet, phytase, diet by phytase interaction, age or blocking group. There was a slight numerical difference between the phytase levels at day 120, with the added phytase having higher calcium concentrations. However, the difference was not significant statistically or physiologically.

The plasma phosphorous concentration was similar across all groups at day 0 with the average being 2.49 mg/dL. There were no significant differences between diet, phytase, diet by phytase interaction, age or blocking group. However, there was a slight numerical difference between phytase groups, as the weanlings that were not receiving phytase had slightly higher plasma phosphorous levels at the start. At day 60 and 120, there were no differences between diet, phytase, diet by phytase interaction, age or blocking group. However, the day was a factor

Table 4. Plasma mineral concentrations of weanling horses fed variable levels of phosphorous with or without phytase.[a]

		Low-P		Adequate-P					
Item:	Phy[b], FTU/kg:	0	1500	0	1500	SEM	P[c]	Phy	Diet x Phy
Calcium(mg/dL):									
Day 0:[d]		10.13	9.70	8.89	9.79	.77	NS	NS	NS
Day 60:		11.63	11.46	12.96	11.23	.87	NS	.1642	NS
Day 120:		11.36	11.87	10.49	11.97	.63	NS	.0616	NS
Phosphorous(mg/dL):									
Day 0		2.53	2.39	2.76	2.26	.29	NS	.1608	NS
Day 60		1.85	1.70	1.79	2.17	.31	NS	NS	NS
Day 120		2.19	2.04	2.16	2.01	.44	NS	NS	NS

[a] Data are means of four horses per treatment, except the Adequate-P, 0 Phytase which contained three.
[b] Phy= Phytase
[c] P= Diets containing varying levels of phosphorous
[d] NS= not significant, P >.20

with the horses at day 60 having a phosphorous concentration of 1.88 mg/dL and 2.10 mg/dL at day 120. However, this should not have been a factor physiologically.

Digestibility Results

Energy digestibility was not affected by the experimental diets(Table 5). The weanlings had average energy digestibility of 51.21% and 53.95% on days 60 and 120, respectively. The weanlings were not affected by diet, phytase, diet by phytase interaction, age, blocking group or day. The error values were fairly high, but that is likely due to animal variability.

Actual intake of calcium averaged 28.47g and 45.75g at day 60 and 120 respectively. The increase in amount corresponds to increase in feed consumption due to growth. Calcium digestibility percentages were greatly affected by the ingestion of sand that became apparent once the study was complete. Several of the day 60 digestibilities were negative, which is likely not a correct value. The error value of 15.15 is high and thus these values are difficult to compare. The day 120 calcium digestibilities were slightly closer to the

Table 5. Nutrient digestibility of weanling horses fed varying levels of phosphorous, with or without phytase.[a]

		Low-P		Adequate-P					
Item:	Phy[b], FTU/kg:	0	1500	0	1500	SEM	P[c]	Phy	P x Phy
Energy digestibility (%):									
Day 60[d]		47.17	55.18	51.73	50.74	8.90	NS	NS	NS
Day 120		58.11	58.23	58.03	41.43	12.35	NS	NS	NS
Calcium digestibility (%):									
Day 60		-6.18	-3.75	31.63	2.95	15.15	.1268	NS	NS
Day 120		47.57	61.59	44.60	33.02	14.23	NS	NS	NS
Phosphorous digestibility (%):									
Day 60		34.47	38.84	40.23	26.27	8.39	NS	NS	NS
Day 120		44.68[e]	40.75[e]	9.48[f]	26.04[g]	5.57	.0011	.1536	.0496

[a] Data are means of four horses per treatment, except the Adequate-P, 0 Phytase which contained three.
[b] Phy= Phytase
[c] P= Diets containing varying levels of phosphorous
[d] NS= not significant, P >.20
[e,f,g] Means within a row with different superscripts differ (P< .05).

expected values of around 50% Ca digestibility (NRC, 2007). The average calcium digestibility at day 120 was 46.70%. Statistically, diet, phytase, diet by phytase interaction, age or blocking

group was not significant, yet numerically the addition of phytase to the low-P diet increased the % digestibility of Ca.. Again, the error term is high and sand consumption was likely a factor.

Phosphorous intake averaged 20.07g and 19.76g at day 60 and 120, respectively. Both of these values are close to the NRC recommendations of 20.9-21.7g/day for growing weanlings (NRC, 2007). However, at day 120, the values are slightly lower than the recommended values. Phosphorous digestibility at day 60 was not affected by the differences in diet or phytase. The weanlings had an average digestibility of 34.95% at day 60. Day 120, however, was affected by diet differences. The horses on the low phosphorous diet had higher digestibilities than those on the adequate diet. There was no statistical significance to any variation between the phytase levels. However, a diet by phytase interaction did exist. The two low phosphorous groups were statistically the same, and higher than the adequate phosphorous diet. Within the adequate phosphorous diets there was a difference. The horses that received phytase had higher digestibility at 26.04% than those that did not receive phytase supplementation, which only had a digestibility percentage of 9.48%.

Discussion

Phytase supplementation in pigs has shown an increase in bone strength, as well calcium and phosphorous digestibility (Cromwell et al., 1995; Kim et al., 2005) Therefore, the addition of phytase to the equine diet was studied in order to obtain whether bone strength would be affected in the horse as well. Although other studies had not seen an increase in phosphorous digestibility (Van Doorn et al., 2004; Hainze et al., 2004), there was some evidence that phytase could increase calcium digestibility (Van Doorn et al., 2004). Therefore, sixteen weanlings were used to test the effect of phytase on bone minerals as well as mineral digestibility and plasma mineral concentrations.

Growth results for this study were not greatly affected by any of the diet-phytase combinations. There was a slight effect due to diet differences. The horses on the adequate-P diet had higher end weights than the low-P diet. However, these horses had start weights that were slightly greater than the others, yet not at a significant level. Therefore, it is not unexpected for them to finish heavier. Average daily gain was not different between the groups. While in pigs gain is improved by phytase, it was not in the weanling horses studied.

Individual animal variability was a key factor during this project, as is evident in the starting bone growth data. The starting calcium levels of the splint bone and third metacarpal were highly variable. The variability could not be attributed to age or initial blocking group, which suggests that the percent of calcium in the bones varied between individual horses and that genetics were likely a factor. Beyond the variability of the initial data, there was no effect due to phytase or the diets on the calcium content of either bone. There was a diet by phytase interaction in percent calcium of MCIII. The added phytase decreased the bone calcium content of the horses ingesting the low-P diet, yet increased the calcium levels of those that were ingesting the adequate-P diet. An explanation of these results is not easily apparent. The two diets however were not very different in phosphorous concentration and the low-P diet toward the end of the study contained .947% Ca compared to the .487% in the adequate-P diet. Therefore, perhaps the horses on the low-P diet performed better due to the high calcium content of the feed. Additionally, the addition of phytase to the adequate-P diet may have brought the horses up to a similar level as the low-P horses. However, there is no explanation as to why the low-P with added phytase diet was not also higher in bone Ca percentage. Phosphorous content in the MCIII was not affected by either diet or phytase. There was a slight phytase effect in the splint bone; phosphorous decreased with the addition of phytase. Since the splint bone is

vestigial, perhaps it serves as a mineral reservoir for the animal, and thus a decrease in mineral content would not be greatly significant. Overall in the MCIII, bone minerals of the horses on the low-P diet were decreased by the addition of phytase, while increased by the addition of phytase to the adequate-P diets.

Plasma minerals were not affected by diet or phytase. A change in plasma minerals was not expected, as their concentrations are very tightly regulated. Calcium is thought to be the most tightly regulated ion in the body (Copp, 1960). Therefore, phytase addition should not affect the blood minerals of the animal. Plasma calcium did increase across all treatments over time, but not with any physiological significance. Phosphorous concentration decreased at day 60, but returned to the basal level by day 120. This decrease may have been associated with the systemic shock of the study itself, as the initial 60 days were associated with the healing of the surgical sites. Again, however, this decrease is not large enough to be physiologically significant.

The original internal marker that was going to be used for this project was acid insoluble ash. However, while the weanlings' intakes were closely monitored, while they were turned out on dry lot during the day, they consumed sand and dirt. Due to the large amounts of sand that were consumed by the weanlings in unknown quantities, the use of acid insoluble ash was not feasible. Therefore, indigestible acid detergent fiber was used instead. Although there has been no previous research in horses to support the success of this procedure in this species, it is commonly used in cattle research (Cochran et al., 1986; Stanley et al., 1993). In this study the calculated fecal output amounts, which ranged from 1.22kg to 4.36kg DM, were within the expected amounts, thus making the internal marker an acceptable assay compared to the acid insoluble ash. The calcium digestibility percentages at day 60 were indicative of the large volumes of sand ingested. With percentages -6.18, -3.75, and 2.95% for the low-P without

phytase, low-P with phytase, and adequate-P with phytase, respectively, it is apparent that not all of the calcium ingested by the animals was measured. However, by day 120, the percent digestibility of calcium had returned to a more appropriate amount, which was not significantly different between diets or phytase levels.

Energy digestibility was not affected by the different diets or phytase levels. Additionally, the energy digestibility values were consistent with previous studies, as far as mixed ration diets. Hintz et al. (1971) showed that carbohydrate digestion, which is the primary energy source in equine diets (NRC, 1989), is generally around 51% in a grain and forage mixed ration. The diets were formulated to have the same amount of energy, so it was not expected that there would be any differences. Dry matter digestibility averaged 48.19 and 52.96% at day 60 and day 120, respectively. These averages are similar to other reported average dry matter digestibilities (Van Doorn et al., 2004), which also helps justify the use of this marker.

Phosphorous digestibility was also not different between any of the treatments at day 60, which is consistent with previous research (Van Doorn et al., 2004; Patterson et al., 2003; Hainze et al., 2004). However at day 120 there was a difference between the two diets. The horses on the low-P diets had significantly higher phosphorous digestibility than those on the adequate-P diet. Both diets were similar in P content, with .34 and .39% P in the adequate-P and low-P concentrate rations, respectively. The low-P diet, however, did not have any added inorganic phosphate. Even with these deviations from the intended diets, it is not apparent as to why the low-P horses performed better. The phytase did increase the percent digestibility of P in the adequate-P horses from 9.48 to 26.04%. The percent digestibility of the horses that did not receive phytase seems abnormally low, and perhaps there was also interaction of the consumed sand with these numbers.

Additionally, one of the weanlings did not complete the studied. The individual horse, that was on the adequate-P diet, with no added phytase, died of colitis approximately halfway through the study. His death was not related to the experiment itself, and his original data was excluded from all results.

While phytase did not significantly improve bone minerals, digestibility or plasma mineral concentrations, this study did show the substantial differences between the splint bone and MCIII. Therefore, the splint bone is not a good model for bone composition of loading bones.

Implications

Results of this study do not support the use of phytase as a good way to improve calcium and phosphorous digestion. The inherent phytase of the equine digestive tract is probably sufficient for the horse in breaking down phytate. In addition, there is no evidence to support that phytase greatly alters bone composition. More research concerning phytase and horses is needed, yet with greater animal numbers and more of a controlled environment.

The use of indigestible acid detergent fiber as an internal marker is very promising. Research needs to be conducted comparing this method against other common marker methods used in horses in order to examine its effectiveness.

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CHAPTER 4

CONCLUSION

This study was conducted to evaluate the effectiveness of phytase as a way to increase bone mineral content and increase nutrient digestibility. However, there was no change in bone composition due to the diets or phytase. However, this experiment has shown the difference between the composition of the third metacarpal and the splint bone of the horse. The calcium and phosphorous content between the two bone differs substantially ($P < .0001$), and thus the splint bone is not a good model for general bone composition in the horse. Plasma minerals were not affected by the different diets or phytase. Digestibility of energy was similar amongst all weanlings, as was expected due to the isocaloric nature of the diets. Phosphorous digestibility was higher in the horses on the low-P diet, and phytase did improve phosphorous digestibility of the adequate-P diet. Calcium digestibility did not differ between diet or phytase addition, but the numbers were not likely correct. The horses on the study consumed sand that was not accounted for when they were turned out. Therefore, it is hard to vouch for the validity of the digestibility data.

A novel use of indigestible acid detergent fiber as an internal marker was implemented in this project. While the data seemed to support the effectiveness its use for this study, further research needs to be conducted in order to determine whether it is a good marker to use in equine nutritional studies, compared to other established internal markers and against total collection values.