PHOSPHORUS DIGESTIBILITY AND PHYTATE DEGRADATION IN LONG YEARLINGS AND MATURE HORSES

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2013

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Dr. Laurie Lawrence, Major Professor
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PHOSPHORUS DIGESTIBILITY AND PHYTATE DEGRADATION IN LONG YEARLINGS AND MATURE HORSES

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By
Ashley Lauren Fowler
Lexington, KY

Director: Dr. Laurie Lawrence, Professor of Animal Science
Lexington, KY

2013

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ABSTRACT OF THESIS

PHOSPHORUS DIGESTIBILITY AND PHYTATE DEGRADATION IN LONG YEARLINGS AND MATURE HORSES

Much of the phosphorus (P) in grain-based concentrates fed to growing horses is in the form of phytate-P. Little is known about the ability of young horses to degrade phytate-P or whether age affects mineral digestion in horses. The objective of this study was to examine the effect of age on P, calcium (Ca), and magnesium (Mg) digestibility and phytate-P degradation. Four long yearling geldings and 4 mature geldings were fed a diet of alfalfa cubes, timothy cubes and a pelleted concentrate. The diet contained 0.28% total P and 17.4% of that P was in the phytate form. There was a 14-d diet adaptation period followed by a 4-d fecal collection period. There was no difference in apparent P digestibility between the 2 age groups (P > 0.05). Phytate-P disappearance was 94.8% and did not differ between ages (P > 0.05). Apparent Ca digestibility tended to be higher in mature geldings (P = 0.0526), but apparent Mg digestibility did not differ between ages (P > 0.05). Long yearlings have the same ability to digest P and Mg as mature geldings, but more research is needed to determine the effect of age on Ca digestibilities.

KEYWORDS: environmental impact, fecal collection, growing horse, mineral, organic phosphorus

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December 4, 2013
PHOSPHORUS DIGESTIBILITY AND PHYTATE DEGRADATION IN LONG YEARLINGS AND MATURE HORSES

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LIST OF ABBREVIATIONS

ADF Acid detergent fiber
ADFD Acid detergent fiber digestibility
ADG Average daily gain
Al Aluminum
ATP Adenosine triphosphate
Ba Barium
BAP Bone alkaline phosphatase
BW Body weight
Ca Calcium
Ca:P Calcium to phosphorus ratio
DE Digestible energy
DM Dry matter
DMD Dry matter digestibility
DMI Dry matter intake
DNA Deoxyribonucleic acid
Fe Iron
FGF23 Fibroblast growth factor-23
FTU Unit of phytase activity
HPO$_4^{2-}$ Divalent hydrogen phosphate ion
H$_2$PO$_4^{-}$ Monovalent dihydrogen phosphate ion
IVEDMD In vitro enzymatic dry matter disappearance
IVDMD In vitro dry matter disappearance
K Potassium
$Lpa$ Low phytic acid
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>Mg</td>
<td>Magnesium</td>
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<tr>
<td>Mn</td>
<td>Manganese</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>N</td>
<td>Nitrogen</td>
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<tr>
<td>Na</td>
<td>Sodium</td>
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<tr>
<td>Na\textsuperscript{+}/K\textsuperscript{+}ATPase</td>
<td>Sodium-potassium adenosine triphosphatase</td>
</tr>
<tr>
<td>Na\textsuperscript{+}/P IIb</td>
<td>Sodium-phosphate transporter IIb</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral detergent fiber</td>
</tr>
<tr>
<td>NDFD</td>
<td>Neutral detergent fiber digestibility</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>OM</td>
<td>Organic matter</td>
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<tr>
<td>P</td>
<td>Phosphorus</td>
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<tr>
<td>Phytate-P</td>
<td>Phosphorus in the phytate form</td>
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<tr>
<td>PiT-2</td>
<td>Phosphate transporter 2</td>
</tr>
<tr>
<td>PiUS</td>
<td>Inorganic phosphate uptake stimulator</td>
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<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
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<tr>
<td>Si</td>
<td>Silicon</td>
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<td>SLC20</td>
<td>Solute carrier family 20</td>
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<tr>
<td>SLC34</td>
<td>Solute carrier family 34</td>
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<tr>
<td>VFA</td>
<td>Volatile fatty acid</td>
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<td>Zn</td>
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CHAPTER 1: INTRODUCTION

Phosphorus (P) is a very important mineral for life, as it is the second most abundant mineral in the body and is present in nearly every cell (Swenson and Reece, 1993). However, recent environmental concerns have been raised about P being a water pollutant and also a dwindling non-renewable resource. Therefore, more attention has been placed on maximizing P utilization efficiency in the animal industry in order to preserve this limited resource and minimize excretion into the environment.

Organic P or phytate, makes up around two-thirds of the total amount of P in grains and is also present in smaller amounts in forages (Common, 1940; Eeckhout and de Paepe, 1994). In the digestive tract phytate-P must be liberated by the action of phytase, which is produced mainly by the microbes in the gastrointestinal tract. Therefore the organic form of P is less available to monogastric animals that lack large amounts of microbes to liberate the P from the phytate molecule. Inorganic P is often added to animal feeds because it is thought to be a more available form for the animal to utilize. However, there has been debate about how available organic P is to the horse. Hintz et al. (1973) found that the P in wheat bran, which is presumed to be mostly phytate-P, was half as available as inorganic P. However, other authors have reported phytate-P to have the same digestibility as inorganic P (Hainze et al., 2004, Van Doorn et al., 2004a). Only one study reported the actual degradation of phytate-P in mature horses and found the disappearance to be around 94% (Lavin et al., 2013). Inorganic P may be unnecessary in animal feeds if organic P is already available; this would lead to a decreased P intake and a reduced dependence on mining the already diminishing supply of rock phosphate.
While it appears that phytate-P is able to be degraded by the adult horse, there have been no studies that examine phytate-P digestibility in growing horses compared to mature horses. In poultry, it has been suggested that phytate-P utilization increases with increasing age, as the chick’s digestive capability changes as the animal matures (Olukosi et al., 2007). It has been shown that growing horses have higher total P digestibilities than mature horses, but the amount of phytate was not taken into consideration in that experiment (Cymbaluk et al., 1989). It is unclear if the growing horse would be able to break down phytate-P and utilize it as efficiently as a mature horse.

By better understanding the digestibility of P by horses who are consuming organic P, more accurate requirements can be calculated which may reduce P intake and excretion. In addition, knowing how much phytate can be degraded by a growing horse may provide insight into the forms of P that can be included in the diets of these animals.
CHAPTER 2: LITERATURE REVIEW

**Phosphorus metabolism in horses**

Phosphorus is a very important mineral for living organisms, as it has more known functions in the body than any other mineral. It is the second most abundant mineral in the body, behind Ca. Approximately 75% of the body’s stores of P are found in the bones and teeth in the form of apatite salt and Ca phosphate. In addition to providing skeletal support, P is involved in energy metabolism in the form of ATP, building cell membranes as phospholipids, buffering body fluids, phosphorylating proteins for activation and building DNA as a component of nucleotides (Swenson and Reece, 1993). The intake of dietary P helps to support these processes in new tissue growth as well as replacing the P that is lost to cell turnover.

Once P is ingested, it must then be absorbed. The horse has a unique flux of P into and out of the gut. Schryver et al. (1972) first studied P absorption and secretion in horses. In their study P was secreted into the first half of the small intestine and an almost equal amount was absorbed in the latter half of the small intestine. More P was secreted into the cecum, possibly to buffer the volatile fatty acids (VFAs) that are produced in the hindgut by the fermentation process. Unlike in other animals where P absorption happens in the small intestine, the main site of P absorption in the horse is in the dorsal large colon and the small colon (Schryver et al., 1972; Swenson and Reece, 1993).

The mechanism for P transport across the intestinal lining of the horse has not received a lot of attention. One study reported that ouabain, an inhibitor of the Na⁺K⁺ATPase, significantly inhibited P transport across equine jejunal tissue *in vitro*.
This finding suggests that P transport in the horse depends on a Na⁺-dependent mechanism. In fact, increasing the salt intake of horses from 1 to 5% increased P retention from 3 to 7 mg/kg BW and increased true P absorption from 28 to 40% (Schryver et al., 1987). It is possible that the increased amount of sodium facilitated more P transport across the intestinal mucosa. In other species, two main P transporters have been identified in the intestinal membrane. The first transporter belongs to the SLC34 family and is called Na⁺/P IIb. The second belongs to the SLC20 family and is called PiT-2. The Na⁺/P IIb transporter transports 3 Na ions with 1 P ion into the enterocyte (Hartmann et al., 1995). It appears to transport both divalent phosphate (HPO₄²⁻) and monovalent phosphate (H₂PO₄⁻) independent of pH (Busch et al., 1995; Hilfiker et al., 1998). The PiT-2 transporter transports 2 Na ions with 1 P ion into the enterocyte (Ravera et al., 2007). It is inhibited by alkaline pH, which indicates that it prefers to transport the monovalent form of phosphate (H₂PO₄⁻) that predominates at a lower pH (Bai et al., 2000). These transporters have not been identified or characterized in horses, but it is likely that they exist in the horse because they are present in many other species.

Phosphate transport into intestinal enterocytes is regulated by a few different factors. In rats, a low P diet has been shown to increase the mRNA levels of a membrane-bound protein involved in P transport, PiUS, and therefore upregulate Na⁺-dependent P transport (Katai et al., 1999). This upregulation in transport may be a mechanism to ensure sufficient P absorption when P intake is not adequate. Vitamin D has been shown to be an important nutrient for P transport. Vitamin D deficient rats showed a decrease in Na⁺-dependent P transport, but rats that were repleted with vitamin D showed an increase
in PiT-2 mRNA and regained normal P transporting capabilities (Katai et al., 1999). In horses, vitamin D supplementation increases the digestibility of P, potentially due to the increase in P transporters (Hintz et al., 1973; Breidenbach et al., 1998a).

There are a few factors that can hinder the availability of P to be absorbed in horses. The presence of phytate-P most likely has the greatest negative influence on P digestibility and will be discussed in a later section. Kapusniak et al. (1988) demonstrated that elevated amounts of dietary Al can decrease P digestibility in ponies, potentially due to the formation of insoluble phosphates with Al. High amounts of Ca in the diet may negatively affect P digestibility as well. Van Doorn et al. (2004b) found that ponies fed 148 mg Ca/kg BW/d had significantly higher apparent P digestibilities than ponies fed 316 and 535 mg Ca/kg BW/d. Cymbaluk et al. (1989) calculated a regression equation relating fecal P to P intake and found the R-squared value to be 0.53. When fecal Ca was added to the equation, the R-squared value improved to 0.82, which suggests that the excretion of fecal P may be related to how much fecal Ca is excreted.

In addition to dietary factors, the environment has also been shown to influence P digestibility. Horses housed in a cold barn during the winter had significantly lower P digestibilities than horses housed in a heated barn (Cymbaluk et al., 1990). Cold temperatures are known to decrease diet digestibility in many species, but it is still somewhat unclear on how this occurs and to what extent (NRC, 1981). An increase in rumen motility and a subsequent decrease in retention time have been reported in ruminants in response to cold temperatures, which leads to a decrease in diet digestibility (Westra and Christopherson, 1976; Kennedy et al., 1977). The horses used by Cymbaluk et al. (1990) showed a numerical decrease in retention time when housed in cold
temperatures, but the differences were not significant. It is possible that this numerically smaller retention time in the cold-housed horses contributed to a decrease in P digestibility.

The effect of exercise and confinement on P balance has varying results. Buchholz-Bryant et al. (2001) demonstrated that horses had greater P balances when they were sedentary than when they were performing an ascending aerobic exercise regimen. Nielsen et al. (1998) reported that 2 yr old Quarter Horses had no statistical difference in P retention when they were sedentary or exercised, although there appeared to be a numerical decrease in retention after the horses began exercising. In contrast, a review of the available literature found that there was no difference in mean daily P retention in exercised and sedentary horses (Lawrence et al., 2003). However, other factors such as diet, exercise regimen, age, and experimental methods could influence P balances of the different studies independent of the effect of exercise.

Age can also affect P digestibility in horses. Cymbaluk et al. (1989) reported that P absorption decreased as horses aged from 8 to 24 mo. However, the composition of the diet changed as the horses aged and the amount of P present in the diet decreased from 145.7 mg/kg BW to 83.5 mg/kg BW. These factors could confound the results of the study. In a later experiment, Cymbaluk et al. (1990) showed that P digestibility decreased from 52.2% in 8 mo old horses to 34.8% in 12 mo old horses fed the same diet adjusted for changing body weights. Mature ponies fed the same diet as the growing horses had even lower P digestibility values ranging from 23.5% to 32.1%. The reason for this decline could be due to changing P transporter capabilities. In rodents, intestinal type Na⁺/P IIb transporter gene expression decreases with increasing age, thus decreasing the
capability to transport P (Xu et al., 2002). A study performed with rabbit brush border membrane vesicles also observed increased Na\(^+\)-dependent P transport activity in younger animals compared to older animals (Borowitz and Granrud, 1992). A decline in transporter activity with age leads to less P being absorbed and lower P digestibilities. Lower digestibilities for older horses means that when calculating daily P requirements, different P digestibilities should be used for horses of different ages. The NRC (2007) uses a digestibility value of 45% for all growing horses (0 to 24 mo) and a lower digestibility value of 35% for mature horses due to differences in diet composition. However, it is possible that age-related changes in P transporters as well as different diet compositions play a role in P absorption in horses of differing ages.

Once P is absorbed, it can be utilized by the horse for a variety of functions, secreted back into the gastrointestinal tract, or excreted in the urine. In mature horses, absorbed P is mainly used to replace dermal losses and for gastrointestinal secretions. In growing horses, the majority of the absorbed P is used for new tissue growth, especially bone development. In addition to providing skeletal support, bones also serve as a store for Ca and P. In all animals, bone is constantly being broken down and rebuilt due to the activity of osteoblasts (bone mineralization) and osteoclasts (bone resorption). This bone remodeling process is hormonally regulated and varies due to the animal’s need for Ca and P and the physiological state of the body.

Insulin is one hormone that specifically can affect bone remodeling. Insulin binds to an insulin receptor on osteoblasts which decreases the expression of osteoprotegerin (Opg) and leads to increased bone resorption by osteoclasts (Karsenty and Ferron, 2012). The activated osteoblasts then release osteocalcin that stimulate the beta cells in the
pancreas to produce more insulin, resulting in a feed-forward loop (Karsenty and Ferron, 2012). In rodents, low insulin secretion and insulin resistance are linked with lower serum total osteocalcin concentrations (Botolin et al., 2005). Due to the feed-forward loop involving insulin and osteocalcin, it is expected that both insulin and osteocalcin concentrations will increase over time. However, leptin is a hormone secreted by adipose tissue that has been shown to reduce the amount of biologically active osteocalcin in the body, thus keeping the insulin-osteocalcin feed-forward mechanism in check (Hinoi et al., 2008). All of these hormones together help regulate some aspects of bone metabolism.

In addition to regulating bone metabolism directly, the body needs to regulate Ca and P metabolism, which are the main components of bone. There are three main hormones that affect P metabolism in most animals: vitamin D, parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF23) (Bergwitz and Jüppner, 2010). Vitamin D can be ingested in the diet or can be synthesized in the skin when exposed to sunlight. When skin is exposed to the sun, 7-dehydrocholesterol is converted to previtamin D₃, which is then transformed to vitamin D₃ through a heat-dependent process (Wallis et al., 2008). Vitamin D₂ and vitamin D₃ from the diet are transported into venous circulation to be deposited in fat cells for storage (McDowell, 1989). The liver and kidney hydroxylate these vitamins into their active metabolite forms when they are needed (McDowell, 1989). Vitamin D can improve the absorption of Ca and P from the intestines and is also important for good bone health. It is responsible for increasing bone formation and resorption (Bergwitz and Jüppner, 2010). Vitamin D deficiencies are associated with low bone mineral density and osteomalacia, or rickets (Holick, 2004).
Parathyroid hormone is released from the parathyroid gland in response to high dietary and serum P as well as low serum Ca (Poole and Reeve, 2005; Jüppner, 2011). It acts to restore normal circulating concentrations of Ca and P in the blood. In the kidney, PTH acts to increase Ca reabsorption, decrease renal P reabsorption and increase 1α-hydroxylase activity which produces the active form of vitamin D₃, 1,25-dihydroxycholecalciferol (Albright and Ellsworth, 1929; Murayama et al., 1998). The vitamin D metabolite, 1,25-dihydroxycholecalciferol, then acts to increase Ca and P absorption from the intestines as mentioned above. Continuous exposure to PTH also acts to increase bone resorption to help return blood Ca to normal levels (Uzawa et al., 1995), while intermittent exposure results in increased bone mineral density (Dobnig and Turner, 1995).

Fibroblast growth factor-23 is produced by osteoclasts and osteoblasts and is released in response to high blood P levels or high 1,25-dihydroxycholecalciferol levels (Perwad et al., 2005; Jüppner, 2011). This hormone acts to lower blood P levels by decreasing renal reabsorption of P to increase the amount of P that is excreted in the urine (Bergwitz and Jüppner, 2010). Because FGF23 has not been examined in equines, it is not known if this hormone would have the same effects in the horse. Horses do not normally have large amounts of P in their urine (Schyver et al., 1971b), so it is unknown if FGF23 would influence urinary P excretion the same way it does in other species. FGF23 also decreases the amount and activity of brush-border membrane Na⁺-dependent P transporters available for P absorption in the intestine (Perwad et al., 2007). FGF23 suppresses expression of 1α-hydroxylase, thereby decreasing the amount of 1,25-dihydroxycholecalciferol produced, indirectly decreasing the amount of P absorbed from
the intestine (Perwad et al., 2007). The net result is a decrease in the circulating levels of P.

In horses, the Ca and P homeostatic system is somewhat different than most other animals. Vitamin D, PTH and FGF23 may react and be regulated differently in the horse than in other species. In the horse, serum Ca concentrations are much higher than in other species whereas serum P and 1,25-dihydroxycholecalciferol are much lower (Breidenbach et al., 1998b). The low level of circulating 1,25-dihydroxycholecalciferol likely indicates that this hormone plays a different role in the Ca and P metabolism of horses compared to other animals. Breidenbach et al. (1998a) reported that the digestibility of Ca was not improved in horses supplemented with vitamin D and renal Ca excretion increased markedly, contrary to what occurs in most other animals. The horses supplemented with vitamin D in this study also exhibited improved P digestibility, increased renal P excretion and higher plasma levels of P, similar to what occurs in other animals. The renal loss of P in vitamin D supplemented horses used in this study was greater than the amount that was being absorbed from the intestines and the authors suggested that vitamin D may increase bone mobilization as well. Horses can develop soft tissue calcification with excess vitamin D supplementation, which further suggests that vitamin D does play a role in bone metabolism (Harrington and Page, 1983). Serum P levels are not as tightly regulated in horses as they are in other species, suggesting that the regulatory mechanisms for maintaining P homeostasis in horses may not be as pronounced as described in other species (Breidenbach et al., 1998b). However, because circulating Ca levels are tightly regulated in the equine, regulating hormones may exert
their effects in response to low or high blood Ca, which could indirectly affect P metabolism.

There are a variety of factors that affect the absorption and metabolism of P in the body, most of which relate to the needs and physiological state of the animal. The horse has some unique aspects of P absorption and metabolism compared to other species; however it is still unclear why this difference exists. More research is needed in horses to further elucidate mechanisms involved in P transport and homeostasis.

**Phosphorus requirements in horses**

Accurate estimates of daily P requirements are necessary to ensure adequate intakes, but also to help prevent wasting of this limited resource. In mature horses, the P requirement is calculated using estimates of fecal endogenous losses and the true digestibility of P (NRC, 2007). Schryver et al. (1971b) found that the fecal endogenous loss of the mature horse was 10 mg/kg BW and that this value did not change with varying intakes. The true digestibility of P for an adult horse has been estimated to be around 35%. Therefore for a 500-kg mature horse, the NRC (2007) equation for calculating the requirement would be (0.01 g P needed/0.35 digestibility coefficient) * 500 kg BW with a resulting P requirement of 14.3 g.

For growing horses, the requirement is based on two components: growth and maintenance. The growth component is the amount of P that is required per kg of BW gain, adjusted for digestibility. It has been estimated that growing horses deposit 8 g P/kg BW gain (Schryver et al., 1974). The digestibility of P in diets fed to growing horses is estimated to be 45% (NRC, 2007). A higher digestibility is used for growing horses
because they are fed diets with added inorganic P, while mature horses typically eat mostly plant sources of P (NRC, 2007). The maintenance component is calculated similarly to the mature horse, by using estimates of fecal endogenous losses and digestibility.

It has been suggested that the fecal endogenous losses of growing horses are higher than those of mature horses (Kichura et al., 1983; Cymbaluk et al., 1989). The NRC (2007) uses a value of 18 mg/kg BW for the fecal endogenous losses of growing horses based solely on the work done by Cymbaluk et al. (1989). Using this information, the NRC (2007) equation used to calculate P requirements for the growing horse is \( (0.018 \text{ g P needed}/0.45 \text{ digestibility coefficient} \times \text{kg BW}) + (8 \text{ g P needed}/0.45 \text{ digestibility coefficient} \times \text{kg gain/d}) \). However, previous versions of “The Nutrient Requirements of Horses” (NRC, 1989) and more recent studies have suggested that the fecal endogenous losses of growing horses are more similar to those of mature horses. Kichura et al. (1983) found that in yearling horses, high amounts of dietary Ca (200% of requirement) increased endogenous fecal P loss. Cymbaluk et al. (1989) fed growing horses varying levels of Ca, ranging from 41% to 203% greater than their respective requirements. It is possible that these high levels of Ca caused greater fecal endogenous P losses in this study. Ogren et al. (2013) used a regression equation based on P intake levels and excretion levels and suggested that fecal endogenous losses of growing horses are around 10 mg/kg BW. Oliveira et al. (2008) used radiolabelled P isotopes and suggested fecal endogenous losses are around 8.42 mg/kg BW.

If the fecal endogenous losses of growing horses are truly lower than the NRC (2007) suggests, then the requirements listed by the NRC (2007) for growing horses may
be too high. However, requirements for growth are also calculated using a higher true digestibility than for mature horses. As mentioned above, this is based on the assumption that the P in diets fed to growing horses will come from inorganic P sources. If the P in growing horse diets comes only from organic sources, then a lower true digestibility value may be necessary to calculate requirements if organic P is truly not as available as inorganic P. A lower true digestibility combined with a lower fecal endogenous loss value could result in a similar daily P requirement to the NRC (2007).

**Sources of phosphorus in equine feeds**

Total P content in equine feeds can vary depending on the forage type and grain type. Table 2-1 shows P concentrations of different forages commonly fed to horses in Kentucky. Fowler et al. (2012) examined P concentrations in pasture grasses in Kentucky and found the range to be 0.326 to 0.465%. Lawrence et al. (2006) looked at P concentrations in cool season grass pastures on Kentucky horse farms and found the average concentration to be 0.370%. The DairyOne feed library showed that the average P concentration in pasture is typically higher than it is in hays (DairyOne Forage Lab, 2013). Table 2-2 shows the P concentration in common grains and grain by-products as reported by several studies. Average total P ranged from 0.28% (corn) to 1.69% (rice bran). There is a much larger range of total P in grains and grain by-products than in common forages.

P is present in equine diets in two forms, organic and inorganic. Inorganic P makes up the majority of the total P in forages, such as hay and pasture, and is also present in grains to varying extents. Inorganic P is also included in diets in the form of
monocalcium phosphate, dicalcium phosphate, monosodium phosphate or defluorinated phosphate. Organic P, or phytate, makes up an average of two-thirds of the total P in grains and can also be present in smaller amounts in some forages, such as alfalfa (Common, 1940; Eeckhout and de Paepe, 1994). The amount of phytate-P in feed ingredients varies depending on the type of grain and processing. In common grains and grain by-products, average phytate-P content ranges from 0.19% (corn) to 1.31% (rice bran; Table 2-2). Around 60 to 78% of the total P is in the phytate-P form in these feed ingredients. Byproducts usually have a greater percentage of phytate-P than whole grains, and protein sources usually have a lower amount of phytate than byproducts and whole grains. Previous research has found a positive relationship between total P and phytate-P concentrations in grains, indicating that the more total P in the seed, the more phytate will be present as well (Eeckhout and de Paepe, 1994).

Due to the presence of organic P in these feed ingredients and because the organic form of P is thought to be less available to horses, inorganic P is often added to horse feeds to increase the P content. However, feeds may not need to be supplemented with inorganic P to meet requirements of most horses even when P requirements are high. For a mature 500-kg horse at maintenance, the P requirement is 14 g (NRC, 2007). If this horse is consuming 10 kg (2% of BW) of an average legume hay daily, then daily P intake will be 27 g P from the hay alone (Table 2-1). This amount greatly exceeds the daily P requirement for a horse at maintenance without the addition of any grain to the diet. For a mature 500-kg horse in moderate work, the P requirement is 21 g (NRC, 2007). Horses in moderate work perform activities such as polo, ranch work and frequent showing. If this horse eats 2.25% of BW in feed per day and consumes legume hay and
oats in a ratio of 75:25, he would be eating 8.44 kg of hay and 2.81 kg of oats. This diet would provide 29 g P/d, which more than meets the P requirement for this horse (Table 2-1; Table 2-2). For a mature 500-kg horse in heavy or very heavy exercise, the P requirement increases to 29 g (NRC, 2007). Horses in heavy and very heavy exercise are performing more strenuous activities such as 3-day eventing and racing. If this horse eats 2.5% of BW in feed per day and consumes legume hay and oats in a 60:40 ratio, he would be eating 7.5 kg of hay and 5 kg of oats. This diet would provide 38.5 g P/d, which exceeds the daily P requirement for horses in heavy work (Table 2-1; Table 2-2). The needs of both of these classes of working horses can be met using forage and grain products alone, without the addition of inorganic P to the diet.

Hainze et al. (2004) demonstrated that a diet containing alfalfa hay and oats with no added inorganic P was able to adequately meet P requirements for growing horses. These authors also observed that feeding diets containing inorganic P exceeded the horses’ requirements and resulted in excess water soluble P excretion in the manure. Adding inorganic P to diets may not only be unnecessary, it also could be harmful to the environment. Water soluble P is a major environmental issue because it is the most likely form of P to run off into water bodies and cause eutrophication. By avoiding the addition of inorganic P to the diet, the potential for P runoff could be reduced.

**Measuring phosphorus digestibility**

P requirements are calculated using P digestibility. In order to calculate accurate P requirements that meet the needs of the animal without providing excess, it is important to obtain accurate P digestibilities for a variety of feedstuffs. There are several ways to
measure P digestibility. *In vivo* methods are considered the gold standard because they account for all the factors that could affect digestibility within the animal. Methods can include the total collection of feed and feces or the use of an indigestible marker to measure digestibility. However, *in vivo* methods are time consuming, expensive and labor intensive. An *in vitro* method to estimate P digestibility could be easier and less expensive, but suitable *in vitro* methods have not been developed for estimating dietary P digestibility in horses.

Using *in vivo* methods for measuring P digestibility in an animal requires knowing how much P the animal ingests and how much P is excreted in feces. Analyzing how much P is in the feed and recording how much feed the animal eats daily allows the researcher to calculate P intake. Collection of feces is essential to determine how much P was digested in the gastrointestinal tract. All the fecal material from a 24-h time period is collected using metabolism crates or collection bags to measure total fecal output and total P concentration. Apparent digestibility of P is calculated as \[\frac{(P \text{ intake} - P \text{ excretion})}{P \text{ intake}}\] X 100.

Alternatively, apparent digestibility can be estimated using an indigestible marker. In this method, either an internal indigestible component of the diet is used or an external indigestible marker is applied to the feed. The use of internal or external markers allows researchers to collect spot samples of feces throughout the day to estimate how much feces and total P are excreted. Examples of internal markers that have been utilized in horses are acid insoluble ash, indigestible neutral detergent fiber, \(n\)-alkanes and yttrium and examples of external markers are \(\text{Cr}_2\text{O}_3\) and ytterbium (Sales, 2012).
Intake and excretion of these markers and P is measured. The apparent digestibility of P is calculated as 100 – [(marker intake/marker feces) * (P intake/P feces) * 100].

True digestibility is similar to apparent digestibility, but is corrected for fecal endogenous P losses that are excreted, but did not come directly from dietary sources. True digestibility can be calculated using estimates of fecal endogenous losses published in NRC (2007), however a more accurate way to account for fecal endogenous losses is to measure them directly using a radiolabelled P isotope. Being able to measure fecal endogenous losses is useful for calculating accurate true P digestibilities. An isotope of P, $^{32}$P, is injected either intravenously or intramuscularly. Radioactivity is then measured in feces and plasma specific activity is measured. Endogenous fecal P losses are calculated using the following equation: 

$$\text{Fecal radioactivity} \int_{0 \text{ days}}^{x \text{ days}} \frac{\text{Plasma specific activity}}{dt}\text{.}$$

True digestibility can then be calculated using the following equation: 

$$[(P \text{ intake} - (P \text{ excretion} - \text{fecal P endogenous loss}))/(P \text{ intake})] \times 100.$$

Some studies collect urine to measure total P excretion and calculate retention, but the amount of P in equine urine is usually relatively insignificant with low P intakes. Schryver et al. (1971b) reported that less than 2 mg P/kg BW was excreted in the urine when intake was less than 50 mg P/kg BW. When intakes rose over 90 mg P/kg BW, urinary P excretion increased to around 19 mg/kg BW. Similarly, Van Doorn et al. (2004a) found that urinary P was less negligible when dietary P was 37.4 mg/kg BW, but urinary P rose to 2% of P intake with intakes of 91.8 mg/kg BW. Depending on the level of dietary P fed to the horses and the purpose of the study, collection of urine may not be necessary.
Many studies have measured in vivo apparent and true digestibility of P in horses. Table 2-3 shows results for studies performed with horses that examined P digestibility. A wide range of P intakes have been fed, ranging from 7.6 to 200 mg/kg BW. There is also a wide range of apparent P digestibilities, from 55.6% to 75.4%. Regression was used to investigate the relationship between P intake and apparent digestibility for growing horses and mature horses (Figure 2-1; Figure 2-2). While there is a positive relationship between intake and digestibility, the correlation is poor for both age groups with an R-squared value of 0.45 for growing horses and 0.24 for mature horses. The correlation between P intake and P digestibility is greater for growing horses than mature horses suggesting that P intake may play a larger role in affecting P digestibility for younger horses compared to mature horses. However, these relatively low R-squared values mean that there are many more factors besides P intake that affect P digestibility. These factors may include the presence of phytate in the diet, Ca levels, vitamin D supplementation, environment, animal, age, and study methods. All of these influencing variables make it difficult to predict P digestibility when looking at the diet.

In addition, using conventional in vivo methods to measure P digestibility may not be the most accurate due to P recycling within the gut. Schryver et al. (1972) discovered that P flows in and out of the gastrointestinal tract. It is unclear whether the P that is being secreted into the tract is P that has been absorbed and then immediately resecreted, or if it is endogenous P from other sources. The definition of digestibility is “the percentage of a foodstuff taken into the digestive tract that is absorbed by the body” (Merriam-Webster, 2013). If nutrients are being absorbed and then resecreted and measured as excretion, there is no way of knowing if it was ever absorbed, leading to
inaccurate estimates of digestibility. Schryver et al. (1972) also noted that the amount of P absorbed and secreted in different locations varied with diet type and dietary P. This observation means that feeding different diets could result in different amounts of error in digestibility. More research is needed to explain how P is recycled within the gastrointestinal tract and what factors affect recycling so that correct P digestibilities can be measured.

*In vitro* methods attempt to imitate digestion within the animal. These methods are usually able to accurately mimic digestion processes, but are unable to measure the absorption of nutrients that would occur in the animal. Care is needed when interpreting *in vitro* results because there are some factors that may influence absorption, but not digestion. Lowman et al. (1999) developed an *in vitro* procedure to estimate DM digestibility of various feeds by mimicking hindgut fermentation in horses. These authors incubated feedstuffs at the horse’s body temperature with equine feces as a source of microbial inoculum. Abdouli and Attia (2007) developed a two-stage *in vitro* technique to determine OM digestibility in horses by mimicking small intestine digestion followed by hindgut fermentation. Small intestinal digestion was simulated by incubating the feedstuffs with pepsin and amylase at the horse’s body temperature. The hindgut fermentation step was performed similar to Lowman et al. (1999). However, there are no published techniques for determining P digestibility *in vitro* in horses. Methods in other species have been established for estimating P digestibility. Liu et al. (1997) incubated feed samples commonly fed to pigs with pepsin and pancreatin to determine P digestibility. In cattle, Morse et al. (1992) obtained rumen fluid from cannulated animals and incubated the feedstuffs with the fluid to simulate ruminal
digestion of P. Similar methods could be utilized for determining P digestibility in horses, using either a one-step or a two-step method to simulate hindgut fermentation only, or small intestine digestion and hindgut fermentation together. Although an in vitro method cannot simulate the factors that affect absorption or P recycling, they could increase the knowledge about the susceptibility of feed ingredients to various digestive processes.

**Phytate-P in plants**

In plants, P occurs mainly in 2 forms: phytate-P and inorganic P. The storage form of P in plants is called phytic acid, or myo-inositol 1,2,3,4,5,6-hexakisphosphate. The mixed salt of phytic acid is commonly known as phytate. Inorganic P is more susceptible to leaching out of the seed than P bound to phytic acid, due to its higher water solubility (Raboy, 2009). Therefore phytic acid is necessary to ensure that the seed does not lose its store of P before it is ready for germination. Phytic acid is found mainly in seeds as a storage form of P for later growth, but it has also been found to be in pollen, spores, roots, stems and leaves (Roberts and Loewus, 1968; Jackson et al., 1982; DeMaggio and Stetler, 1985; Campbell et al., 1991; Brerlay and Hanke, 1996). Within the seed, phytate plays a key role in signaling processes, regulates gene expression, and acts as a store of inositol, phosphate, K, Mg, Ca, Mn, Fe, and Zn for later use during growth (Lott et al., 2000; Raboy, 2009). Phytate helps young seedlings grow by providing them with biosynthetic needs and myo-inositol, which serves as a cell wall component (Oatway et al., 2001). In small grains, phytate is mainly found in the aleurone layer. In cereal grains, phytate is found in the germ and aleurone layer, except for in corn, where it is mainly located in the germ (Raboy, 2007).
Phytate is synthesized in plant cells where it is stored, packaged by the endoplasmic reticulum, and moved to protein bodies for storage (Greenwood and Bewley, 1984). The packaged phytate inside the protein bodies then forms electron-dense, larger spheres called globoids. The negative charge of the phosphate ions attracts positively charged cations, such as K, Ca, and Mg to bind, forming bridges that connect phytate molecules together developing the globoid. Phytate accumulates in the globoid as the seed matures, reaching a maximum amount when the seed is ripe (Oatway et al., 2001).

The accumulation of phytic acid in seeds is affected by many factors. When plants are grown in soil with higher levels of P, phytic acid accumulates in greater amounts in the seeds (Miller et al., 1980a). It has also been shown that cultivar, location and harvest year can affect the phytic acid content in the seed (Miller et al., 1980b; Steiner et al., 2007). However, it is likely that the effect of location and harvest year is due more to the amount of precipitation in each location or year. Ockenden et al. (1997) has in fact shown that the amount of phytate in seeds is positively correlated with rainfall.

Phytate is broken down into inorganic P and myo-inositol by an enzyme called phytase. Plants mostly produce 6-phytase, which cleaves the 6-phosphate off of the myo-inositol molecule first before cleaving other phosphate groups. Microbes mostly produce 3-phytase, which begins hydrolysis at the 3-phosphate (Oatway et al., 2001). Plant phytase is stored in the aleurone layer next to the phytate molecules and is activated by germination to begin the breakdown of phytate, releasing inorganic P for use (Tronier et al., 1971; Bartnik and Szafrańska, 1987). Phytase levels vary among plant species and there does not seem to be a correlation between the amount of phytate or total P and the
amount of phytase in the plant (Eeckhout and de Paepe, 1994). Phytase activity ranges from trace amounts in maize, oats, and sorghum to activities over 5,000 U/kg in rye grain and wheat bran (Weremko et al., 1997).

**Phytate in animals**

Phytate has been shown to bind other positively charged nutrients, mainly K and Mg but also Ca, Mn, Zn, Ba and Fe (Lott et al., 2000). Out of all the minerals, Zn appears to form the most stable and insoluble complex with phytate (Oatway et al., 2001). In pigs, phytate has been shown to decrease Zn availability causing signs of Zn deficiency, but supplemental Zn reversed the symptoms of Zn deficiency (Oberleas et al., 1962). Phytate also forms an insoluble complex with Ca, which can inhibit not only P availability, but also Ca availability (Taylor, 1965). In laying hens, increasing dietary Ca concentrations decreased both phytate degradation and also P digestibility (Van der Klis et al., 1997). Adding phytase to these diets improved both phytate degradation and P digestibility. In a study done with growing piglets, phytase improved the amount of both Mg and Ca that were retained when fed a diet containing 77% of P in the phytate form (Pallauf et al., 1994). The fact that phytase improves Ca and Mg digestibility indicates that those minerals were binding to phytate but became more available after phytase hydrolyzed the phytate molecule. In horses, replacing inorganic sources of P with phytate-P in the diet resulted in a decrease in apparent digestibility of Ca from 42.4% to 26.4% (Van Doorn et al., 2004a).

Phytate can also interfere with protein digestibility by binding with the protein itself, inhibiting proteolytic enzymes, or influencing amino acid transporter activity. It
has been documented *in vitro* that phytate binds protein at a pH below the protein’s isoelectric point, forming insoluble complexes and inhibiting protein digestion (Fontaine et al., 1946). It is likely that protein-phytate complexes form in the acidic environment of the stomach of monogastrics. *In vivo* studies in pigs and chickens have shown that the presence of phytate reduces protein digestibility and the addition of phytase to the diet improves protein availability (Mroz et al., 1994; Ravindran et al., 1999; Rutherford et al., 2002). In *in vitro* experiments, phytate has been shown to inhibit the activity of pepsin (Knuckles et al., 1985; Vaintraub and Bulmaga, 1991). This inhibitory effect decreased to almost 0 when pH approached 4.0 – 4.5 (Vaintraub and Bulmaga, 1991). Vaintraub and Bulmaga (1991) found that phytate had no effect on the activity of trypsin, in contrast to other experiments that show that phytate is capable of inhibiting trypsin (Singh and Krikorian, 1982; Deshpande and Damodaran, 1989). Methodological differences between the experiments, such as pH and protein source, could potentially account for these differing results. Phytate has also been shown to decrease Na digestibility in broilers (Ravindran et al., 2006). It has been suggested that Na is secreted into the gut, most likely in the form of sodium bicarbonate, to help buffer the negatively charged phytate molecule (Cowieson et al., 2004). By dragging Na into the gut lumen, the ability of the Na\(^+\)-dependent transporters to transport glucose and amino acids could be compromised.

Similarly, phytate has also been suggested to inhibit starch digestion. Knuckles and Betschart (1987) saw that phytate inhibited digestion of starch by salivary amylase and *Bacillus subtilis* amylase. In humans, an inverse relationship between glycemic index and phytate intake was observed (Yoon et al., 1983). The negative effect of phytate on starch degradation suggests that phytate interacts with the starch granules and/or amylase
to decrease glucose liberation from the starch molecule, therefore decreasing the glucose response to a meal. Furthermore, these same authors found that *in vitro* incubation of phytate with human saliva prior to the addition of starch inhibits starch degradation. There was little effect on starch degradation when phytate was added at the same time as the saliva, indicating that phytate may directly inhibit amylase. In diabetic mice, the inclusion of 1% dietary phytic acid decreased fasting and non-fasting blood glucose samples and the inclusion of at least 0.5% dietary phytic acid improved glucose tolerance, further showing the effect of phytate on carbohydrate metabolism (Lee et al., 2006).

**Phytate in horses**

Initially, it was believed that phytate-P was less available to horses than inorganic P. Hintz et al. (1973) found that the P in wheat bran is half as available as inorganic P. Because wheat bran contains around two-thirds of its P in the phytate form, the difference in digestibilities between the two diets was attributed to the presence of phytate. However, recent evidence suggests phytate-P is more available than previously thought. Van Doorn et al. (2004a) reported that horses fed a diet with monocalcium phosphate as the main source of P had the same P digestibility as horses fed a diet containing 71% of P in the phytate form. It has also been demonstrated that a forage only diet had the same coefficient of total tract P digestibility as diets containing oats, corn and oats or corn and wheat middlings (Hainze et al., 2004). The P in the forage used in that study was mostly inorganic P, whereas the P in the grains and grain by-products had a higher proportion of P in the phytate form, indicating that inorganic P has a similar digestibility to phytate-P. Oliviera et al. (2008) reported that growing yearlings fed five diets with different feed
ingredients had P bioavailabilities ranging from 41% to 58%. The digestibilities for most of the diets were higher than expected indicating that the P in the ingredients was relatively available, even if it was in the phytate form. However, these studies do not directly measure the digestibility of phytate-P; they can only suggest its availability relative to diets with inorganic P. In order to determine if the horse is capable of hydrolyzing phytate, direct measurements of phytate disappearance need to be made.

Matsui et al. (1999) found that phytate-P was degraded in the upper small intestine and lower large intestine. When the horses were fed a high phytate diet, 28% of the phytate disappeared by the time the digesta had reached the upper small intestine and 82% disappeared by time it had reached the lower large intestine. Disappearances in the large intestine are attributed to the phytase activity of the microbes residing in the hindgut. These large disappearances show that horses have the capability to degrade phytate, but the extent of how much of the liberated P is absorbed remains unclear. A more recent study demonstrated that total tract phytate disappearance for a mixed diet was 96%, even though apparent total P digestibility was 24% (Lavin et al., 2013). Almost all of the phytate-P was liberated from the phytate molecule, but because of the relatively low total P digestibility value, it is assumed that most of the liberated P was excreted. It is possible that the P liberated in the lower large intestine is not being absorbed because the lower large intestine is also the main site of P absorption (Schryver et al., 1972). Depending on passage rate, it may be difficult for P to be absorbed in the same location that it is being liberated.
Hydrolyzing phytate in monogastric animals

Animals produce limited quantities of phytase themselves, so the P from phytate is largely unavailable to them. There are four sources of phytase available to the animal for phytate breakdown. These are endogenous small intestinal mucosal enzymes, intrinsic plant phytases, microfloral enzymes, and exogenous phytases (Selle and Ravindran, 2008).

Mucosal enzymes in the small intestine of the rat, chicken, calf and man have been found to have phytase and alkaline phosphatase activity (Bitar and Reinhold, 1972). Alkaline phosphatase is a phosphomonoesterase that hydrolyzes phosphate esters, producing free inorganic P (Plimmer, 1913). Its function in mammals is not yet fully known, but it has been shown to assist in intestinal P transport (Moog and Glazier, 1972). Both phytases and phosphatases were located in the mucosa in all species examined; however, the presence of phytate inhibited phosphatase activity. In pigs, it was found that the jejunum had the highest intestinal phytase capability (Hu et al., 1996). The efficiency was greatest against inositol triphosphate (IP3) and decreased with increasing phosphorylation up to inositol hexaphosphate (IP6). These authors suggested that the intestinal phytases may work in conjunction with phytases from other sources to further hydrolyze the lower inositol phosphates.

Intrinsic plant phytases have also been shown to assist in phytate breakdown. Pointillart (1991) reported that pigs fed a diet containing rye bran, which has a high intrinsic phytase activity, had better P utilization, bone strength, and growth compared to pigs eating a corn based diet containing a similar amount of P and phytate-P but with low
intrinsic phytase. Han et al. (1997) found that pigs eating a basal diet supplemented with wheat bran retained more P and excreted less fecal P than pigs eating a basal diet supplemented with inorganic P. Pigs fed the basal diet with supplemental microbial phytase instead of wheat bran showed somewhat greater improvements in P retention and excretion, but they also showed increased bone breaking strength at 50 kg BW. While intrinsic phytase may not perform as well as microbial phytase in all cases, it still is able to elicit significant effects with regards to phytate breakdown.

Certain microbes that are present in the gut of animals produce phytase enzymes that liberate inorganic P for absorption by the host (Yanke et al., 1998). Because cattle have a large rumen full of microbes that are capable of producing phytase, phytate-P is considered to be mostly available to them (Morse et al., 1992). However some aspects of the diet, such as high Ca:P ratios and rapid passage rates, can prevent total hydrolysis of phytate in the rumen and phytase supplementation can further improve phytate degradation in cattle (Kincaid et al., 2005). Monogastric animals, such as the pig and chicken, are not able to utilize phytate as completely as cattle because they lack sufficient amounts of these phytase-producing microbes that are crucial for phytate breakdown before the site of P absorption (Pointillart et al., 1991; Cowieson et al., 2006). However, it has been noted in pigs that the amount of phytate present is the feces is relatively low compared to the phytate in the diet, suggesting that phytate hydrolysis is occurring in the hindgut due to microbial activity (Baxter et al., 2003; Leytem et al., 2004).

Exogenous phytases are fed to monogastric animals to improve the availability of P when fed diets high in phytate-P. Phytases are most commonly derived from bacteria, such as *Escherichia coli*, or from fungi, such as *Aspergillus niger* (Selle and Ravindran,
Exogenous phytases are more effective at breaking down phytate than intrinsic plant phytases (Eeckhout and de Paepe, 1991; Zimmermann et al., 2002). In pigs, the addition of phytase to a diet containing phytate improves P digestibility, ADG, and bone density (Jongbloed et al., 1992; Harper et al., 1997). In laying hens, phytase improves P absorption and bone mineralization (Van der Klis et al., 1997). It has also been demonstrated that fish fed phytase have improved weight gain, increased bone ash, and decreased fecal P excretion (Jackson et al., 1996). Researchers have also improved the capability of phytase to withstand conditions within the gastrointestinal tract. Kim et al. (2006) altered the amino acids in the substrate-binding site and subsequently shifted the optimum pH of an Aspergillus niger phytase from pH 5.5 to 3.8. A lower pH is more similar to the pH of the stomach, which would allow the phytase to work earlier in the gastrointestinal tract. Pigs fed the altered phytase had greater ADG, higher plasma P concentrations and lower alkaline phosphatase activity than pigs fed the wild type phytase. This more efficient phytase has the potential to even further optimize P utilization.

**Phytase in horses**

Because phytase has been shown to improve P availability in monogastric animals, several studies have examined the effects of phytase supplementation in horses. However, exogenous phytase supplementation seemed to have no effect on P availability in horses. It should be noted that conditions in the experiments reviewed below that examined phytase efficacy in horses may not have been ideal to observe an effect of phytase.
Morris-Stoker et al. (2001) fed mature horses diets consisting of a 50:50 mix of matua grass hay and a mixed grain concentrate with phytase or without phytase. They fed the phytase at a rate of 1 g/kg concentrate and the assumed activity was 200 units/g of phytase. The concentrate ingredients and phytate level of the diet were not reported. P intakes of horses in both treatment groups averaged 38.9 mg/kg BW, which is 33.3% greater than the NRC (2007) P recommendations. There were no differences in P excretion or P digestibility between the two groups. The authors suggested that the level of phytase supplementation was insufficient to elicit effects on P digestibility. The lack of effect of phytase could also be because the amount of phytate-P in the diet was low, or that the horses were being fed in excess of their P requirement. A high level of P in the diet could mask the effects of phytase because the horse is absorbing the available P it needs without requiring additional phytase activity. Feeding a level of P in the diet below the horse’s requirement could potentially demonstrate an effect of phytase on P digestibility.

Patterson et al. (2002) fed mature horses a diet consisting of 50% native prairie grass hay and 50% of a textured concentrate containing corn, oats and soybean meal. Phytase was added to the diets at 0, 300, 600, and 900 FTU/kg of ration. P intakes ranged from 47.42 mg/kg BW to 55.18 mg/kg BW and exceeded the requirements of the mature horses by over 66% (NRC, 2007), which could potentially veil any effects of the phytase. There was no difference in P excretion, balance or digestibility among the treatments. The Ca:P ratio has been shown to affect phytase efficacy in pigs, and this effect could be a problem in horses. Qian et al. (1996) found that as the Ca:P ratio increased from 1.2:1 to 2.0:1, phytase efficacy decreased linearly. The Ca:P ratio used in this study was 2.4:1,
which is greater than the 1.2:1 ratio that was found to have maximum phytase effects in pigs.

Hainze et al. (2004) fed yearling geldings 4 diets with and without 6 g of a commercially available phytase (specific activity =1500 PU/g). All horses received Coastal bermudagrass hay at a rate of 0.015 * BW per day. In addition, horses received either alfalfa cubes, whole oats, a textured sweet feed containing corn, oats and soybean meal or a pelleted concentrate containing wheat middlings, corn and dehydrated alfalfa. P intakes were different among the diets, ranging from 46.6 mg/kg BW for the oat diet and 100.6 mg/kg BW for the pelleted concentrate diet. These intakes represent 76% to 164% of the NRC (2007) requirements for growing yearlings. There was no effect of phytase on P digestibility, but horses fed the sweet feed diet tended to have lower fecal P and fecal insoluble P when phytase was added. The pelleted concentrate diet resulted in significantly higher fecal P excretion than the oat and the sweet feed diets and sweet feed resulted in a higher fecal P excretion than the oats. The authors attribute these results to the high amount of phytate present in the oats and corn that are the main ingredients of the sweet feed, but because there was no effect of phytase for the oats only diet, this explanation seems unlikely. In addition, phytate content of the diets were not evaluated, so it is not clear which diet truly had high or low amounts of phytate. Ca intakes were not reported in this study, but it is possible that Ca:P ratios were above 1.2:1 as well.

Van Doorn et al. (2004a) fed mature horses four diets. The control diet contained a low amount of P with low amounts of phytate. The rest of the diets had a greater amount of P, but contained either monocalcium phosphate, phytate without phytase or phytate with phytase. Phytase was included at a rate of 1000 FTU/kg concentrate. P
intake was 37.4 mg/kg BW for the control diet and averaged 90.5 mg/kg BW for the other three high P diets. The high P diets exceeded NRC (2007) P requirements for mature horses by 205%. Phytase did not affect excretion of P or P digestibility. However, it did improve Ca digestibility and decrease the amount of Ca excreted. Ca intakes in this study were 69 g/d with a Ca:P ratio of 1.6:1. This ratio is the closest any of the studies have come to a Ca:P ratio of 1.2:1, but effects of phytase on P digestibility were still not seen. The lack of effect of phytase could be due to the high level of dietary P provided to the horses. In addition, the authors reported that the analyzed phytase activity fed to the horses was lower than the targeted activity level, resulting in treatments with less phytate degrading capability than planned for the concentration of phytate used in the diet.

Lavin et al. (2013) fed mature horses three diets with or without phytase. The diets consisted of a control diet, a high P diet, and a forage only diet. Phytase activity in the control diet was 440 FTU/kg, 530 FTU/kg in the high P diet and 3,400 FTU/kg in the forage diet. The targeted activity for the control and high P diets were 800 FTU/kg and the targeted activity for the forage only diet was 8,000 FTU/kg. Phytate levels averaged approximately 1.2 g/kg for the control and high P diets and 0.4 g/kg for the forage diets. P intakes ranged from 39 mg/kg BW for the control diet with phytase to 59 mg/kg BW for the high P diet without phytase. These values exceed the P requirement by 31 to 99%. There was no difference among the diets for P digestibility, P balance or P excretion. This study did not observe an increase in Ca digestibility with the addition of phytase to the diet, but Ca intakes were much higher than the amounts fed in the study done by Van Doorn et al. (2004a). Ca intakes in this study ranged from 86.8 g/d to 116.1
g/d and the Ca:P ratio was greater than 4.7:1 in all diets. With this high Ca:P ratio, it might be difficult to see any effects of phytase. This study was the only study that measured total tract phytate disappearance and found it to be 93% across all diets fed. These authors suggest that because horses already have the ability to break down phytate, supplemental phytase is not helpful. However, cattle can break down phytate quite easily, and they have shown improvement in P digestibility and decreased fecal P excretion with the addition of phytase in some cases (Kincaid et al., 2005; Knowlton et al., 2007). It is likely that the high Ca:P ratio and the excess P in the diet masked the effects of phytase in this study.

Only one study examined the effect of phytase on a diet that was deficient in P (Hainze et al., 2004). All the rest of the studies examined here fed diets that are at least 100% of the horses’ requirements. It is difficult to formulate a diet that meets all other nutrient requirements but is low in P, which could limit the ability of the researchers to feed a low P diet. It also appears that most studies included a relatively low amount of phytate-P in the diet, which would make it difficult to observe any effects of phytase. The Ca:P ratio has also proved to be problematic in these studies. Of all the experiments examining phytase efficacy in horses, Van Doorn et al. (2004a) performed the only experiment that formulated a diet with a Ca:P ratio less than 2:1. However, because of the high amounts of P in the diet, it is unclear if the phytase would have an effect with a low Ca:P ratio. It is possible that none of the experiments looking at phytase efficacy in horses had all of the right conditions to elicit an effect of phytase. It may have been difficult to formulate a practical diet for horses that meets all the conditions to elicit an effect of phytase.
Phosphorus and the environment

Recently, P has been a topic of interest with regards to the environment. Phosphorus is excreted mainly in the manure and if not properly managed, both the inorganic phosphate and the organic form of P can make their way into water bodies causing eutrophication. Eutrophication occurs when phytoplankton feed on excess N and P in the water and undergo population expansion. When these cyanobacteria die, their decomposition reduces oxygen in the water which can kill fish and other aquatic organisms causing disruption of the normal aquatic ecosystem (Correll, 1996). Inorganic P supports growth of many types of aquatic phytoplankton, most notably *Pfiesteria piscicida* (Burkholder et al., 1992). This dinoflagellate releases a toxin that can kill fish and has been implicated in the fish kills in the Chesapeake Bay (Lewitus et al., 1995).

Organic P has been shown to support the growth of the phytoplankton species *Phaeocystis pouchetii, Skeletonema castatum*, and *Nitzschia closterium*, further adding to the phytoplankton blooms (Chu, 1946).

In addition to being an environmental pollutant, P is also a limited resource. Raw phosphate is obtained through rock mining. Rock phosphate is used in agriculture mostly for fertilizer, but can also be added to commercial animal feeds. Currently, there are only a handful of active P mines in the world and the supply is dwindling. The world is predicted to run out of P within the next 40-150 years (McGill, 2012). However, like the oil crisis has shown, restrictions, regulations and economic issues related to limited resources usually start occurring well before the resource is depleted. Therefore, it is essential to start thinking about ways to recycle and reduce the use of P before the shortage causes major problems with modern agriculture systems.
Methods to reduce phosphorus excretion

As P gains more attention as a limited resource and an environmental pollutant, more emphasis will be placed on conserving this nutrient and reducing its excretion. There are three main ways to reduce the environmental impact of using P in animal nutrition: (1) the use of low phytic acid (lpa) genotype crops or crops co-expressing fungal phytase, (2) precisely formulating rations and feeding diets high in inorganic P and low in phytate-P, and (3) using exogenous phytase (Knowlton et al., 2004). The use of exogenous phytase was described in a previous section.

Low phytic acid crops have been developed in an attempt to reduce the amount of the less available phytate-P in plants commonly fed to animals. There are many lpa genotypes of maize, wheat, rice, barley and soybean (Rasmussen and Hatzack, 1998; Larson et al., 2000; Raboy et al., 2000; Wilcox et al., 2000; Guttieri et al., 2004). In lpa crops, the amount of phytic acid is reduced, while the amount of inorganic P is increased resulting in little to no change in seed total P (Raboy, 2009). However, phytic acid is an important storage molecule for the seed and decreasing its concentration could have detrimental effects for the developing seedling. Studies have indicated that lpa mutants have decreased yields and decreased stress tolerance compared to their wild-type siblings, but these decreases in performance are closely related to reductions in phytic acid (Bregitzer and Raboy; 2006). It would be ideal to select a mutant that has a moderate decrease in phytic acid so that acceptable performance will be maintained, while still improving the nutritional value of the crop. Despite the reductions in field performance, lpa crops have shown promise in improving nutrient utilization by humans and animals. In humans, lpa crops have improved Ca and Zn absorption compared to normal phytate
crops (Hambridge et al., 2004; Hambridge et al., 2005). Chicks show increased P retention and decreased fecal P excretion when fed lpa corn (Li et al., 2000; Waldroup et al., 2000). Growing pigs fed a diet containing a lpa corn hybrid had increased bone breaking strength and decreased P excretion compared to pigs fed normal corn diet that had higher total P (Veum et al., 2001). Pigs on the lpa diet also had increased Ca absorption and retention and increased N absorption and retention. Crops with low phytic acid show promise in improving animal performance and efficiency, as well as decreasing P waste and excretion. There was discussion about releasing these lpa crops for commercial use in the early 2000’s, but it seems there is still no commercial company producing lpa crops (Dr. Gary Cromwell, University of Kentucky, Lexington, KY, personal communication).

In addition to breeding for low-phytic acid crops, scientists have also engineered seeds to co-express a fungal phytase gene to boost intrinsic phytase activity (Drakakaki et al., 2005). Broilers fed transformed soybeans expressing fungal phytase performed similarly to broilers fed an exogenous phytase, indicating that this method of improving P availability is just as effective as phytase supplements (Denbow et al., 1998). This approach does not reduce the amount of phytate in the seed at all, therefore reducing the risks associated with lpa crops, such as lower yields. However, there is no commercially available crop containing a fungal phytase and current public concerns over genetically modified crops may be a potential roadblock for this approach. Due to its accessibility and inexpensive price-tag, phytase seems to be the top choice among animal producers when it comes to improving P availability to animals compared to mutant or genetically engineered crops.
Precise ration formulation is a more practical tool that animal producers can utilize to decrease P supplementation and subsequent wasting of this nutrient. Increasing P intake leads to increased P excretion, which is undesirable for environmental reasons (Schryver et al., 1971b; Morse et al., 1992; Rodehutscord et al., 2000). If producers strive to meet P requirements instead of exceed them, the amount of P needed in the diet is reduced and the amount excreted in the manure is also reduced. Animals have differing requirements based on their physiological state. Animals that are growing, lactating, gestating, or exercising have increased P requirements compared to mature sedentary animals. Therefore it is essential to evaluate the ration to make sure that it meets but does not exceed the P requirements of the animal based on its individual needs. However, overfeeding of P is common in many animal production systems for a few reasons. Feed labels on commercial feeds have a wide range of acceptable variation for P content, which can lead to wide variation in the amount of P in the feed. In Kentucky, the acceptable range of variation for actual P concentration compared to the guaranteed analysis is -15% to +2 units. This variation can lead to imprecise ration formulation and potentially overfeeding of P. Byproduct feeds, such as wheat bran and rice bran, are commonly included in animal rations because they are cheap and readily available. However, these feeds are usually higher in P content and in phytate content than whole grains because phytate is typically stored in the aleurone layer of the grains (Raboy, 2007). When the bran is removed from the grain, the P becomes concentrated in this byproduct. Because these byproducts are also high in phytate-P, additional inorganic P is sometimes added to feeds to increase the “available P” in the total ration. Avoiding byproduct feeds, or feeding less of them is one way to reduce the amount of phytate-P in
the diet. By feeding feeds that are lower in phytate-P, the amount of inorganic P needed will be reduced. Alternatively, if phytate-P is available for use by the animal, the need for supplemental inorganic P will also be decreased.

**Summary and rationale**

Phytate is a form of P used for storage in plants. It is mainly found in the seeds of plants and is present in many of the grains commonly fed to horses, such as oats, barley, and wheat (Eeckhout and De Paepe, 1994). Phytate has been shown to be less available than inorganic forms of P to monogastric animals, such as swine and poultry (Pointillart, 1991; Cowieson et al., 2006). The phytate form of P has also been suggested to be less available to horses than inorganic P sources (Hintz et al., 1973). In contrast, some researchers have found that phytate is readily broken down in the horse’s gastrointestinal tract (Matsui et al., 1999; Lavin et al., 2013). It is speculated that the microbes in the hindgut produce phytase which hydrolyzes the bond between the phosphate and the inositol ring. Most studies examining phytate degradation in horses have used mature animals and it is unknown if the growing animal has the same ability to break down phytate compared to an adult animal. The amount of phytate in the horse diet can be quite high when a large amount of concentrate is fed. Therefore, determining how available P is to the horse, especially the growing horse, is important.

There have been few studies looking at the difference in mineral digestibility between long yearlings and mature horses. Because long yearlings are growing, they require more nutrients in their diet. Due to their increased requirement, it has been suggested that they will have a higher digestibility of nutrients than mature horses.
(Cymbaluk et al., 1989). In addition, it appears that common forages, grains and protein sources may contain enough P to minimize the need for additional inorganic P, if the P in the feed ingredients is available. A better understanding of nutrient digestibilities among different ages of horses fed diets without added inorganic P could lead to more accurate recommendations for nutrient requirements, less nutrient wasting and more efficient animals.

In this study, digestibility of DM, NDF, ADF, P, phytate, Ca and Mg were measured in long yearlings and mature geldings. Markers of bone turnover were measured to examine the differences between the age groups. A method for estimating P digestibility in vitro was also developed.

**Objectives**

The objective of this study was to compare digestibility of P, phytate, Ca and Mg in long yearling and mature horses. The hypothesis was that the mature horses would be able to degrade more of the phytate P than the long yearlings, but the long yearlings would have higher digestibilities of total P, Ca and Mg.
Table 2-1. Concentration of P in forages commonly fed to horses in Kentucky

<table>
<thead>
<tr>
<th>Forage</th>
<th>% P, average(^1)</th>
<th>% P, range(^1)</th>
<th>Sample location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timothy grass</td>
<td>0.326</td>
<td>0.30 – 0.36</td>
<td>Kentucky</td>
<td>Fowler et al., 2012</td>
</tr>
<tr>
<td>Tall fescue grass</td>
<td>0.350</td>
<td>0.34 – 0.36</td>
<td>Kentucky</td>
<td>Fowler et al., 2012</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>0.375</td>
<td>0.36 – 0.40</td>
<td>Kentucky</td>
<td>Fowler et al., 2012</td>
</tr>
<tr>
<td>Perennial ryegrass</td>
<td>0.455</td>
<td>0.41 – 0.48</td>
<td>Kentucky</td>
<td>Fowler et al., 2012</td>
</tr>
<tr>
<td>Festulolium grass</td>
<td>0.465</td>
<td>0.45 – 0.49</td>
<td>Kentucky</td>
<td>Fowler et al., 2012</td>
</tr>
<tr>
<td>Cool-season grass</td>
<td>0.370</td>
<td></td>
<td>Kentucky</td>
<td>Lawrence et al., 2006</td>
</tr>
<tr>
<td>pasture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Legume pasture</td>
<td>0.360</td>
<td>0.274 – 0.447</td>
<td>United States</td>
<td>DairyOne Forage Lab, 2013</td>
</tr>
<tr>
<td>Bermudagrass pasture</td>
<td>0.314</td>
<td>0.241 – 0.386</td>
<td>United States</td>
<td>DairyOne Forage Lab, 2013</td>
</tr>
<tr>
<td>Legume hay</td>
<td>0.273</td>
<td>0.223 – 0.323</td>
<td>United States</td>
<td>DairyOne Forage Lab, 2013</td>
</tr>
<tr>
<td>Bermudagrass hay</td>
<td>0.197</td>
<td>0.145 – 0.249</td>
<td>United States</td>
<td>DairyOne Forage Lab, 2013</td>
</tr>
<tr>
<td>Oat hay</td>
<td>0.204</td>
<td>0.140 – 0.268</td>
<td>United States</td>
<td>DairyOne Forage Lab, 2013</td>
</tr>
</tbody>
</table>

\(^1\)DM basis
Table 2-2. Concentration of P and phytate-P in common equine feed ingredients

<table>
<thead>
<tr>
<th>Feed</th>
<th>% total P</th>
<th>% Phytate-P</th>
<th>% Phytate-P of total P</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>0.28 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>83</td>
<td>Düngelhoef et al., 1994</td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0.19</td>
<td>68</td>
<td>Eeckhout and de Paepe, 1994</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>0.21</td>
<td>66</td>
<td>Jongbloed and Kemme, 1990</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>0.17</td>
<td>73</td>
<td>Meschy et al., 2005</td>
</tr>
<tr>
<td></td>
<td>0.26</td>
<td>0.17</td>
<td>66</td>
<td>Nelson et al., 1968</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.28 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>71 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Barley</td>
<td>0.37</td>
<td>0.22</td>
<td>60</td>
<td>Eeckhout and de Paepe, 1994</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>0.28</td>
<td>64</td>
<td>Jongbloed and Kemme, 1990</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>0.19</td>
<td>56</td>
<td>Nelson et al., 1968</td>
</tr>
<tr>
<td></td>
<td>0.42</td>
<td>0.29</td>
<td>63</td>
<td>Steiner et al., 2007</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.39 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>61 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>0.42</td>
<td>0.31</td>
<td>74</td>
<td>Düngelhoef et al., 1994</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>0.22</td>
<td>67</td>
<td>Eeckhout and de Paepe, 1994</td>
</tr>
<tr>
<td></td>
<td>0.41</td>
<td>0.29</td>
<td>71</td>
<td>Jongbloed and Kemme, 1990</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>0.18</td>
<td>55</td>
<td>Meschy et al., 2005</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.20</td>
<td>67</td>
<td>Nelson et al., 1968</td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>0.29</td>
<td>73</td>
<td>Steiner et al., 2007</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.37 ± 0.02</td>
<td>0.25 ± 0.02</td>
<td>68 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Rye</td>
<td>0.36</td>
<td>0.22</td>
<td>61</td>
<td>Eeckhout and de Paepe, 1994</td>
</tr>
<tr>
<td></td>
<td>0.36</td>
<td>0.24</td>
<td>67</td>
<td>Steiner et al., 2007</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.36 ± 0</td>
<td>0.23 ± 0.01</td>
<td>64 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>Oats</td>
<td>0.36</td>
<td>0.21</td>
<td>59</td>
<td>Eeckhout and de Paepe, 1994</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>0.19</td>
<td>56</td>
<td>Nelson et al., 1968</td>
</tr>
<tr>
<td></td>
<td>0.37</td>
<td>0.25</td>
<td>67</td>
<td>Steiner et al., 2007</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.36 ± 0.01</td>
<td>0.22 ± 0.02</td>
<td>61 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>Wheat bran</td>
<td>1.16</td>
<td>0.97</td>
<td>84</td>
<td>Eeckhout and de Paepe, 1994</td>
</tr>
<tr>
<td></td>
<td>0.92</td>
<td>0.63</td>
<td>69</td>
<td>Meschy et al., 2005</td>
</tr>
<tr>
<td></td>
<td>1.37</td>
<td>0.96</td>
<td>70</td>
<td>Nelson et al., 1968</td>
</tr>
<tr>
<td></td>
<td>0.88</td>
<td>0.79</td>
<td>90</td>
<td>Steiner et al., 2007</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>1.08 ± 0.11</td>
<td>0.84 ± 0.08</td>
<td>78 ± 5.2</td>
<td></td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>0.80</td>
<td>0.53</td>
<td>66</td>
<td>Eeckhout and de Paepe, 1994</td>
</tr>
<tr>
<td></td>
<td>1.20</td>
<td>0.96</td>
<td>80</td>
<td>Jongbloed and Kemme, 1990</td>
</tr>
<tr>
<td></td>
<td>0.47</td>
<td>0.35</td>
<td>74</td>
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Table 2-3. Digestibility of P in horses from various studies

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<sup>1</sup>Apparent digestibility is calculated as (Intake P – Fecal P)/Intake P
<sup>2</sup>True digestibilities were calculated using an estimate of fecal endogenous P loss of 10 mg/kg BW
<sup>3</sup>True digestibilities were calculated using fecal endogenous losses measured in the study
Figure 2-1. Relationship between P intake and apparent P digestibility for growing horses

\[ y = 24.489 \ln(x) - 79.569 \]

\[ R^2 = 0.4492 \]
Figure 2-2. Relationship between P intake and apparent P digestibility for mature horses

\[ y = 16.832\ln(x) - 57.88 \]

\[ R^2 = 0.2404 \]
A method to determine *in vitro* P digestibility was developed based on procedures developed by Earing et al. (2010) to measure dry matter digestibility of equine feeds. The method simulates large intestinal digestion using a batch system with equine feces as the inoculum. The Ankom Daisy™ Incubator (Ankom Technology, Macedon, NY) was used by Earing et al. (2010) and in the current study. This incubator holds four incubation jars and rotates the jars continuously while maintaining a constant temperature. Sealed filter bags containing ground feed samples are incubated in the jars with a slurry of feces and buffers. At the end of the incubation time, bags are rinsed, dried and weighed to determine *in vitro* DMD. A series of modifications were made to these procedures to evaluate the effect of incubation time, sample size, buffer type, and pre-digestion on measures of P digestibility.

**Effect of incubation time on in vitro phosphorus digestibility**

The procedure of Earing et al. (2010) was modified for this experiment by using larger sample sizes and analyzing the fiber bags for P post-digestion (Appendix 1). Samples of alfalfa hay, orchardgrass hay, straw, rolled oats, and wheat bran were ground through a 1 mm screen and then 0.75 g of each sample was placed in an Ankom F57 filter bag (Ankom Technology, Macedon, NY) and sealed. Table 3-1 shows the P concentrations in the feed samples as measured by using a colorimetric assay (Appendix 3). Two incubation jars were used and each jar contained triplicate samples of each feed and two blank bags containing no feed to calculate a correction factor for each jar. These bags were placed in incubation jars with 1500 ml of Ankom A buffer, 300 ml of Ankom
B buffer (Appendix 2) and fresh feces. Feces were obtained from mares and yearlings that were consuming cool season grass pasture, hay and an oat-based concentrate. Immediately following defecation, fecal material that did not touch the ground was collected, double-bagged in plastic bags, purged with CO₂, and transported to the lab in a cooler containing warm water bags to maintain a temperature close to 37.5 °C. After fecal addition, the pH of the jars was adjusted to 7.00 ± 0.02 using Ankom’s A and B buffers as needed. One jar was incubated in the Ankom Daisy™ Incubator (Ankom Technology, Macedon, NY) at 37.5°C for 48 h and one jar was incubated for 72 h to determine the effect of time on digestibility. After incubation, bags were removed from the jars, rinsed with cold water, and dried in a forced air 55°C oven for 24 h or until no further weight loss was noted. Bags were then reweighed to determine IVDMD. Bags were ashed at 600°C overnight, boiled in 10 ml of 6 N HCl acid for 20 min, filtered into a 100 ml volumetric flask, filled with 0.2% K ionization buffer and analyzed for P using a colorimetric assay (Appendix 3). A paired t-test was used to evaluate the effect of incubation time on P digestibility (SAS 9.2, SAS Institute, Cary NC).

Results from this experiment are shown in Table 3-2. Bags incubated for 72 h had a greater IVDMD than bags incubated for 48 h (P < 0.05). Previous work has shown 48 h to be a sufficient amount of time to incubate 0.25 g samples with no increase in IVDMD at 72 h (Earing et al., 2010). However, in this experiment, the large sample size (0.75 g) used may have been too large to allow for enough surface area for complete digestion by 48 h. A larger sample size was chosen for the first experiment to ensure that there was enough measurable P in the filter bags after incubation in order to calculate an accurate P
digestibility. However, a smaller sample size may allow for more complete DM digestion after 48 h while still containing P amounts above the lower limit of detection.

There was a trend for incubation time to affect *in vitro* P digestibility for orchardgrass, oats, wheat bran, and straw with values ranging from 15.15 ± 17.97% for straw at 48 h to 97.43 ± 0.40% for wheat bran at 72 h (*P* = 0.067). Compared to values reported *in vivo* for these feeds, the *in vitro* P digestibilities appeared to be very high. An unexpected result was a negative P digestibility of -215.78 ± 17.85% and -243.92 ± 2.64% at 48 h and 72 h, respectively, for alfalfa. The reason for this result is unknown, although alfalfa is much higher in Ca than the other feeds which may be important. It is possible that the Ca in the alfalfa binds to the P that is in the Ankom buffer forming an insoluble complex and P therefore accumulates within the filter bag.

**Effect of incubation time with smaller sample sizes**

The next experiment examined the effect of feed sample size on IVDMD and P digestibility because the last experiment demonstrated that a 0.75 g sample may be too large to allow for complete digestion. The same method was used as described above, with the exception that both jars contained 0.25 g samples instead of 0.75 g samples. Again, one jar was incubated for 48 h and one jar for 72 h. A paired t-test was used to evaluate the effect of incubation time on P digestibility (SAS 9.2, SAS Institute, Cary NC).

Table 3-3 shows the results for this experiment. There was a trend for the 0.25 g samples incubated for 72 h to have greater IVDMD than the samples incubated for 48 h (*P* = 0.056). Other researchers have determined that 48 h is sufficient time for DM
digestibility using 0.5 g sample sizes (Earing et al., 2010). Previous work has also demonstrated that 0.25 g samples incubated for 48 h more closely approximated in vivo estimates of DM digestibility than larger sample sizes (Lattimer et al., 2007). It appears that 0.25 g samples incubated for 48 h would be the ideal conditions to estimate in vivo DM digestibility and these conditions were used in all further experiments.

There was no effect of incubation time on P digestibility using 0.25 g samples, either including or excluding the negative values ($P > 0.05$). Positive P digestibilities ranged from 44.78 ± 5.40% for oats at 48 h to 91.44 ± 0.88% for wheat bran at 72 h. Again, alfalfa had a negative P digestibility, but straw also had a negative value. It is unknown why straw had two very different P digestibilities between the experiments. The use of a buffer without P in it may be warranted due to the negative P digestibilities.

Variation between experiments when using the same method was an issue in this experiment and in the following experiments. It is possible that using feces from different horses could affect the results. The time of collection of the feces relative to the time of feeding the horse could also influence activity of the microbes (Desrousseaux et al., 2012), which could lead to variation in IVDMD and P digestibility.

**Effect of a non-phosphorus containing buffer**

The use of a non-phosphorous buffer was examined in this experiment. One jar contained the previously used Ankom buffers and one jar contained a Bis-Tris buffer with no P (Brask-Pederson et al., 2011; Appendix 1). Each jar contained three bags each (0.25 g) of the same feeds used previously and two blank bags. A sample size of 0.25 g and an incubation time of 48 h were used. The pH adjustment for the Bis-Tris buffer was
done using 2 N HCl instead of the Ankom buffers because of the P present in the Ankom buffers. The pH was not adjusted until after the feces were added, and the HCl caused some bubbling to occur in the fecal slurry. It was unknown if the direct addition of HCl disrupted the fecal microbes in the jar. Bags were incubated for 48 h, then rinsed, dried and assayed for P. A paired t-test was used to evaluate the effect of buffer type on P digestibility (SAS 9.2, SAS Institute, Cary NC).

Table 3-4 shows results for this experiment. The buffer type did not have an effect on IVDMD ($P > 0.05$). The lack of statistical difference could be due to higher standard errors for the IVDMD of the feedstuffs incubated in both buffers compared to previous experiments. In addition, forages (alfalfa, orchardgrass, and straw) had numerically lower IVDMD in the Bis-Tris buffer compared to the Ankom buffer and grain products (oats and wheat bran) had higher IVDMD. However, alfalfa had an IVDMD of 56.64 ± 3.61% in the Bis-Tris buffer in the current experiment and had an IVDMD of 58.32 ± 2.29% in the previous experiment. IVDMD for wheat bran in the Bis-Tris buffer is also very similar to IVDMD in the previous experiment (70.48 ± 1.13% in the previous experiment; 71.94 ± 3.12% in the current experiment). Similarities between the two buffer types indicate that there is likely no effect of the buffer type on the fermentative capacity of the microbes in the incubation jar. Straw had a negative IVDMD in the Bis-Tris buffer and the reason for this is unknown.

Buffer type also did not influence P digestibility across all feeds ($P > 0.05$), but all feeds had positive values in the Bis-Tris buffer. In the previous experiments, alfalfa had a negative P digestibility but use of the Bis-Tris buffer resulted in a P digestibility of 49.07 ± 2.90. The lowest P digestibility in the Bis-Tris buffer was oats (32.11 ± 3.82%)
and the highest P digestibility was orchardgrass (79.01 ± 0.29%). When a t-test was used to compare the effect of buffer type on individual feedstuffs, the grain products had lower P digestibilities with the Bis-Tris buffer compared to the Ankom buffer ($P < 0.05$), while the alfalfa and orchardgrass had higher P digestibilities ($P < 0.05$). Because the buffer type had opposite effects on the forage and grain products, an overall effect of buffer type on P digestibility was not observed.

**Effect of adjusting the pH prior to fecal addition**

The next experiment examined the effect of adjusting the pH before fecal addition on IVDMD and P digestibility. The method was the same as the previous experiment, with one jar containing the Ankom buffer and one jar containing the Bis-Tris buffer. However, the pH of the Bis-Tris buffer was adjusted with 2 N HCl before the feces were added. No bubbling occurred when the feces were added to the buffer. pH was also taken at the end of the 48 h incubation. A paired t-test was used to evaluate the effect of buffer type on P digestibility (SAS 9.2, SAS Institute, Cary NC).

At the end of the 48 h incubation, the Ankom buffer had a pH of 6.45, while the Bis-Tris buffer had a pH of 6.77. The Bis-Tris buffer did a better job of keeping the pH close to 7.00, creating a more stable environment for the microbes. Table 3-5 shows results for this experiment. There was no difference in IVDMD between the two buffers ($P > 0.05$). The grain products had numerically lower P digestibilities with the Bis-Tris buffer, while the forages had numerically higher P digestibilities but there was no difference between buffers across all feed ($P > 0.05$). When using a t-test to compare the effect of buffer type on individual feedstuffs, alfalfa had a higher P digestibility with the
Bis-Tris buffer \((P < 0.01)\), while oats and wheat bran had lower P digestibilities \((P < 0.01)\). P digestibilities from the previous experiment where pH was adjusted after fecal addition were numerically lower than P digestibilities in the current experiment where pH was adjusted before fecal addition. These results may indicate that adjusting the pH with HCl after the feces were added inhibited the ability of the microbes to digest P from the feed samples.

In the horses, enzymatic foregut digestion happens before microbial fermentation in the hindgut occurs. Some protein and starch breakdown occurs in the small intestine, thus changing the composition of the feed before it reaches the hindgut. A method that accounts for foregut digestion may better approximate IVDMD and P digestibility in the horse.

**Effect of pre-digestion**

The next experiment looked at the effect of pre-digesting the feed samples before incubating them with feces. The pre-digestion step was created to mimic small intestine digestion of the feeds before they reach the large intestine to be fermented. This method is modified from Abdouli and Attia (2007) which uses feeds incubated in pepsin and amylase solutions to mimic pre-cecal digestion. The only change to their method is that a Bis-Tris buffer was substituted for a phosphate buffer when incubating with the amylase. A detailed description of this 2-step method is available in Appendix 1. Four samples \((0.3 \text{ g})\) of the same feeds used in previous experiments were placed into Ankom bags and sealed. The bags were then placed in beakers and incubated at 37.5°C in a water bath first with a pepsin solution for 2 h and then with amylase for 4 h. The bags were then rinsed
with ethanol, acetone, and distilled water. Two bags per feed sample were dried, weighed, and analyzed for P after pre-digestion and the remaining bags were placed in the Daisy incubator with the Bis-Tris buffer and fresh feces. At the same time, another jar was prepared containing 0.25 g samples in bags that had not been pre-digested. A paired t-test was used to evaluate the effect of pre-digestion on P digestibility (SAS 9.2, SAS Institute, Cary NC).

Table 3-6 shows results for the pre-digestion step only as well as the 1-step (fermentation only) and the combined 2-step methods (pre-digestion and fermentation). Alfalfa and wheat bran had the greatest in vitro enzymatic dry matter disappearance (IVEDMD) for the pre-digestion step only. Wheat bran is typically high in starch and alfalfa is typically high in crude protein. The composition of these two feeds would make them most susceptible to enzymatic digestion of protein and starch in the pre-digestion step. The P digestibility of the feeds that were only subjected to the pre-digestion step were relatively high, indicating that the enzymes play a big role in liberating P.

Overall, IVDMD was greater for the 2-step method than for fermentation alone (P < 0.05). Although, straw and wheat bran had very small differences in IVDMD between the two methods. While a small difference for wheat bran was surprising, it was expected that feeds low in crude protein or starch, such as straw, would not benefit as much from having an additional enzymatic digestion step. Because the feeds in the 2-step method were incubated with additional enzymes, it is not surprising that IVDMD is greater for the 2-step method. However, the difference in IVDMD for feeds between the 1-step and 2-step methods is not equal to the pre-digestion step’s IVEDMD. It could be possible that some of the DM components are soluble and leach out of the fiber bag in the pre-
digestion step. It is also possible that the pre-digestion enzymes break down a lot of the DM before the microbes are added and do not leave as much feed for the microbes to ferment.

The 2-step method also had greater P digestibility than the 1-step method ($P <0.05$). In the fermentation only method, P digestibility is also relatively similar to pre-digestion only, indicating that the microbes are efficient at digesting P from feeds. It is possible that the addition of enzymes allowed for greater P liberation from the feeds during the 2-step method, either by increasing surface area before the microbes were introduced or by breaking any bonds with P and protein or starch that could interfere with further digestion. The phytate molecule can bind to starch and protein, forming insoluble complexes (Fontaine et al., 1946; Knuckles and Betschart, 1987). By breaking down starch and protein in a pre-digestion step, it is possible that the phytate-bound P is more available for breakdown by the microbes.

**Comparing in vivo results to 1-step and 2-step methods for a forage only diet**

P digestibilities for *in vitro* methods were higher than expected when looking at *in vivo* results. Typically, P digestibility *in vivo* is relatively low. The NRC (2007) uses a true P digestibility value of 35% for mature horses receiving diets containing organic P and 45% for growing and lactating horses receiving diets with organic and inorganic P. Pagan (1994) reports an average true P digestibility of 25.2% over 120 diets. However, estimating true P digestibility in an animal takes into consideration estimates of fecal endogenous losses, ability of the animal to absorb the P, P transporter regulation, and P recycling within the gastrointestinal tract. *In vitro* incubations do not account for these
factors; these methods only suggest that P from these sources is available for absorption to the horse. In these *in vitro* experiments, any phytate present in the grain products is presumably being broken down by microbial action in the fecal material. For example, wheat bran contains about 76% of its total P in the phytate form (Eeckhout and de Paepe, 1994). In the last experiment, wheat bran had a high *in vitro* P digestibility (82 to 99%), indicating the phytate is able to be broken down by the microbes in the hindgut and P is able to be liberated. However, this does not estimate the true digestibility and absorption of the P from wheat bran, which is why it is important to compare *in vivo* results to *in vitro* methods.

The next experiment compared *in vivo* results to *in vitro* methods. A coastal bermudagrass hay from University of Florida that was used in an *in vivo* P digestibility trial was subjected to both the 1-step and the 2-step *in vitro* digestion methods.

The IVDMD value for 1-step method was 25.36% and for the 2-step method it was 29.97%. P digestibility for the 1-step method was 69.52% and 72.97% for the 2-step method. The *in vitro* method estimates true availability, whereas the *in vivo* method measures apparent digestibility. The University of Florida researchers reported their apparent P digestibility for the bermudagrass hay to be 50.3% (Warren, University of Florida, Gainesville, FL, personal communication). Correcting for fecal endogenous losses, the true P digestibility was estimated to be 76%. Based on this sample, the *in vitro* method is fairly accurate at predicting P digestibility for this forage. However, additional studies are needed to validate the method.
Table 3-1. Phosphorus concentration in *in vitro* feed samples

<table>
<thead>
<tr>
<th>Feed</th>
<th>% P, DM basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa hay</td>
<td>0.27</td>
</tr>
<tr>
<td>Orchardgrass hay</td>
<td>0.47</td>
</tr>
<tr>
<td>Straw</td>
<td>0.26</td>
</tr>
<tr>
<td>Rolled oats</td>
<td>0.54</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Table 3-2. Effect of incubation time using 0.75 g samples on IVDMD and P digestibility (mean ± SEM, n=3)

<table>
<thead>
<tr>
<th>Feed</th>
<th>IVDMD (%) 48 h</th>
<th>IVDMD (%) 72 h†</th>
<th>P digestibility (%) 48 h</th>
<th>P digestibility (%) 72 h†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>54.91 ± 1.18</td>
<td>58.98 ± 1.34</td>
<td>-215.78 ± 17.85</td>
<td>-243.92 ± 2.64</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>41.73 ± 0.68</td>
<td>50.93 ± 0.67</td>
<td>54.51 ± 17.97</td>
<td>78.36 ± 0.73</td>
</tr>
<tr>
<td>Straw</td>
<td>12.29 ± 0.99</td>
<td>23.86 ± 0.34</td>
<td>15.15 ± 12.39</td>
<td>50.45 ± 3.07</td>
</tr>
<tr>
<td>Oats</td>
<td>72.91 ± 1.44</td>
<td>75.90 ± 1.17</td>
<td>65.12 ± 2.66</td>
<td>77.77 ± 0.88</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>62.42 ± 1.07</td>
<td>68.58 ± 0.29</td>
<td>93.23 ± 1.29</td>
<td>97.43 ± 0.40</td>
</tr>
</tbody>
</table>

†Mean IVDMD across all feeds for 48 h and 72 h differ (P < 0.05)

Table 3-3. Effect of incubation time using 0.25 g samples on IVDMD and P digestibility (mean ± SEM, n=3)

<table>
<thead>
<tr>
<th>Feed</th>
<th>IVDMD (%) 48 h</th>
<th>IVDMD (%) 72 h†</th>
<th>P digestibility (%) 48 h</th>
<th>P digestibility (%) 72 h†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>58.32 ± 2.29</td>
<td>58.04 ± 1.40</td>
<td>-296.18 ± 14.87</td>
<td>-430.40 ± 12.84</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>49.09 ± 0.74</td>
<td>55.33 ± 0.59</td>
<td>49.96 ± 2.62</td>
<td>46.74 ± 7.17</td>
</tr>
<tr>
<td>Straw</td>
<td>12.16 ± 2.01</td>
<td>20.15 ± 1.70</td>
<td>-20.12 ± 15.41</td>
<td>-19.61 ± 12.16</td>
</tr>
<tr>
<td>Oats</td>
<td>82.92 ± 1.78</td>
<td>85.60 ± 0.87</td>
<td>44.78 ± 5.40</td>
<td>69.38 ± 4.81</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>70.48 ± 1.13</td>
<td>73.24 ± 0.55</td>
<td>90.64 ± 0.64</td>
<td>91.44 ± 0.88</td>
</tr>
</tbody>
</table>

†Mean IVDMD across all feeds for 48 h and 72 h tend to differ (P = 0.056)
Table 3-4. Effect of buffer type on IVDMD and P digestibility (mean ± SEM, n=3)

<table>
<thead>
<tr>
<th>Feed</th>
<th>IVDMD (%) Ankom buffer</th>
<th>IVDMD (%) Bis-Tris buffer</th>
<th>P digestibility (%) Ankom buffer</th>
<th>P digestibility (%) Bis-Tris buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>64.13 ± 2.61</td>
<td>56.64 ± 3.61</td>
<td>-219.18 ± 25.75</td>
<td>49.07 ± 2.90</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>49.43 ± 2.58</td>
<td>44.35 ± 2.09</td>
<td>62.76 ± 2.07</td>
<td>79.01 ± 0.29</td>
</tr>
<tr>
<td>Straw</td>
<td>31.78 ± 2.39</td>
<td>-9.91 ± 2.85</td>
<td>37.11 ± 1.40</td>
<td>72.31 ± 3.52</td>
</tr>
<tr>
<td>Oats</td>
<td>72.76 ± 3.89</td>
<td>88.29 ± 3.23</td>
<td>72.74 ± 3.80</td>
<td>32.11 ± 3.82</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>60.80 ± 2.83</td>
<td>71.94 ± 3.12</td>
<td>94.94 ± 0.19</td>
<td>67.80 ± 1.77</td>
</tr>
</tbody>
</table>

Table 3-5. Effect of buffer type on IVDMD and P digestibility when pH is adjusted after fecal addition (mean ± SEM, n=3)

<table>
<thead>
<tr>
<th>Feed</th>
<th>IVDMD (%) Ankom buffer</th>
<th>IVDMD (%) Bis-Tris buffer</th>
<th>P digestibility (%) Ankom buffer</th>
<th>P digestibility (%) Bis-Tris buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>60.91 ± 0.31</td>
<td>61.66 ± 0.70</td>
<td>-125.73 ± 11.85</td>
<td>62.12 ± 0.52</td>
</tr>
<tr>
<td>Orchardgrass</td>
<td>51.50 ± 1.05</td>
<td>46.20 ± 0.43</td>
<td>84.39 ± 0.833</td>
<td>89.80 ± 2.29</td>
</tr>
<tr>
<td>Straw</td>
<td>10.10 ± 0.84</td>
<td>11.80 ± 0.50</td>
<td>73.11 ± 2.32</td>
<td>88.73 ± 3.35</td>
</tr>
<tr>
<td>Oats</td>
<td>83.31 ± 1.52</td>
<td>74.67 ± 1.06</td>
<td>92.22 ± 1.37</td>
<td>29.52 ± 3.70</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>69.66 ± 0.48</td>
<td>64.83 ± 0.27</td>
<td>98.42 ± 0.66</td>
<td>69.83 ± 1.40</td>
</tr>
</tbody>
</table>

Table 3-6. Effect of pre-digestion step on in vitro enzymatic dry matter disappearance (IVEDMD), IVDMD and P digestibility (mean ± SEM)

<table>
<thead>
<tr>
<th>Feed1</th>
<th>IVDMD (%)3</th>
<th>IVDMD (%) ferm. step2</th>
<th>IVDMD (%) 2-step3*</th>
<th>Enzymatic P digestibility (%)3</th>
<th>P digestibility (%) ferm. step2</th>
<th>P digestibility (%) 2-step3**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>35.08 ± 1.1</td>
<td>53.25 ± 0.9</td>
<td>66.21 ± 5.4</td>
<td>49.41 ± 3.6</td>
<td>48.37 ± 3.7</td>
<td>58.76 ± 27.1</td>
</tr>
<tr>
<td>OG</td>
<td>18.03 ± 0.8</td>
<td>45.60 ± 0.6</td>
<td>55.12 ± 0.1</td>
<td>68.08 ± 14.0</td>
<td>85.12 ± 0.4</td>
<td>92.94 ± 0.3</td>
</tr>
<tr>
<td>S</td>
<td>8.18 ± 0.7</td>
<td>19.77 ± 1.3</td>
<td>20.28 ± 0.4</td>
<td>85.66 ± 2.4</td>
<td>87.34 ± 2.2</td>
<td>88.48 ± 0.02</td>
</tr>
<tr>
<td>O</td>
<td>9.44 ± 0.2</td>
<td>72.44 ± 1.0</td>
<td>82.53 ± 1.1</td>
<td>57.53 ± 5.6</td>
<td>50.97 ± 2.9</td>
<td>82.18 ± 3.0</td>
</tr>
<tr>
<td>WB</td>
<td>30.07 ± 0.3</td>
<td>64.25 ± 0.3</td>
<td>65.30 ± 1.3</td>
<td>86.98 ± 4.0</td>
<td>82.22 ± 1.3</td>
<td>98.73 ± 0.9</td>
</tr>
</tbody>
</table>

1A = alfalfa, OG = orchardgrass, S = straw, O = oats, WB = wheat bran
2n=3
3n=2
*Mean IVDMD across all feeds for fermentation step and 2-step differ (P < 0.05)
**Mean P digestibility across all feeds for fermentation step and 2-step differ (P < 0.05)
CHAPTER 4: SURVEY OF PHOSPHORUS IN COMMERCIAL HORSE FEEDS IN CENTRAL KENTUCKY

As discussed in Chapter 2, the P requirement of many non-breeding mature horses can be met with the use of forage alone. Additionally, the P requirement of working horses can be met with the use of forage and cereal grains without added inorganic P. Consequently, the need to add additional inorganic P to the concentrate may be small. It was hypothesized that commercially available feeds provide more P than is required by mature horses.

To investigate this hypothesis, 28 commercial feeds available in central Kentucky were sampled for P content. Feeds from multiple feed mills with varying P concentrations on the labels and varying purposes (e.g. performance horse feed, foal starter, pleasure horse feed, etc) were sampled. Feeds from Buckeye Nutrition, Burkmann Feeds Hallway Feeds, Legends Horse Feeds, McCauley Bros., Nutrena, Producer Feeds, Purina, Southern States Cooperative, Triple Crown, and Woodford Feed were used in this survey. One bag of each feed was opened and 3 to 4 handfuls of feed were removed from each of the top, middle and bottom sections of the bag. This representative sample was ground through a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) to pass through a 1 mm screen. P concentration was then evaluated using a gravimetric method (Appendix 4).

Table 4-1 shows the detailed list of all the feeds sampled, the minimum P concentration on the label, the analyzed P concentration and the first three ingredients on the feed label, and whether the feed contained any source of inorganic P. The guaranteed minimum P concentration in the feeds that were sampled ranged from 0.3 to 0.8%. The
analyzed concentrations ranged from 0.47 to 0.83%. Inorganic P sources were listed on the labels of more than 67% of the feeds evaluated. All but one of the feeds without added inorganic P listed wheat middlings as the first, second or third ingredient. The one feed that did not list wheat middlings in the first three ingredients listed grain products, plant protein and grain by-products, which could include wheat middlings. Wheat middlings contain approximately 0.80% total P, which is relatively high compared to other feed ingredients (Eeckhout and de Paepe, 1994). It is possible that wheat middlings provide enough P and adding additional inorganic P was unnecessary in these feeds.

Feeds that did not contain added inorganic P have P concentrations that are the same or even greater than feeds with added P. The guaranteed minimum concentration of P for feed without inorganic P ranged from 0.3 to 0.7% and the analyzed concentration ranged from 0.47 to 0.83%. These results suggest that P-adequate concentrates can be formulated without inorganic P, especially if the organic P is already available to the horse.

Only five of the sampled feeds had analyzed P concentrations below the feed label values. Twenty-three of the feeds were above the feed label, with a pleasure horse feed from manufacturer 8 having the highest percentage over the feed label of 76%. Six feeds have overages of less than 10% and 16 have overages less than 25%. Regulations for Kentucky animal feed state that the acceptable analytical variations for P in animal feed are -15% and +2 units (Harrison, 2012). For example, if the feed label was 0.4% P, the acceptable range is 0.34 to 2.4%. Notices of violation are issued for overages, but there is no fine imposed. Violations are rare and are usually a result of mistakes in formulation or mislabeling (Dr. Glen Alan Harrison, University of Kentucky Division of Regulatory Services, Lexington, KY, personal communication). However, an overage
allotment of 2% seems like a large range, especially when precise ration formulation of a limited nutrient requires accurate values. Rations are formulated for horses using the values provided on the feed label, and an actual concentration in excess of the value on the label would lead to oversupplementation of P, leading to excess P excreted.

It is possible that the needs of the horse could be met without the addition of inorganic P, especially if the organic P form is available to the horse. Research has suggested that phytate, or the organic form of P, is able to be broken down by the horse (Lavin et al., 2013; Matsui et al., 1999). Some studies even suggest that phytate-P is as available to the horse as inorganic P (Schryver et al., 1971b; Van Doorn et al., 2004a). If phytate-P truly is available to the horse, the added inorganic P becomes an unnecessary cost and it could contribute to excess excretion of P in the manure, as well as wastage of raw phosphate. Further research is needed to confirm the availabilities of different forms of P to the horse so that feeds can be better tailored to meet their needs, while being environmentally conscious.
<table>
<thead>
<tr>
<th>Feed type</th>
<th>Manufacturer</th>
<th>Form¹</th>
<th>1st ingredient</th>
<th>2nd ingredient</th>
<th>3rd ingredient</th>
<th>Label P, min %</th>
<th>Analyzed P, %</th>
<th>% over label</th>
<th>Forms of inorganic P²</th>
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<tr>
<td>Pleasure</td>
<td>8</td>
<td>T</td>
<td>Oats</td>
<td>Corn</td>
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<td>Corn</td>
<td>Rice bran</td>
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<tr>
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<td>Plant protein</td>
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<td>Plant protein</td>
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<td>Oat mill prod</td>
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<td>0.83</td>
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</table>
Table 4-1 (continued)

1P = pelleted, T = textured
2Forms of inorganic P included monocalcium phosphate, dicalcium phosphate, deflourinated phosphate, and monosodium phosphate
CHAPTER 5: PHOSPHORUS DIGESTIBILITY IN MATURE HORSES AND LONG YEARLINGS

Little is known about the difference in P digestibility between long yearlings and mature horses. Because P digestibilities are used to calculate daily P requirements, it is important to have accurate values for specific age groups. The NRC (2007) uses a P digestibility of 35% to calculate daily requirements for mature horses and a P digestibility of 45% is used for all growing horses. The reason for the difference in these digestibilities is attributed to the different forms of P fed to growing horses and mature horses. Mature horses consume mostly organic P and growing horses usually have inorganic P added to their rations. However, very little is known about phytate degradation in the gastrointestinal tract of these two age groups. One study has shown that mature horses can degrade around 94% of ingested phytate (Lavin et al., 2013), but no work has been done in younger animals. Therefore, the objective of the current study was to examine P digestibility and phytate degradation in long yearling geldings and mature geldings.

Materials and Methods

Horses and general management

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (Protocol No. 2008-0311). The study was performed in October and November of 2012 at the University of Kentucky’s Maine Chance Farm. Four mature geldings (1 Standardbred, 1 Quarter Horse, 1 Thoroughbred-Paint cross and 1 Thoroughbred; age, 10.5 ± 7.5 yr; weight, 535 ± 43.6 kg) and four long
yearling Thoroughbred geldings (age, 19 ± 1 mo; weight, 478 ± 61.1 kg) were used in this study. The experiment was performed in two blocks. Two mature horses and two long yearlings were used in each block. Table 5-1 shows the breed, age, weight and block of the horses used. Each block consisted of a 14-d adaptation period and a 4-d total fecal collection to measure DM, NDF, ADF, P, phytate, Ca, and Mg digestibility. Serum and plasma samples were collected the day before fecal collection started to measure plasma Ca and serum P, as well as insulin, serum bone alkaline phosphatase, and osteocalcin. Saliva was collected before and after feeding on d 2 and d 3 of collection to measure salivary P.

Prior to the start of the experiment, horses were maintained in fields containing cool-season grasses. Mature horses received 2-kg of an oat-based concentrate and yearlings received 3-kg of the same concentrate per d. Horses were vaccinated for rabies (EquiRab; Intervet, Summit, New Jersey), eastern equine encephalitis, western equine encephalitis, tetanus, West Nile virus, equine influenza, and rhinopneumonitis in the spring preceding the trial (Prestige V + WNV, Intervet, Summit, New Jersey). All horses were again vaccinated for equine influenza and rhinopneumonitis (Calvenza 03; Boehringer-Ingelheim, Ingelheim am Rhein, Germany) in August 2012. They were maintained on a regular de-worming schedule for control of internal parasites and the two oldest geldings had their teeth floated prior to the start of the study. Horses were brought into the barn for a few hours each day to get them acclimated to the stalls before adaptation began.
Diets and adaptation period

Prior to the start of the study, a number of candidate feed ingredients were analyzed for total P and phytate-P. The goal was to feed a diet that was low in total P and had greater than 50% of its total P in phytate form. Table 5-2 shows the total P and phytate-P of all the ingredients that were screened. A pelleted concentrate was then custom formulated with selected ingredients (McCauley Bros. Inc., Versailles, KY). The final ingredient composition of the pelleted concentrate is shown in Table 5-3. In addition to a pelleted concentrate, horses also received timothy cubes and alfalfa cubes. Two batches of timothy cubes were used. The first block of horses received batch 1 and the second block received batch 2. Feed was sent to Dairy One (Ithaca, NY) for nutrient analysis. Using the Dairy One nutrient values and initial body weights (Table 5-1), the diet was formulated to meet all nutrient requirements, except P, for growing or mature horses (NRC, 2007). All horses received a diet consisting of 50% timothy cubes, 35% pelleted concentrate and 15% alfalfa cubes. Each horse received a different amount of total feed in order to provide a level of P that was 5% below their requirement (NRC, 2007). The nutrient composition of each feed is shown in Table 5-4.

During the 14-d adaptation phase, the horses were acclimated to rubber matted stalls (4.5 m x 4.5 m) and to wearing Bun-Bag fecal collection harnesses (Bun-Bag, Sagle, ID). Horses stayed in stalls during the night and were turned out in dry lots during the day. They were given ad libitum access to water in the stalls and the dry lots. Horses were fed their pelleted concentrate twice daily and the alfalfa and timothy hay cubes were fed in the afternoon. Three days before the start of the collection phase, they were housed in the stalls 24 h/d. They were hand-walked for 15 min twice daily and they were fed
half of their diet every 12 h. Feed intake was measured daily. Each horse’s body weight was recorded three times per week using a large animal platform scale (Trancell Technology TI-500BWL, Buffalo Grove, IL).

Fecal collection period

During the 4-d collection phase, horses were housed individually in their same stalls with rubber mat floors and hand-walked for 15 min twice daily. They were fed every 12 h and intake was recorded. Orts were weighed every 24 h and saved for future analysis. Horses were given *ad libitum* access to water and consumption from the water bucket was measured daily. The tare weight of each horse’s empty water bucket was recorded. The buckets were then filled with water and the weight of the water was recorded. Water buckets were weighed at least every 12 h to record the weight of the water consumed. Water volume was estimated assuming that 1 kg water = 1 L water. Water was added as needed throughout the collection period and the weight was always recorded. Water samples were taken daily for later analysis. Temperature data was obtained for the days during fecal collection (Meteorological data provided by University of Kentucky AG Weather and Climatological Database, Spindletop station).

A complete timeline of events is shown in Figure 5-1. Horses were fitted with Bun-Bag fecal collection harnesses beginning at 2:00 pm on d 1 of the collection period and monitored 24 h/d to ensure that all feces were collected in the bags. Bun-bags were removed at 2:00 pm on d 5 of collection for a total collection period of 96 h. Bun-bags were emptied every 1 to 2 h into labeled tubs for each horse. The tubs were lined with garbage bags so they could be closed between collection times. The Bun-bags were then
rinsed with water and allowed to dry. A clean Bun-bag was attached immediately following the removal of the soiled one to prevent loss of feces. In the instance of feces falling out of the Bun-bag, fecal material was collected and weighed, but not mixed in with the rest of the collected feces. Feces were compiled for each horse and then thoroughly mixed at the end of a 24 h period. A subsample, 10% of the total fecal weight per d, was collected and frozen for later analysis to measure and calculate DM digestibility, NDF digestibility, ADF digestibility, P digestibility, phytate digestibility, Ca digestibility, and Mg digestibility.

Feed and fecal sample analysis

All feed samples, refusals and feces were dried in a 55° forced-air oven for 24 h or until there was no further loss of weight to determine DM. Samples were then ground with a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) to pass through a 1 mm screen. Samples of feed, refusals and feces were analyzed for phytate by anion exchange (Latta and Eskin, 1980; Appendix 5). Duplicate samples were weighed out and each duplicate was run through two anion exchange columns to account for variation between columns. The amount of P in feed, feces and refusals was determined by a gravimetric assay (Shaver, 2008; Appendix 4). One horse had very high fecal total P values, so those samples were re-run to ensure there was no error in the procedure. The CV between the two runs was 2.1%, so the values for the first set of samples that were analyzed for that horse were used in calculations. Duplicate samples of feed, feces and refusals were analyzed for NDF and ADF using the Ankom 200 Fiber Analyzer (Ankom Technology, Macedon, NY; Appendix 6). Samples of the water that was offered to the horses were sent to the Kentucky Geological Survey laboratory to be analyzed for total P.
using a colorimetric assay (ASTM D515-88). Duplicate samples of feed, refusals, and
feces were analyzed for Ca and Mg using atomic absorption (Appendix 7).

In the case of orts, the analyzed amount of nutrient in the refusal was subtracted
from the amount of nutrient offered in the diet to obtain a more accurate nutrient intake.
Also, the amount of P in the water was included in the nutrient intake for P. Apparent
digestibility was calculated for DM, NDF, ADF, phytate, P, Ca, and Mg using the
following equation: \[(\text{nutrient intake} - \text{nutrient excreted in feces})/\text{nutrient intake}\] * 100.
True digestibility was calculated for P, Ca and Mg using the following equation:
\[\{(\text{nutrient intake} - (\text{nutrient excreted in feces} - \text{fecal endogenous loss}))\} * 100.\] Estimates
of fecal endogenous losses were obtained from NRC (2007). In the case of P, retention
was calculated as (intake – fecal excretion) because urinary P is very low when P intake
is less than 50 mg/kg BW (Schryver et al., 1971b).

Blood and saliva sampling

Blood samples were taken on d -1 of the adaptation period to determine serum P
and plasma Ca levels, insulin, BAP and osteocalcin. Samples were taken pre-feeding, 1
h, 2 h, 4 h, and 6 h post-feeding. Whole blood from each horse was collected from the
jugular vein into a plain tube to obtain serum and a tube containing lithium heparin to
obtain plasma (BD Vacutainer, BD, Franklin Lakes, NJ). Both tubes were centrifuged
within 1 h of collection at 3000 rpm for 20 min at 16 °C. The serum or plasma
supernatant was pipetted into 1.5 ml microcentrifuge tubes and frozen at -20 °C within 2
h of collection. Samples were transferred to -80°C freezer for storage within 9 d of
collection. To determine serum P concentrations, duplicate serum samples were first
deproteinated by adding 1 ml of serum to 5 ml of 6% trichloroacetic acid (TCA), centrifuged at 2,500 rpm for 10 min and then P concentrations were determined using a colorimetric assay (Fiske and Subbarow, 1925; Appendix 3). Plasma Ca was determined by diluting duplicate plasma samples with a La$_2$O$_3$ and HCl solution and then using atomic absorption to determine Ca concentration (Appendix 7). Insulin concentrations were determined with duplicate serum samples using a radioimmunoassay (Coat-A-Count® Insulin, Siemens Healthcare Diagnostics, Erlangen, Germany; Appendix 8). Duplicate serum samples were analyzed for BAP and osteocalcin using immunoassays (Microvue BAP EIA Kit, San Diego, CA; Microvue Osteocalcin EIA Kit, San Diego, CA; Appendix 9). For intra-assay variation, the CV for osteocalcin was 3.1% and the CV for BAP was 6.8%.

Saliva was collected on d 2 and d 3 of collection. Samples were taken 1 h pre-feeding and 6 h post feeding to determine salivary P concentration (Appendix 10). A commercially available cotton swab containing citric acid (Salivette, Sarstedt Ag & Co., Nümbrecht, Germany) was tied to a chifney bit (Figure 5-2) and inserted into the horse’s mouth for 1 min (Figure 5-3). Two swabs per horse were used to ensure ample saliva was collected for analysis. Samples were immediately refrigerated at 4$^\circ$C until P analysis. Analysis of samples occurred within 3 d of collection. Tubes containing the Salivettes were centrifuged at 1,000 rpm for 15 min at room temperature. Recovered saliva was then analyzed for P using a colorimetric assay (Appendix 3). A complete dataset for all horses was not obtained due to difficulties getting enough saliva for analysis from some horses.
**In vitro diet digestibility**

Samples (0.25 g) of the same alfalfa cubes, timothy cubes and pelleted concentrate offered to the horses were ground to pass through a 1mm screen and weighed into Ankom F57 filter bags. Bags were then subjected to the 1-step fermentation method as described in Appendix 1. Calculations to determine IVDMD and *in vitro* P digestibility were performed.

**Statistical analysis**

The effect of age on DMD, NDFD, ADFD, P digestibility, Ca digestibility, Mg digestibility, and phytate digestibility was determined using ANOVA with age and block as main effects (SAS 9.2, SAS Institute, Cary NC). Each group of four horses (two mature geldings and two long yearling geldings) that were collected at the same time was treated as a block. Serum osteocalcin, bone alkaline phosphatase, insulin, blood Ca and blood P were evaluated using repeated measures ANOVA with age, block, and time as main effects (SAS 9.2, SAS Institute, Cary NC). A paired t-test was used to evaluate the difference between *in vivo* and *in vitro* methods on IVDMD and P digestibility (SAS 9.2, SAS Institute, Cary NC). Results were considered significant when \( P < 0.05 \) and a trend when \( 0.05 < P < 0.1 \).

**Results and Discussion**

**Body weights and ADG**

The average weight of the yearlings was 478 ± 58.9 kg and the average weight of the mature geldings was 541 ± 45.9 kg. Individual weights for horses in Block 1 are
shown in Table 5-5 and weights for horses in Block 2 are shown in Table 5-6. The ADG of the long yearlings was calculated using body weights recorded during the last week of the adaptation period and the 4-d collection period (Table 5-7). The mean ADG for the long yearlings was -0.083 ± 0.40 kg/d. Based on the initial body weights and the age of the long yearlings, the mean ADG is expected to be 0.32 ± 0.03 kg/d (NRC, 2007). Hintz et al. (1979) also found that ADG of long yearlings raised on a commercial Thoroughbred farm was 0.27 kg/d, which is higher than the values recorded in this study. The diets were formulated to meet the horses’ daily DE requirements, however, the DE that was calculated for each component of the diet may have been overestimated. Overestimation of DE would lead to formulation of a DE deficient diet. The mature geldings also lost weight over the course of the study, which could indicate that DE was limiting in diets for both the growing horses and the mature horses.

**DM, NDF and ADF digestibility**

There were limited amounts of refusals during the collection period, with only one horse in each block refusing small amounts of feed. Intakes were not adjusted in response to the refusals during the collection period because the horses did not have significant amounts of refused feed during the adaptation period and a change to the diet during the collection period was not ideal. The long yearlings had a higher absolute DMI (kg) than the mature geldings ($P < 0.01$; Table 5-8) and a higher relative DMI (kg/kg BW) than the mature geldings ($P < 0.0001$; Table 5-8). The horses were fed the same diet, but in different amounts based on the requirements of each horse. Because growing horses require more nutrients than mature horses, the long yearlings received more feed to meet their daily requirements.
Although the long yearlings had greater DM intakes, there was no difference in DMD between the two age groups (Table 5-8; *P* > 0.05). DMD by the long yearlings was 52.48 ± 1.54% and was 55.37 ± 5.51% by the mature geldings. Little information is available about DMD in long yearlings. In yearlings, the DMD of a Matua bromegrass hay, which has NDF and ADF concentrations similar to the timothy cubes used in this study, was 51% (LaCasha et al., 1999). The DMD of a native prairie grass hay and a corn and oat concentrate consumed by mature horses was 59.0% (Patterson et al., 2002). Earing et al. (2010) reported a DMD by mature horses of 55.1% for a timothy and oat diet, which is a diet most similar to the one used in the current study. These studies agree with the DMD for both age groups reported in this study.

Long yearlings consumed more NDF and ADF than mature horses, and the long yearlings also excreted more NDF and ADF than mature horses (Table 5-9; *P* < 0.01). The NDFD was 39.06% for long yearlings and 44.19% for the mature geldings (*P* = 0.0917; Table 5-9). The ADFD was 24.46% for long yearlings and 31.55% for the mature geldings (*P* = 0.0630; Table 5-9). Earing (2011) fed mature horses and yearlings a timothy cube and oat based diet and found that NDFD by mature horses was 36.9% and 47.7% for the yearlings. The NDFD by mature horses in the current study was higher but NDFD by long yearlings was lower than the horses used by Earing (2011). Mean NDFD for a meadow hay and barley diet (70 hay:30 barley) eaten by mature ponies was 40.4% and mean ADFD was 36.1% (Drogoul et al., 2001), which are similar to values seen in the mature horses in the current study. LaCasha et al. (1999) reported that Matua bromegrass consumed by yearlings had a mean NDFD of 47% and a mean ADFD of 20%, and Cymbaluk et al. (1990) reported that a pelleted concentrate and brome hay diet
consumed by yearlings had an NDFD of 36.0% and an ADFD of 12.8%. The variation between the studies could be due to varying NDF and ADF content in the diets, differing intakes, or other dietary factors that could affect microbial fermentation, such as starch amount. High amounts of starch have been shown to inhibit cellolytic growth in vivo and could alter non-structural carbohydrate digestibility (Medina et al., 2002). In contrast, low amounts of starch added to a low quality forage has been shown to improve DM digestibility of the forage (Kienzle et al., 2002). The improvement in digestibility of the forage is thought to be due to an increase in easily fermentable substrate for the microbes and an improved nitrogen content to support the growth of the microbial population.

In order to see a difference in NDFD for this study, a sample size of 12 would be necessary (Cochran and Cox, 1957). To see a difference in ADFD, a sample size of 34 would be needed because the variance of ADFD is much greater than that of NDFD (Cochran and Cox, 1957). Due to the labor involvement, cost of sample processing and time necessary to do digestibility studies, it would be difficult to collect that many horses to see a difference for NDFD or ADFD.

Water intake

The long yearlings drank more absolute water (L) than the mature geldings (\( P < 0.01; \text{Table 5-10} \)) and the long yearlings drank more relative water (L/kg BW) than the mature geldings (\( P < 0.001; \text{Table 5-10} \)). However, on a per kg DMI basis, there was no difference in water intake between the age groups (\( P > 0.05; \text{Table 5-10} \)). The NRC (2007) suggests that at an ambient temperature of 20°C, a mature horse at maintenance eating a dry diet should drink close to 0.05 L water/kg BW per day or around 3.3 L
water/kg DMI. The mature horses in the current study drank much less water than suggested by the NRC (2007) on an absolute basis, but they drank a comparable amount in relation to DMI (0.022 ± 0.0006 L/kg BW and 3.38 ± 1.09 L/kg DMI). The estimated water intake of a yearling at 20°C is 0.06 L/kg BW or 3.2 L/kg DMI (NRC, 2007). Again, the long yearlings in the current study drank less water on an absolute basis, but a similar amount in relation to DMI (0.038 ± 0.002 L/kg BW and 3.03 ± 0.78 L/kg DMI). Pagan et al. (1998) found that horses eating a mixed diet drank 3.68 L/kg DMI, which is slightly higher than the amount consumed by the horses in this study (3.38 ± 1.09 L/kg DMI and 3.03 ± 0.78 L/kg DMI for mature geldings and long yearlings, respectively). It has been demonstrated that horses eating a hay only diet drink more water than when consuming a hay and grain diet (Danielsen et al., 1995). High amounts of fiber in the diet have also been shown to increase water consumption on an absolute basis and in relation to DMI (Warren, 2000). The DMI of the horses in this study were lower compared to other studies (Cymbaluk et al., 1989; Pagan et al., 1998), which could influence the total amount of water consumed. Differences in diet composition and type could account for the differences among studies.

While there was no effect of age on water intake/kg DMI, there was an effect of block on water intake/kg DMI ($P < 0.01$). Different ambient temperatures can affect water intake by horses, with colder temperatures leading to a decrease in intake (Cymbaluk, 1990). The horses in Block 1 drank 4.76 ± 0.46 L water/kg DMI and the horses in Block 2 drank 1.66 ± 0.46 L water/kg DMI. The average temperature during the days when the horses in Block 1 were collected was 10.0°C, with a high of 15.5°C and a low of 3.9°C. The average temperature during the days when the horses in Block 2 were
collected was 4.4°C, with a high of 12.2°C and a low of -2.8°C. Cymbaluk et al. (1990) reported that growing horses housed in 15.5°C drank 2.70 L/kg DMI and growing horses housed in 6.2°C drank 2.60 L/kg DMI. The horses in Block 1 drank more water than the horses in the Cymbaluk et al. (1990) study and the horses in Block 2 drank less. It seems unlikely that the small temperature variation seen in the current study caused the horses to vary so much between blocks. However, it is possible that the horses used by Cymbaluk et al. (1990) were adapted to the cold weather by the time the study occurred and were able to adjust their water intakes. The horses in the current study were experiencing the first cold exposure of the season which might influence water intake. Another study reported that mature horses housed outside in a 3 to 15°C environment drank an average of 26.8 kg of water, or 3.51 kg water/kg DMI (Fonnesbeck, 1968). While the ambient temperature is similar to the temperature during Block 2, the water intake of the horses in the current study was much less than the intakes observed by Fonnesbeck (1968). These horses may have also have been adapted to the colder weather, which may have caused a greater water intake than the horses in the current study.

*Phosphorus intake, excretion, and digestibility*

Values for P intakes, P excreted, P retained, apparent and true P digestibilities are shown in Table 5-11. On a daily basis, the long yearlings consumed more P than the mature geldings ($P < 0.01$). On average, the long yearlings consumed 25.2 g P/d (53 mg/kg BW) and the mature geldings consumed 15.9 g P/d (30 mg/kg BW). For 18 mo old horses maturing to 600 kg, the NRC (2007) recommends feeding 24.7 g/d, which is very similar to the amount fed to the long yearlings. The average P requirement for the mature geldings based on their initial BW is 15.8 g/d, which is also very similar to the amount
they were fed. Other studies measuring P digestibility by mature horses utilize P intakes ranging from 13 to 200 mg/kg BW, based on feed ingredients and purpose of the study (Table 2-3). The amount of P fed to the mature geldings is close to the lower part of this wide range, but nevertheless it is very close to the P intake recommended by the NRC (2007). The range of P intakes for growing horses is 47 to 156 mg/kg BW, which is a smaller range than that for mature horses (Table 2-3). The amount of P fed to the long yearlings in the current study also is close to the lower part of this range, but is close to the intake of P recommended by the NRC (2007).

While the long yearlings ingested more P than the mature geldings, they also excreted more than the mature geldings ($P = 0.0015$). These results agree with the findings of Schryver et al. (1971b) who showed that ponies that were fed greater levels of P excreted more. A review of many P digestibility studies also showed that P excretion increased with increasing intake (Lawrence et al., 2003). It is important to consider P excretion into the environment as well as meeting P requirements when formulating diets for mature and growing horses.

P retention was calculated as P intake minus fecal P excreted. For the long yearlings, mean P retention was $3.93 \pm 0.61$ mg/kg BW and mean P retention for all 4 mature geldings was $-1.69 \pm 2.23$ ($P = 0.0059$). There was one gelding with very high P excretion compared to the other three geldings, although his fecal P excretion value was not classified as an outlier. If this gelding is removed, P balance for the mature geldings ($n = 3$) becomes $0.37 \pm 1.20$ and there is no longer a tendency for the long yearlings to have greater retention ($P = 0.10$). Because mature horses are not growing and P is only
needed to replace mostly fecal endogenous and some external losses (hair, skin, hooves, etc), it would be expected that retention would be close to 0, as seen in this study.

The mature horses in this study all lost weight during the last week of adaptation and during the collection period. Because P is present in almost every cell, it is possible that weight loss contributed to a greater P excretion for the mature horses. In addition, studies in humans have reported that bone mineral density decreases with weight loss (Villareal et al., 2006), which would presumably mean that P from the bones was being mobilized and potentially excreted. Table 5-12 shows individual weight loss, P excretion, and P retention for the mature geldings. There was no clear relationship between the magnitude of weight loss and the amount of P excreted or retained. For all the horses except one (Kilo), an increase in weight loss is associated with an increase in P retention. It is unknown if weight loss and weight gain has the potential to influence P retention in mature horses.

Lavin et al. (2013) reported that mature horses eating a diet that supplied 39 to 59 mg P/kg BW had balances that were close to 0. However, many other studies have reported that retention values for mature horses are greater than 0. Hintz et al. (1973) reported that mature ponies fed wheat bran retained anywhere from 6 to 24 mg P/kg BW. These authors fed diets that contained 66 to 121 mg P/kg BW. It is interesting to note that increasing P intake seems to increase the amount of P retained in mature horses. It is possible that the horses used by Hintz et al. (1973) were gaining weight during the study and P was being deposited in the new tissue. Other possible explanations for the location of the retained P include sweat, hair, hooves, and dandruff.
A positive P balance for the long yearlings is expected as these animals are still growing and depositing P in bone and new tissues. The NRC (2007) estimates that growing horses deposit and retain 8 g P/kg gain. On average, the long yearlings in this study were losing 0.083 kg/d and should be retaining -1.39 mg P/kg BW•d⁻¹ (-0.664 g P/d) based on this estimate of retention. Their actual retention was higher than this estimate (3.93 mg P/kg BW•d⁻¹). Nielsen et al. (1998) reported that 2 yr old horses in training eating 20.4 to 27.3 g P/d retained 2.5 to 6.8 g P/d whereas the long yearlings in the current study were eating 25.23 g P/d and retained 1.83 g P/d (3.93 mg/kg BW). In another study using exercised long yearlings eating 38.9 to 71.1 mg P/kg BW, P retention was reported as 1.11 to 3.78 mg P/kg BW (Ogren et al., 2013). Oliviera et al. (2008) fed yearlings 79.68 to 113.08 mg P/kg BW and reported P retention as 25.20 to 47.43 mg/kg BW. The various age differences among these studies may account for some differences in P retention, as older horses are not growing as rapidly as younger horses and do not require as much P for growth. It also appears among these studies that retention might increase with increasing intake in growing horses as well.

There was no difference in apparent or true P digestibility between the 2 age groups (P > 0.05). Apparent P digestibility was -5.77% for mature geldings and 7.44% for long yearlings. These values are similar to findings by Buchholz-Bryant et al. (2001) who reported that growing horses receiving 133% of their NRC (1989) requirements had an apparent P digestibility of 6.4%, while mature horses receiving 133% of their NRC (1989) P requirements had an apparent P digestibility of 0.26%. In another study, apparent P digestibility by long yearlings fed 38.9 mg P/kg BW was 2.9% and true P digestibility was 26.5%, which are similar values to the ones reported for long yearlings.
in the current study (Ogren et al., 2013). Van Doorn et al. (2004a) fed mature ponies 37.4 mg P/kg BW and reported an apparent P digestibility of 2.4% and a true P digestibility of 28.0%, which are very similar to the mature geldings in the current study. However, the diet fed by Van Doorn et al. (2004a) contained very small amounts of phytate-P (1.2 mg/kg BW), which indicates the phytate-P is not the only variable that can cause low P digestibility.

As mentioned previously, one mature gelding appeared to have higher P excretion than the other three geldings. When the mature gelding with high P excretion was removed from the dataset, the average apparent P digestibility by the mature geldings increased from -5.77 ± 7.75% to 1.16 ± 4.10% and true P digestibility increased from 28.04 ± 7.51% to 34.97 ± 4.15%. With this one horse removed, there was still no difference in P digestibilities between the two age groups ($P > 0.05$). However, when this horse is removed, the average true P digestibility is the same value of 35% that the NRC (2007) uses for calculating requirements for mature horses.

Measuring true P digestibility in horses is complicated because it is difficult to know if the excreted P was truly unavailable for absorption or if it was once absorbed and then recycled back into the gastrointestinal tract. Schryver et al. (1972) found that P is secreted into the upper small intestine and the cecum and absorbed in the lower small intestine and the lower large colon. The amount of P that was secreted and absorbed appeared to be influenced by P intake and/or the diet type. These authors fed three diets containing 0.26% (alfalfa), 0.32% (alfalfa-corn), and 0.42% (corn) P. Ponies fed the alfalfa diet had greater amounts of P secreted into the upper small intestine, less P absorbed in the dorsal colon and more P absorbed in the small colon. However, this
effect could be due to the lower amount of total P in the diet, or it could be due to the form of P provided in each of the diets. Further investigation on P recycling and factors that affect it is warranted.

True digestibility is important to consider because it is used to calculate daily P requirements. When calculating true P digestibility, an endogenous fecal P loss of 10 mg/kg BW is used for mature horses and 18 mg/kg BW is used for growing horses (NRC, 2007). However, recent research has suggested that the endogenous fecal losses of growing horses may not be greater than mature horses. Ogren et al. (2013) used long yearlings in training and suggested that fecal endogenous P losses were 10 mg/kg BW. Oliveira et al. (2008) reported that rapidly growing yearlings had fecal endogenous P losses of 8.42 mg/kg BW. If the endogenous fecal P loss of 10 mg/kg BW is used to calculate true digestibility by the long yearlings in the current study, the true P digestibility decreases from 41.25% to 26.3%. The NRC (2007) uses a true P digestibility value of 45% to calculate requirements for growing horses, which is similar to the true P digestibility of long yearlings in the current study when 18 mg/kg BW is used. However, Oliveria et al. (2008) also reported that true P digestibilities by long yearlings were 41 to 57% when using fecal endogenous loss values closer to 10 mg/kg BW. Conversely, Ogren et al. (2013) reported that forage only diets fed to long yearlings had true P digestibilities around 19.4 to 26.5%, which agrees with true P digestibilities from the current study using the lower value for fecal endogenous losses. It is difficult to determine if the differences in P digestibility among studies are due to the use of different estimates of fecal endogenous losses to calculate true digestibility, or if other factors are influencing digestibility, such as age, diet, or environment. More research on the actual
fecal endogenous losses of long yearlings and other ages is necessary to calculate accurate true digestibilities.

True P digestibility is estimated to be 45% for growing horses because they consume diets containing inorganic P as the main source of P (NRC, 2007). Mature horses have an estimated true P digestibility of 35% because the main source of P in their diets is typically phytate-P which is thought to be less available. Because both of the age groups in this study had calculated true P digestibilities lower than 30%, it was of interest to determine the disappearance of phytate-P to see if inclusion of organic P in the diet reduced P availability.

Phytate disappearance

Values for phytate-P intakes, amounts excreted and disappearances are shown in Table 5-13. The percentage of phytate-P/total P in the diet that was fed to both age groups was 17.4%, which is much less than the targeted percentage of 50%. The pelleting process can decrease phytate content (Skoglund et al., 1997), which may be why there was a decrease in the phytate percentage of the final pelleted concentrate. It is difficult to formulate a diet for horses that meets all the requirements, is palatable, and is also very high in phytate-P. However, the ingredients used in the pelleted concentrate are ingredients that are very commonly fed to horses, so the concentrate still represents a diet regularly fed to horses in the industry.

Phytate-P disappearance was calculated as the amount of phytate-P that was consumed versus the amount of phytate-P that was excreted in the feces. Mature geldings consumed 4.60 mg/kg BW of phytate-P and the long yearlings consumed 8.26 mg/kg BW
The amount of phytate-P excreted in the feces was 0.21 mg/kg BW for mature geldings and 0.47 mg/kg BW for long yearlings ($P = 0.0039$). Phytate-P disappearance averaged 94.8% across both age groups ($P > 0.05$). Lavin et al. (2013) also reported that phytate disappearance was 94% in mature horses ingesting an average of 10 mg/kg BW of phytate. It appears that horses can degrade almost all of the phytate supplied in the diet. Similarly, only trace amounts of phytate-P have been detected in the manure of pigs fed a barley diet that contained half of its total P bound to phytate (Leytem et al., 2004). It is known that pigs do not utilize phytate-P as effectively as inorganic P, but they still have the ability to hydrolyze the P bonds in their large intestine.

In cattle, ruminal microbes hydrolyze phytate-P quite efficiently, and total tract phytate disappearance has been reported as high as 94 to 99% (Clark et al., 1986; Morse et al., 1992). However, because the hydrolysis of the phytate molecule occurs prior to the site of absorption in ruminants, the liberated P is more available for absorption in cattle than in monogastric animals. Regardless of digestive tract physiology, it appears that cattle, pigs, and horses have a high capacity to break down phytate-P across the total gastrointestinal tract, but differ in their ability to make use of the liberated P.

It is important to note that the method used to detect phytate only measures the amount of the phytate molecule; it does not measure the amount of liberated P that would be absorbed. The majority of phytate-P breakdown is attributed to microbial phytase activity in the hindgut. The main site of P absorption is in the large and small colon (Schryver et al., 1972). Because phytate-P breakdown and P absorption occurs in the same place, it is possible that the all liberated P is not able to be absorbed. Therefore, while phytate-P may be hydrolyzed in the gastrointestinal tract, all of liberated P may not
be absorbed and could be excreted in the manure. Weir et al. (2013) found that excretion of water soluble P, or inorganic P, in horses was increased when phytate made up a higher portion of the diet. It appears that these horses were able to break down the phytate molecule, but were not able to absorb the liberated inorganic P and therefore excreted it into their manure, increasing the fecal water soluble P content. The inability of the liberated phytate-P to be absorbed may also explain the low P digestibilities seen in the current study. Alternatively, the horses could have absorbed the liberated P and recycled it back into the gut to be excreted which would also cause a low P digestibility.

The low amount of phytate measured in the feces in this study could also be due to effects of sample handling. It is also possible that microbial breakdown of phytate occurred after fecal excretion, while the fecal samples were waiting to be frozen. However, environmental conditions during the study period may not have been conducive for microbial activity in the feces after fecal excretion. The feces were stored at ambient temperature in a closed black plastic garbage bag in a tub during the 24-h periods between sub-sampling. The mean ambient temperature during the first collection week was 8.3°C and during the second week it was 5.8°C, which is well below the average horse’s body temperature of 37.5°C. In addition, fecal balls were immediately broken up and exposed to air when they were removed from the BunBag, inhibiting further activity of any anaerobic microbes. It seems unlikely that significant phytate breakdown could occur after fecal excretion, but this needs to be confirmed.

Mature geldings and long yearlings have similar digestibilities of P and similar phytate-P disappearances, but long yearlings have higher P retention than the older horses. Due to the similar P digestibility and similar phytate disappearances, it may not
be necessary to use two different P digestibilities for long yearlings and mature horses when calculating requirements. More research is needed in younger horses to determine phytate disappearance and P digestibility when phytate is included in the diet. While phytate-P is able to be hydrolyzed in the horse’s gastrointestinal tract, total P digestibility remains low. It is possible that phytate-P is liberated after the site of P absorption, leading to P excretion and low P digestibilities. In addition, the presence of phytate in the diet has been shown to affect the digestibility of other minerals, such as Ca and Mg. It was of interest to examine the digestibility of these minerals in mature geldings and long yearlings as well.

*In vitro DM and phosphorus digestibility of the diet*

*In vitro* DMD and P digestibility were determined for the diet fed to the horses using the 1-step fermentation method. *In vivo* DMD for the diet was 55.50% and IVDMD was 55.51%. The 1-step method was very accurate at estimating DMD for this mixed diet. *In vivo* true P digestibility was lower (23%) than *in vitro* (61%). In a diet containing grain products, the phytate-P in the grains may not be absorbed as well as inorganic P. Hintz et al. (1973) showed that diets containing phytate have lower P digestibilities than diets containing inorganic P. However, it has also been demonstrated that phytate-P is broken down by the horse (Lavin et al., 2013, Matsui et al., 1999). This apparent disagreement in the literature could indicate that the P is liberated from the feedstuff, but the horse may not be able to absorb it. The results of this *in vitro* experiment confirm that the P in the grain products is available for digestion, but there may be other factors that prevent the horse from absorbing and retaining it.
Calcium intake, excretion, and digestibility

Values for Ca intakes, Ca excreted, apparent Ca absorbed, apparent and true Ca digestibilities are shown in Table 5-14. On a daily basis, the long yearlings consumed more Ca than the mature geldings ($P < 0.01$). Long yearlings ingested 128.21 mg Ca/kg BW (60.73 g Ca/d) and the mature geldings ingested 72.14 mg Ca/kg BW (38.90 g Ca/d). On average, the mature geldings should be receiving 22.5 g Ca/d based on the NRC (2007) requirements and their initial BW, which is lower than the amount they were receiving. Based on an expected mature weight of 600 kg, the long yearlings should be receiving 44 g Ca/d (NRC, 2007), which is also less than what they were actually being fed. Overall, the amount of Ca in the diet was adequate to meet the Ca requirements of both age groups of horses.

The long yearlings excreted more Ca than mature geldings ($P = 0.0011$). Increasing Ca intake increases Ca excretion (Schryver et al., 1970). However, Schryver et al. (1970) also found that increasing Ca intake increases the amount of Ca absorbed. In the current study, the apparent amount of Ca absorbed from the gastrointestinal tract was calculated as Ca intake minus Ca excretion. The apparent amount of Ca absorbed is reported because fecal endogenous losses are not included in the calculation. Long yearlings apparently absorbed 64.40 mg Ca/kg BW and mature geldings apparently absorbed 30.29 mg Ca/kg BW ($P = 0.0002$). The greater Ca absorption for the long yearlings shows that either the increased Ca intake caused an increase in absorption, or a greater need for Ca due to growth caused an increase in absorption. Cymbaluk et al. (1989) reported that long yearlings absorbed 79.6 mg Ca/kg BW when fed 208 mg Ca/kg BW, which is comparable to what was seen in the current experiment. Buchholz-Bryant
(2001) reported that 2 and 3 yr old horses absorbed 46.8 mg Ca/kg BW when fed 142.5 mg Ca/kg BW and mature horses absorbed 26.9 mg/kg BW when fed 65.0 mg/kg BW. The values for the mature horses are similar, but the growing horses in the current study have slightly higher amounts of absorbed Ca than the 2 and 3 yr olds being fed similar amounts of Ca. Because the horses in the current study are younger, it is possible that they need to absorb more Ca than 2 and 3 yr old horses for development and bone growth. In addition, actual amounts of retained Ca may not be different between studies if urinary Ca is low, but it is impossible to draw conclusions about renal excretion of Ca in the current study as urinary losses were not measured.

Average apparent Ca digestibility by the long yearlings was 50.37% and it was 42.80% by the mature geldings ($P = 0.0526$). True Ca digestibility by the long yearlings was 78.71% and it was 70.57% by the mature geldings ($P = 0.0577$). Pagan (1994) reported that the average apparent Ca digestibility by mature horses was 44.4% and true Ca digestibility was around 75%. Schryver et al. (1971a) observed that the apparent Ca digestibilities by 2 yr old ponies averaged 46% and true Ca digestibilities averaged 68%. These values for mature horses are very close to the digestibilities observed in the current study. For 17 mo old horses, Cymbaluk et al. (1989) reported that the average true Ca digestibility was 50.9%, which is much lower than the true digestibility observed in the current study by long yearlings (78.7%). However, the NRC (2007) estimates true Ca digestibility at 50% for all ages of horses, but mentions that younger horses may be closer to 70%, which more closely estimated the true Ca digestibility calculated in the current study. Both age groups were much greater than the estimated 50% used by the NRC (2007) to calculate Ca requirements. However, because Ca digestibility can be influenced
by many variables and determining a digestibility value to be applied to all groups of horses may be impossible, a low digestibility value was used to calculate requirements by the NRC (2007) in order to avoid Ca deficiencies.

There are many factors that can influence Ca digestibility in horses. High amounts of P and phytate-P have been shown to decrease Ca digestibility. Phytate forms an insoluble complex with Ca that renders Ca unavailable for absorption in the gastrointestinal tract (Taylor, 1965). When feeding horses 1% of the total P as phytate-P, apparent Ca digestibility was 42.4% and decreased to 26.4% when fed 55% of the total P as phytate-P (Van Doorn et al., 2004a). The percentage of P in the phytate form in the current study was only 17.4% for both age groups, so the effect of phytate on Ca digestibility is most likely not as great as seen by Van Doorn et al. (2004a). Schryver et al. (1971a) reported that decreasing the Ca:P ratio from 2:1 to 0.29:1 decreased absorption of Ca from 7 g/100 kg/d to 3.7 g/100 kg/d, respectively. In the current study, the first group of horses were fed a Ca:P ratio of 2.1:1 and the second group was fed a ratio of 2.8:1. This variation in Ca:P ratio was due to the varying nutrient concentrations of the two different batches of timothy cubes fed to the 2 groups of horses. These ratios are greater than the ones fed by Schryver et al. (1971a) and are unlikely to hinder Ca digestibility.

Magnesium intake, excretion, and digestibility

Values for Mg intakes, Mg excreted, apparent Mg absorbed, apparent and true Mg digestibilities are shown in Table 5-15. Long yearlings ingested more Mg than the mature geldings ($P < 0.01$). The long yearlings consumed 43.20 mg Mg/kg BW (20.58 g Mg/d)
and the mature geldings consumed 24.36 mg Mg/kg BW (13.11 g Mg/d). The NRC (2007) recommends that the mature horses in this study should be eating 8.25 g Mg/d, which is less than what they were actually consuming. For the growing horses, the average recommended amount of daily Mg was 6.8 g Mg/d, which is much less than what the long yearlings were eating. The Mg in the diet was sufficient to meet the requirements for both mature horses and the long yearlings.

Long yearlings excreted more than the mature geldings as well \((P = 0.0040)\). Hintz and Schryver (1973) found that increasing Mg intake increased Mg excretion in ponies. However, even though Mg excretion was higher for the long yearlings, the younger horses apparently absorbed more than the mature horses \((P = 0.0184)\). The long yearlings apparently absorbed 18.27 ± 2.59 mg/kg BW and the mature horses apparently absorbed 9.07 ± 0.83 mg/kg BW. Hintz and Schryver (1973) found that when mature ponies were fed 24 mg Mg/kg BW, they apparently absorbed 11 mg Mg/kg BW and when they were fed 46 mg Mg/kg BW, they apparently absorbed 27 mg Mg/kg BW. These values are similar to the mature geldings’ values, but the absorbed amount for the long yearlings is a little lower compared to mature ponies fed a similar amount.

Differences in age could affect the absorption of Mg.

There was no difference in apparent or true Mg digestibility between the two age groups \((P > 0.05)\). The mean apparent Mg digestibility across both age groups was 39.8%. Mean true Mg digestibility was calculated using fecal endogenous losses of 6 mg Mg/kg BW (NRC, 2007). Mean true Mg digestibility was 59.2% by both age groups. Van Doorn et al. (2004a) reported that the apparent Mg digestibility by mature horses was 41% when fed a low P diet, which is very close to the average value determined for the
horses in the current study. Pagan (1994) also reported average apparent Mg digestibility to be around 37% and true Mg digestibility to be 52% for mature horses, which are slightly lower than the values seen in the current study. The NRC (2007) uses a true digestibility value of 40% to calculate Mg requirements for all classes of horses, which is much lower than the values calculated in this study and found in the literature. Using a low true digestibility value could result in overestimated Mg requirements.

Mg digestibility is relatively similar across most studies because it is not influenced by as many factors as the other minerals measured in this study. Mg digestibility was not altered when horses were fed varying Ca levels, elevated Al, or salt (Schryver et al., 1986; Schryver et al., 1987; Van Doorn et al., 2004b). The anti-nutrients, phytate and oxalate, also do not influence Mg digestibility (Hintz and Schryver, 1972b; McKenzie et al., 1981; Van Doorn et al., 2004a). However, increasing the amount of dietary P has been shown to decrease Mg digestibility (Hintz and Schryver et al., 1972b; Van Doorn et al., 2004a). In addition, increased dietary Si has been demonstrated to improve Mg digestibility in horses (O’Connor et al., 2008). Different amounts of P and Si in the diet could account for some variability among studies.

*Serum phosphorus*

Serum P was measured before feeding and post-prandially to examine the influence of age on P levels in the blood when horses were fed a diet containing organic P with no added inorganic P. Across all time points, long yearlings had a mean serum P concentration of 5.43 ± 0.32 mg/100 ml serum and the mature geldings had an average concentration of 3.07 ± 0.32 mg/100 ml serum. Horses in this study had comparable
serum P values to other experiments. Cymbaluk and Christison (1989) reported that serum P concentrations for growing horses fed a low P diet was 6.22 mg/100 ml serum. Breidenbach et al. (1998a) reported that mature horses fed a low P diet had serum P concentrations of 3.34 mg/100 ml serum.

Long yearlings had greater serum P than the mature geldings across all time points measured, from pre-feeding (0 h) to 6 h post-feeding (Figure 5-4; \( P < 0.0001 \)). Pearson (1934) also found that younger horses had greater serum P concentrations than older horses. However, Pearson (1934) also noted that horses ingesting more dietary P had higher serum P concentrations as well. The long yearlings were receiving greater amounts of P than the mature horses and the greater serum P concentrations in the long yearlings reflect this difference. Unlike blood Ca levels, serum P is not very tightly regulated and can be influenced by many factors, such as diet, age, hormones, diseases and activity (Toribio, 2011). Therefore, it is not a good indicator of body stores of P, but may better indicate dietary and physiological status.

It was hypothesized that serum P would increase post-prandially because dietary P intake is known to affect serum P concentration (Pearson, 1934; Toribio, 2011). It was expected that serum P concentration would rise as dietary P was absorbed from the gastrointestinal tract. However, this response was not observed in this study. There was a trend for the serum P concentrations to decrease over time post-feeding (\( P = 0.075 \)), but there was no significant interaction between age and time. The decrease in serum P post-prandially has also been noted by other authors (Greppi et al., 1996; Piccione et al., 2008). However, these authors also note that there seems to be a diurnal variation in serum P that is unrelated to feeding time. More experiments correcting for feeding time
need to be done to determine if serum P is more sensitive to diurnal patterns, meal feeding or a combination of both.

Plasma calcium

Plasma Ca was measured pre-feeding and post-prandially to determine if there was a difference in blood Ca levels between the two age groups and if the Ca levels changed in response to a meal. Across all time points, long yearlings had an average plasma Ca concentration of 12.40 ± 0.38 mg/100 ml plasma and mature geldings had an average concentration of 13.50 ± 0.38 mg/100 ml plasma. Breidenbach et al. (1998b) estimates the range of Ca concentration in the blood to be 11.02 to 13.03 mg/100 ml plasma. The long yearlings have an average plasma Ca concentration that falls into this range, but the mature geldings are slightly higher.

Figure 5-5 shows the concentration of Ca in the plasma of both groups from pre-feeding (0 h) to 6 h post-feeding. There was no effect of age, time or an interaction of age and time for plasma Ca (P > 0.05). Because blood concentrations of Ca are not responsive to factors such as diet and age, it is not surprising that the concentrations are not different across time or between the two age groups (Breidenbach et al., 1998b).

The Ca:P ratio in the blood across all time points for long yearlings averaged 2.30:1 and for mature geldings it averaged 4.44:1. Because both age groups had similar blood Ca levels but the long yearlings had a higher blood P level, the ratio is different for the two age groups. It is interesting to note that the ratio of Ca:P in bone is 2:1 and a similar ratio is seen in the blood of the growing horses in this study.
Salivary phosphorus

Saliva was collected before and after feeding to determine P concentration and examine differences between age groups. There was some difficulty obtaining sufficient samples for analysis from all horses. Table 5-16 shows the P concentrations in saliva from the horses that provided ample saliva. Both groups were sampled 1 h pre-feeding and 6 h post-feeding. Block 1 was sampled at 2 h post-feeding and again 6 h post-feeding. Block 2 was only additionally sampled at 3 h post-feeding. There were no differences in salivary P between the two ages at any time point \((P > 0.05)\). However, due to the number of horses sampled at each time point, the lack of significance between age groups should not discourage further investigation into salivary P.

Concentrations of P in the saliva ranged from 1.11 to 3.14 mmol/L (34.38 mg/L to 92.25 mg/L) with an average value of 2.31 mmol/L (71.54 mg/L). It is estimated that ponies weighing 150 kg secrete 10 to 12 L/d of saliva from both parotid ducts, or 66 to 80 ml/kg BW per day (Alexander and Hickson, 1970). Using a salivary flow of 66 ml/kg BW, the mature geldings in this study could have secreted 2.26 to 3.24 g of P in their saliva every day and the long yearlings could have secreted 1.08 to 2.91 g of P in their saliva daily. If a mature 500-kg horse was fed the NRC (2007) requirement for P (14 g), salivary P would be around 16 to 24% of the daily intake. Because the requirement for long yearlings is greater (23 g/d) and they secreted less saliva than the mature horses, salivary P would only be around 5 to 13% of the intake. Recycling of P into the saliva is a major way of maintaining P homeostasis in ruminants (Horst, 1986). It is possible that horses use saliva as a method of maintaining P homeostasis as well if they are truly
secreting 24% of their dietary P back into their saliva, but more research needs to be done examining this relationship.

The salivary P concentrations seen in this study are much higher than reported by other authors (Alexander, 1966; Eckersall et al., 1984; Eckersall et al., 1985; Van Doorn et al., 2011). Eckersall (1984) reported a concentration closest to the values observed in the current study of 1.75 mmol/L and even this is low. Saliva sample contamination, differences in diet, and different sample collection and analysis methods could affect salivary P concentrations among studies. There was also large variation between horse, day, and sample time in the current study. Large variations are consistent with the observations of Eckersall et al. (1985) who reported that 16% of salivary P variability was due to horse variation and 31% was due to day variation. Differences between studies as well as individual horse variation could contribute to very large differences among studies.

Bone metabolism markers

Measures of bone metabolism were examined pre-feeding and up to 6 h post-feeding to determine any effect of age on markers of bone turnover in horses fed a low P diet. There was a significant difference between age groups for osteocalcin concentrations, with younger horses having greater concentrations ($P = 0.033$). However, there was no effect of time ($P > 0.05$). Figure 5-6 shows osteocalcin concentrations for both groups over time, starting from pre-feeding (0 h) to 6 h post-feeding. The average concentration for long yearlings across time points was $36.48 \pm 1.40$ ng/ml. For mature geldings, the average concentration was $19.60 \pm 0.90$ ng/ml.
Because osteocalcin is an indicator of bone formation, younger horses have been shown to have higher concentrations of osteocalcin than older horses. Lepage et al. (1990) reported that horses between the ages of 1.5 and 2.5 yrs had osteocalcin concentrations of 35.7 ng/ml and horses between the ages of 3.5 to 20 yrs had a concentration of 6.7 ng/ml. The concentration reported for the mature horses is lower than the concentration seen in the current study. This may be due to the wide range of ages used, as 20-yr old horses may have different bone turnover rates than 3.5-yr old horses. Lepage et al. (1992) examined horses from the ages of 36 to 60 mo and found that osteocalcin levels averaged 15.8 ng/ml, which is more similar to the value seen in the current study for mature geldings. It is unlikely that dietary Ca or P affected osteocalcin values among the studies as no correlation has been found between these minerals and serum osteocalcin in humans or bone osteocalcin in pigs (Michaëlsson et al., 1995; Nicodemo et al., 1998).

Long yearlings had higher serum bone alkaline phosphatase (BAP) concentrations than mature geldings ($P = 0.017$). Price et al. (2001) also reported that BAP concentrations decreased as horses aged. BAP is an indicator of bone mineralization, as it is present on the surface of osteoblasts (Price, 1993) and blood BAP accurately reflects osteoblastic activity (Leung et al., 1993). Therefore, it is not surprising that long yearlings would have greater BAP concentrations, as they are still undergoing bone growth. Across all time points, the long yearlings had an average BAP concentration of $135.51 \pm 2.32$ U/L. The mature geldings averaged $67.96 \pm 1.00$ U/L. Price et al. (2001) found that 18 mo yr old horses had BAP concentrations of 111 U/L, which is comparable to the concentration observed in the long yearlings. Hank et al. (1993) reported an average BAP value of $51.2 \pm 18.1$ U/L. The BAP concentration for the mature geldings...
falls in the upper limit of this range. Figure 5-7 shows average BAP concentrations for both age groups over time, from pre-feeding (0 h) to 6 h post-feeding. There was no effect of time on BAP concentrations ($P > 0.05$).

**Insulin**

Insulin and osteocalcin have been shown to be affected by one another. In rodents, low insulin secretion and insulin resistance are linked with lower serum total osteocalcin concentrations (Botolin et al., 2005). It is important to consider all influencing hormones on bone metabolism so as not to confound dietary treatment with any other hormonal processes that may be occurring. Therefore, examining the insulin concentrations at the same time points when osteocalcin was examined was of interest.

On average across all time points, the long yearlings had a serum insulin concentration of $11.91 \pm 2.68$ uIU/mL and the mature geldings had a concentration of $22.26 \pm 4.38$ uIU/mL. There was an effect of time on insulin concentrations ($P = 0.0017$), but there was no effect of age or an age and time interaction ($P > 0.05$; Figure 5-8). Numerically, it appears that mature geldings had higher insulin concentrations that the long yearlings at certain time points. Malinowski et al. (2002) reported that older horses (27.0 yrs old) had significantly greater insulin concentrations post-prandially than younger horses (6.8 yrs old). Insulin rose rapidly after ingesting a meal, peaked around 2 h post-prandially and decreased to almost pre-feeding values 6 h after feeding. This is the expected response of insulin to a meal, although other authors have reported insulin peaks as early as 30 min post-prandially (Malinowski et al., 2002).
When looking at the concentration of osteocalcin over time and insulin over time, osteocalcin stays relatively level and insulin increases post-prandially then returns to baseline. Post-prandial changes in insulin did not appear to alter osteocalcin concentrations.

**Conclusions**

Overall, this study indicates that mature geldings and long yearlings have the same ability to digest P, phytate-P, and Mg but long yearlings tend to have greater true Ca digestibilities. However, additional research is warranted to determine more accurate methods of evaluating true P digestibility in horses and estimating accurate fecal endogenous losses for growing horses. The use of different digestibilities to calculate daily requirements for P may be unnecessary for long yearlings and mature horses. However, it would be more accurate to use separate digestibilities between these two age groups when calculating Ca requirements. Long yearlings also retained more P and absorbed more Ca and Mg. This increase in absorption and retention could be due to an increased requirement for bone growth, as the long yearlings had greater concentrations of bone formation markers than mature geldings.

Horses have the ability to degrade phytate-P, but true and apparent P digestibilities are still low. It is unknown if the liberated P from phytate was not able to be absorbed due to being liberated in the same site of P absorption, or if it was absorbed and then recycled back into the gastrointestinal tract. The horses in this study could have been recycling up to 24% of their dietary P intake back into their saliva, which might include liberated P from phytate. More research is needed to confirm the amount of
salivary P that is secreted on a daily basis and to examine the effect of different forms of dietary P on salivary P recycling.

Feeding diets high in organic P leads to poor P digestibility and contributes to high excretion of total P, much of which is likely water soluble. Liberating the phytate earlier in the gastrointestinal tract may lead to improved P digestibilities. The use of a pH altered phytase that works in the horse’s stomach may help increase phytate-P liberation before the site of P absorption in the hindgut (Kim et al., 2006). If P requirements can then be met with organic P, adding inorganic P to diets may not be necessary thereby decreasing the reliance on the dwindling supply of global phosphate reserves.

While blood insulin had a large response to a meal, Ca, P, osteocalcin and BAP concentrations were relatively stable. This finding shows that it is unnecessary to fast horses before measuring these blood variables as they are not influenced by ingesting a meal. This information could be useful when taking samples from horses that are eating continuously or grazing.
### Table 5-1. Horse information: Block, age, breed, initial body weight at the beginning of the adaptation period, and average body weight across the adaptation and collection periods (d -8 to d 5)

<table>
<thead>
<tr>
<th>Horse</th>
<th>Block</th>
<th>Age</th>
<th>Breed</th>
<th>Initial weight (kg)</th>
<th>Average weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilo</td>
<td>1</td>
<td>17 yrs</td>
<td>Quarter Horse</td>
<td>541.0</td>
<td>537.2</td>
</tr>
<tr>
<td>Easy</td>
<td>1</td>
<td>17 yrs</td>
<td>Standardbred</td>
<td>488.0</td>
<td>474.2</td>
</tr>
<tr>
<td>Leroy</td>
<td>2</td>
<td>4 yrs</td>
<td>Thoroughbred</td>
<td>583.0</td>
<td>560.3</td>
</tr>
<tr>
<td>George</td>
<td>2</td>
<td>4 yrs</td>
<td>Thoroughbred-Paint cross</td>
<td>590.0</td>
<td>571.8</td>
</tr>
<tr>
<td>Moses</td>
<td>1</td>
<td>18 mo</td>
<td>Thoroughbred</td>
<td>537.0</td>
<td>550.8</td>
</tr>
<tr>
<td>Maestro</td>
<td>1</td>
<td>20 mo</td>
<td>Thoroughbred</td>
<td>456.5</td>
<td>462.0</td>
</tr>
<tr>
<td>Eggs</td>
<td>2</td>
<td>18 mo</td>
<td>Thoroughbred</td>
<td>410.0</td>
<td>405.1</td>
</tr>
<tr>
<td>Paddy</td>
<td>2</td>
<td>20 mo</td>
<td>Thoroughbred</td>
<td>512.5</td>
<td>495.6</td>
</tr>
</tbody>
</table>

### Table 5-2. Total P and phytate-P of feed ingredients screened for inclusion in the experimental diet

<table>
<thead>
<tr>
<th>Feed1</th>
<th>% Total P (average ± %CV)</th>
<th>% Phytate-P (average)</th>
<th>(Phytate-P/Total P)*100, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>0.278 ± 1.5</td>
<td>0.08</td>
<td>28.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>0.64 ± 1.9</td>
<td>0.10</td>
<td>16.4</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>0.13 ± 2.9</td>
<td>0.05</td>
<td>39.8</td>
</tr>
<tr>
<td>Defatted rice bran</td>
<td>2.06 ± 0.9</td>
<td>0.82</td>
<td>39.7</td>
</tr>
<tr>
<td>Straight rice bran</td>
<td>1.92 ± 0.1</td>
<td>0.75</td>
<td>39.1</td>
</tr>
<tr>
<td>Beet pulp</td>
<td>0.11 ± 2.6</td>
<td>0.05</td>
<td>48.6</td>
</tr>
<tr>
<td>Alfalfa meal</td>
<td>0.28 ± 12.0</td>
<td>0.22</td>
<td>77.6</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>1.27 ± 0.03</td>
<td>0.43</td>
<td>34.0</td>
</tr>
<tr>
<td>Flax</td>
<td>0.61 ± 0.9</td>
<td>0.51</td>
<td>83.7</td>
</tr>
<tr>
<td>Barley</td>
<td>0.35 ± 2.3</td>
<td>0.35</td>
<td>102.0</td>
</tr>
<tr>
<td>Wheat middlings</td>
<td>1.09 ± 4.9</td>
<td>0.50</td>
<td>45.0</td>
</tr>
</tbody>
</table>

1 All feed samples provided by McCauley Bros. Inc., Versailles, KY
Table 5-3. Ingredients in the pelleted concentrate

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>35</td>
</tr>
<tr>
<td>Barley</td>
<td>24</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>20</td>
</tr>
<tr>
<td>Flaxseed</td>
<td>15</td>
</tr>
<tr>
<td>Molasses</td>
<td>5.2</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>0.5</td>
</tr>
<tr>
<td>McCauley Trace Mineral Premix¹</td>
<td>0.1</td>
</tr>
<tr>
<td>McCauley Vitamin Premix²</td>
<td>0.1</td>
</tr>
<tr>
<td>Pres Toxi-chek³</td>
<td>0.1</td>
</tr>
</tbody>
</table>

¹Provides 26.25 ppm of Cu, 60 ppm of Mn, 0.4 ppm of Se, 75 ppm of Zn.
²Provides 9.7504 IU/kg of vitamin A, 1.76 IU/kg of vitamin D₃, 233.915 IU/kg of vitamin E, 0.099 mg/kg of biotin, 40.773 mg/kg of pantothenic acid, 1.901 mg/kg of vitamin K, 9.713 mg/kg of thiamine, 61.059 mg/kg of niacin, 6.536 mg/kg of riboflavin, 2.889 mg/kg of folic acid, 6.767 mg/kg of pyridoxine, 0.033 mg/kg of vitamin B₁₂.
³Mold inhibitor additive. Lucta USA, Inc., Northbrook, IL.

Table 5-4. Nutrient composition of feeds

<table>
<thead>
<tr>
<th>Feedstuff</th>
<th>Timothy cubes, Batch 1</th>
<th>Timothy cubes, Batch 2</th>
<th>Alfalfa cubes</th>
<th>Pelleted concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE, Mcal/kg¹</td>
<td>2.05</td>
<td>1.98</td>
<td>2.46</td>
<td>3.19</td>
</tr>
<tr>
<td>CP, %¹</td>
<td>10.5</td>
<td>10.5</td>
<td>20.2</td>
<td>12.7</td>
</tr>
<tr>
<td>NDF, %¹</td>
<td>63.9</td>
<td>66.1</td>
<td>37.8</td>
<td>27.1</td>
</tr>
<tr>
<td>ADF, %¹</td>
<td>40.7</td>
<td>42.6</td>
<td>31.6</td>
<td>21.0</td>
</tr>
<tr>
<td>Ca, %¹</td>
<td>0.33</td>
<td>0.54</td>
<td>2.11</td>
<td>0.38</td>
</tr>
<tr>
<td>P, %¹</td>
<td>0.23</td>
<td>0.20</td>
<td>0.23</td>
<td>0.37</td>
</tr>
<tr>
<td>Ca:P</td>
<td>1.43:1</td>
<td>2.70:1</td>
<td>9.17:1</td>
<td>1.03:1</td>
</tr>
<tr>
<td>Phytate-P, %²</td>
<td>0.039</td>
<td>0.026</td>
<td>0.034</td>
<td>0.082</td>
</tr>
<tr>
<td>Mg, %¹</td>
<td>0.17</td>
<td>0.20</td>
<td>0.25</td>
<td>0.23</td>
</tr>
</tbody>
</table>

¹Analysis performed by Dairy One, Ithaca, NY and presented on a DM basis
²Analyzed using anion exchange method (Appendix 5)
Table 5.5. Weights of horses in Block 1 during the last week of adaptation (d -8 to day-1) and collection period (d 1 to d 5)

<table>
<thead>
<tr>
<th>Horse</th>
<th>Day -8</th>
<th>Day -6</th>
<th>Day -3</th>
<th>Day -1</th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilo¹</td>
<td>555</td>
<td>549.5</td>
<td>553</td>
<td>523</td>
<td>523</td>
<td>519.5</td>
</tr>
<tr>
<td>Easy¹</td>
<td>484</td>
<td>477.5</td>
<td>477</td>
<td>468.5</td>
<td>470</td>
<td>468</td>
</tr>
<tr>
<td>Moses²</td>
<td>556</td>
<td>554</td>
<td>555</td>
<td>553</td>
<td>546</td>
<td>541</td>
</tr>
<tr>
<td>Maestro²</td>
<td>463</td>
<td>463.5</td>
<td>463.5</td>
<td>461.5</td>
<td>457</td>
<td>463.5</td>
</tr>
</tbody>
</table>

¹ Mature gelding  
² Long yearling

Table 5.6. Weights of horses in Block 2 during the last week of adaptation (d -8 to day -1) and collection period (d 1 to d 5)

<table>
<thead>
<tr>
<th>Horse</th>
<th>Day -8</th>
<th>Day -6</th>
<th>Day -3</th>
<th>Day -1</th>
<th>Day 2</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leroy¹</td>
<td>577</td>
<td>568</td>
<td>562.5</td>
<td>553</td>
<td>552.5</td>
<td>549</td>
</tr>
<tr>
<td>George¹</td>
<td>581.5</td>
<td>577</td>
<td>572.5</td>
<td>571</td>
<td>568.5</td>
<td>560.5</td>
</tr>
<tr>
<td>Eggs²</td>
<td>404</td>
<td>407.5</td>
<td>404.5</td>
<td>404</td>
<td>403</td>
<td>407.5</td>
</tr>
<tr>
<td>Paddy²</td>
<td>489</td>
<td>492</td>
<td>502</td>
<td>497.5</td>
<td>497</td>
<td>496</td>
</tr>
</tbody>
</table>

¹ Mature gelding  
² Long yearling

Table 5.7. Actual and expected ADG for the long yearling geldings

<table>
<thead>
<tr>
<th>Horse</th>
<th>Actual ADG (kg/d)¹</th>
<th>Expected ADG (kg/d)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moses</td>
<td>-1.25</td>
<td>0.41</td>
</tr>
<tr>
<td>Maestro</td>
<td>0.042</td>
<td>0.28</td>
</tr>
<tr>
<td>Eggs</td>
<td>0.29</td>
<td>0.30</td>
</tr>
<tr>
<td>Paddy</td>
<td>0.58</td>
<td>0.30</td>
</tr>
</tbody>
</table>

¹ ADG calculated using body weights on d -8 and d 5  
² Expected ADG calculated using body weight on d -8 and the age of the horse at the start of the study (NRC, 2007)
Table 5-8. Average DMI, DM excreted and DMD for long yearlings and mature geldings (mean ± SEM; n=4)

<table>
<thead>
<tr>
<th>Age</th>
<th>DMI, kg</th>
<th>DMI, kg/kg BW</th>
<th>DM excreted, kg</th>
<th>DMD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long yearling</td>
<td>8.35 ± 0.41</td>
<td>0.0175 ± 0.0004</td>
<td>3.97 ± 0.24</td>
<td>52.48 ± 1.54</td>
</tr>
<tr>
<td>Mature gelding</td>
<td>5.32 ± 0.26</td>
<td>0.0098 ± 0.0002</td>
<td>2.37 ± 0.18</td>
<td>55.37 ± 5.51</td>
</tr>
</tbody>
</table>

P-value | 0.0024 | < 0.0001 | 0.0047 | 0.4021 |

Table 5-9. Average NDF intake, NDF excreted, NDFD, ADF intake, ADF excreted, and ADFD for long yearlings and mature geldings (mean ± SEM; n=4)

<table>
<thead>
<tr>
<th>Age</th>
<th>NDF Intake, kg</th>
<th>NDF Excreted, kg</th>
<th>NDFD, %</th>
<th>ADF Intake, kg</th>
<th>ADF Excreted, kg</th>
<th>ADFD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long yearling</td>
<td>4.06 ± 0.13</td>
<td>2.49 ± 0.15</td>
<td>39.06</td>
<td>2.27 ± 0.11</td>
<td>1.73 ± 0.11</td>
<td>24.46</td>
</tr>
<tr>
<td>Mature gelding</td>
<td>2.58 ± 0.20</td>
<td>1.43 ± 0.12</td>
<td>44.19</td>
<td>1.45 ± 0.078</td>
<td>0.98 ± 0.080</td>
<td>31.55</td>
</tr>
</tbody>
</table>

P-value | 0.0024 | 0.0039 | 0.0917 | 0.0023 | 0.0038 | 0.0630 |

Table 5-10. Average water intake for long yearlings and mature geldings (mean ± SEM; n=4)

<table>
<thead>
<tr>
<th>Age</th>
<th>Water intake, L</th>
<th>Water intake, L/kg BW</th>
<th>Water intake, L/kg DMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long yearling</td>
<td>17.97 ± 1.00</td>
<td>0.038 ± 0.002</td>
<td>3.03 ± 0.78</td>
</tr>
<tr>
<td>Mature gelding</td>
<td>10.93 ± 0.78</td>
<td>0.022 ± 0.0006</td>
<td>3.38 ± 1.09</td>
</tr>
</tbody>
</table>

P-value | 0.0039 | 0.0003 | 0.5030 |
Table 5-11. Intake, amount excreted, apparent digestibility, true digestibility and retention of P for long yearlings and mature geldings (mean ± SEM; n=4)

<table>
<thead>
<tr>
<th>Phosphorus</th>
<th>Long yearling</th>
<th>Mature gelding</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake, g/d</td>
<td>25.23 ±1.56</td>
<td>15.94 ± 0.63</td>
<td>0.0028</td>
</tr>
<tr>
<td>Intake, mg/kg BW</td>
<td>52.87 ± 1.13</td>
<td>29.55 ± 0.49</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Excreted, mg/kg BW</td>
<td>49.04 ± 1.32</td>
<td>31.29 ± 2.24</td>
<td>0.0015</td>
</tr>
<tr>
<td>Apparent digestibility, %</td>
<td>7.44 ± 1.32</td>
<td>-5.77 ± 7.75</td>
<td>0.1312</td>
</tr>
<tr>
<td>True digestibility, %¹</td>
<td>26.35 ± 1.37</td>
<td>28.04 ± 7.51</td>
<td>0.1444</td>
</tr>
<tr>
<td>Retained, mg/kg BW²</td>
<td>3.93 ± 0.61</td>
<td>-1.69 ± 2.24</td>
<td>0.0599</td>
</tr>
</tbody>
</table>

¹Calculated using fecal endogenous losses of 10 mg P/kg BW for mature horses and 18 mg P/kg BW for growing horses (NRC, 2007)
²Retention calculated as P intake minus fecal P excretion

Table 5-12. Individual weight loss, P excretion, and P retention for mature geldings

<table>
<thead>
<tr>
<th>Horse</th>
<th>Weight loss, kg¹</th>
<th>P excreted, mg/kg BW</th>
<th>P retention, mg/kg BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy</td>
<td>-16</td>
<td>30.10</td>
<td>0.80</td>
</tr>
<tr>
<td>George</td>
<td>-21</td>
<td>30.91</td>
<td>-1.10</td>
</tr>
<tr>
<td>Leroy</td>
<td>-28</td>
<td>37.44</td>
<td>-7.86</td>
</tr>
<tr>
<td>Kilo</td>
<td>-35.5</td>
<td>26.73</td>
<td>2.20</td>
</tr>
</tbody>
</table>

¹ADG calculated using body weights on d -8 to d 5

Table 5-13. Intake, amount excreted and disappearance of phytate-P for long yearlings and mature geldings (mean ± SEM; n=4)

<table>
<thead>
<tr>
<th>Phytate-P</th>
<th>Long Yearling</th>
<th>Mature Gelling</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake, mg/kg BW</td>
<td>8.26 ± 0.28</td>
<td>4.60 ± 0.15</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Excreted, mg/kg BW</td>
<td>0.47 ± 0.05</td>
<td>0.21 ± 0.02</td>
<td>0.0039</td>
</tr>
<tr>
<td>Disappearance, %</td>
<td>94.25 ± 0.66</td>
<td>95.35 ± 0.29</td>
<td>0.1901</td>
</tr>
</tbody>
</table>
Table 5-14. Intake, amount excreted, apparent digestibility, true digestibility and apparent absorbed amount of Ca for long yearlings and mature geldings (mean ± SEM; n=4)

<table>
<thead>
<tr>
<th></th>
<th>Long yearling</th>
<th>Mature gelding</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calcium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake, g/d</td>
<td>60.73 ± 2.30</td>
<td>38.90 ± 2.86</td>
<td>0.0016</td>
</tr>
<tr>
<td>Intake, mg/kg BW</td>
<td>127.44 ± 4.22</td>
<td>71.57 ± 3.07</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Excreted, mg/kg BW</td>
<td>63.03 ± 1.28</td>
<td>41.28 ± 2.99</td>
<td>0.0011</td>
</tr>
<tr>
<td>Apparent digestibility, %</td>
<td>50.37 ± 1.80</td>
<td>42.80 ± 2.08</td>
<td>0.0526</td>
</tr>
<tr>
<td>True digestibility, %</td>
<td>78.71 ± 1.17</td>
<td>70.57 ± 2.96</td>
<td>0.0577</td>
</tr>
<tr>
<td>Apparent absorbed, mg/kg BW</td>
<td>64.40 ± 4.28</td>
<td>30.29 ± 0.92</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

1Calculated using fecal endogenous losses of 20 mg Ca/kg BW for mature horses and 36 mg Ca/kg BW for growing horses (NRC, 2007)

Table 5-15. Intake, amount excreted, apparent digestibility, true digestibility, and apparent absorbed amount of Mg for long yearlings and mature geldings (mean ± SEM; n=4)

<table>
<thead>
<tr>
<th></th>
<th>Long yearling</th>
<th>Mature gelding</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Magnesium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intake, g/d</td>
<td>20.58 ± 1.02</td>
<td>13.11 ± 0.63</td>
<td>0.0023</td>
</tr>
<tr>
<td>Intake, mg/kg BW</td>
<td>43.20 ± 0.85</td>
<td>24.36 ± 0.35</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Excreted, mg/kg BW</td>
<td>24.80 ± 2.56</td>
<td>15.11 ± 0.48</td>
<td>0.0040</td>
</tr>
<tr>
<td>Apparent digestibility, %</td>
<td>42.31 ± 5.94</td>
<td>37.28 ± 2.87</td>
<td>0.4096</td>
</tr>
<tr>
<td>True digestibility, %</td>
<td>56.25 ± 5.90</td>
<td>62.13 ± 2.45</td>
<td>0.2991</td>
</tr>
<tr>
<td>Apparent absorbed, mg/kg BW</td>
<td>18.27 ± 2.59</td>
<td>9.07 ± 0.83</td>
<td>0.0184</td>
</tr>
</tbody>
</table>

1Calculated using fecal endogenous losses of 6 mg Mg/kg BW for both age groups (NRC, 2007)

Table 5-16. Phosphorus concentrations (mM) in saliva collected from long yearlings and mature geldings at various time points (mean ± SEM)

<table>
<thead>
<tr>
<th>Time</th>
<th>Long Yearlings</th>
<th>Mature Geldings</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h pre-feeding</td>
<td>1.11 ± 0.41; n=3</td>
<td>2.17 ± 0.71; n=3</td>
<td>0.2980</td>
</tr>
<tr>
<td>2 h post-feeding</td>
<td>3.14 ± 0.17; n=2</td>
<td>2.87 ± 1.09; n=2</td>
<td>0.8295</td>
</tr>
<tr>
<td>3 h post-feeding</td>
<td>1.13; n=1</td>
<td>2.03 ± 0.58; n=2</td>
<td>---</td>
</tr>
<tr>
<td>6 h post-feeding</td>
<td>1.72 ± 0.49; n=4</td>
<td>2.93 ± 1.12; n=4</td>
<td>0.4325</td>
</tr>
</tbody>
</table>
Figure 5-1. Timeline for adaptation and collection events

- 1. Horses adapted to stalls, diet and BunBags
- 2. Blood samples taken
- 3. Bun-Bags on at 2:00
- 4. Saliva collection
- 5. Bun-Bags off at 2:00
Figure 5-2: Salivette tied to chifney bit

Figure 5-3: Horse wearing chifney bit
Figure 5-4. Serum P concentrations in relation to feeding time

![Graph showing Serum P concentrations in relation to feeding time for Long yearlings and Mature geldings.](image-url)
Figure 5-5. Plasma Ca concentrations in relation to feeding time
Figure 5-6. Osteocalcin concentrations for long yearlings and mature geldings over time
Figure 5-7. Bone alkaline phosphatase concentrations in long yearlings and mature geldings over time
Figure 5-8. Serum insulin concentrations for long yearlings and mature geldings over time.
Appendix 1: In Vitro Phosphorus Digestion


Solutions

Pepsin and 0.1 M HCl Solution:

Dissolve 8.3 ml of stock HCl (12N HCl) into 400 ml of water. Cool and dilute to 1 L. Dissolve 1.25 g of pepsin in 1 L of the 0.1 M HCl. Adjust pH to 2 ± 0.05. Use an enzyme to substrate ratio of 0.05 (w:w).

α-Amylase and 0.1 M Bis-Tris Solution:

Add 20.924g Bis Tris to 1 L of purified water. Adjust pH to 6.8 ± 0.1. Dissolve 0.25 g of amylase in 25 ml of the 0.1 M Bis-Tris buffer. Adjust pH to 6.8 ± 0.1. Use an enzyme substrate ratio of 0.02 (w:w).

Sample and Bag Preparation

Feed samples should be dried in a 55°C oven and ground to pass through a 1 mm screen. Ankom F57 filter bags should be soaked in acetone for 5 min, allowed to dry, labeled with a black permanent marker and weighed to obtain a tare weight.

Procedure

1. Weigh 0.3 g of each feed sample in triplicate, place into pre-labeled Ankom F57 bags and seal using a heat sealer. Weigh and seal 2 empty bags to be used in calculating a correction factor. Put each bag into a 50 ml beaker.
2. Add 12 ml of the pepsin solution per sample to the 50 ml beaker.

3. Incubate beakers in a water bath at 39º C for 2 hours.

4. Remove beakers and adjust pH to 6.8 ± 0.02 using the Bis-Tris buffer (without the amylase enzyme).

5. Add 1 ml of the amylase solution per sample.

6. Incubate at 39º C for 4 hours.

7. Remove bags from beakers.

8. Wash with 96% ethanol (2 x 10 ml), acetone (2 x 10 ml) and distilled water.


**Reagents**

Bis-Tris Buffer (From: Brask-Pederson et al., 2011)

- 10.0812 g NaHCO$_3$ (0.12 M)
- 7.53 g Bis-Tris (0.036 M)
- 0.5965 g KCl (0.0008 M)
- 0.4675 g NaCl (0.0008 M)
- 0.1220 g MgCl$_2$•6H$_2$O (0.0006 M)
- 0.0588 g CaCl$_2$•2H$_2$O (0.0004M M)
- Add all components to 1 L nanopure water and mix.
2N HCl

- Add 166 ml concentrated HCl to a 1 L volumetric flask containing 400 ml of nanopure water. Cool and fill to volume.

_Fecal collection_

Collect freshly voided feces from the horse, taking care not to collect feces that come in contact with the ground. Place feces in a plastic bag, purge with CO₂, and put the bag in a cooler with warm water bags to maintain a temperature around 37.5°C. Immediately transport cooler to the lab.

_Procedure_

Before Feces Arrive:

1. Set up water bath to a constant temperature of 37.5°C
2. Pre-heat 1800mL of the Bis-Tris buffer and the mixing jar in the water bath
3. Turn on Daisy II incubator with the incubation jars inside to allow them to warm up to 37.5°C
4. Place buffer in the incubation jar and adjust to pH 7.00 using 2N HCl
5. Add assigned Ankom F57 filter bags from pre-digestion step.

Note: If only doing fermentation step, follow sample and bag preparation as described above, but only add 0.25 g of feed sample to each filter bag.

6. Place the incubation jar in the pre-warmed incubator

After Feces Arrive:

7. Place fecal samples in the warm water bath and allow time for feces to equilibrate to temperature
8. Weigh out 200 g of feces in weigh dish and transfer to the pre-heated mixing jar
9. Remove desired incubation jar from the incubator and remove 400 mL of buffer solution using a graduated cylinder
10. Add 400 mL of buffer to the mixing jar with the feces
11. Purge the mixing jar with CO₂ for 15 seconds
12. Cap the mixing jar and blend for 30 seconds
13. Transfer the fecal/buffer solution into the incubation jar and stir well
14. Ensure that the pH is 7.00 ± 0.02 and adjust as needed using 2N HCl. Make sure to stir well between pH readings to ensure equal distribution of added solution. Adjustments to pH should be minimal.
15. Purge incubation jar with CO₂ for 30 seconds and return to Daisy incubator
16. Set the timer for 48 hours and begin incubation

Bag Washing

Remove jars from incubator. Dump jar individually into a strainer and collect filter bags. All other contents of the jar can be disposed of. Separate the bags into separate strainers based on content. i.e. forage or concentrate. Rinse bags over a tub with a gentle stream of cold water. Water flow should not disturb contents of the bag. Rinse both sides of the bags until water runs clear in the tub. Place bags on oven drying tray. Make sure they are evenly spaced with no overlapping bags. Place tray with bags in a forced air oven at 55° C for 24 h or until bag weights are consistent. Remove bags from oven, place in dessicator and allow them to cool. Record their weights.
**Calculations**

IVDMD, % = 100 – [((W_3 – (W_1*CF))/W_2)*100]

Where: 
- \( W_3 \) = final post-digestion bag weight 
- \( W_2 \) = feed sample weight, DM basis 
- \( W_1 \) = empty bag tare weight 
- \( CF \) = post-digestion blank bag weight/initial blank bag weight

*In vitro* P digestibility, %

\[
\text{In vitro P digestibility, } \% = \frac{M_1 - [(C_1 * 10 - W_1) * (0.03097/W_2) \div \% \text{ DM feed}]}{M_1} \times 100
\]

Where: 
- \( M_1 \) = mg P in feed in filter bag, DM basis 
- \( C_1 \) = mM P in filter bag with feed inside 
- \( W_1 \) = average mM P in blank bags 
- \( W_2 \) = dry post-digestion bag weight – (bag tare weight * CF)
Appendix 2: Ankom A and B buffers

Buffer Solution A:

- KH$_2$PO$_4$: 10.0 g/L
- MgSO$_4$•7H$_2$O: 0.5 g/L
- NaCl: 0.5 g/L
- CaCl$_2$•H$_2$O: 0.1 g/L
- Urea (reagent grade) 0.5 g/L
- Bring to 1 L with nanopure water

Buffer Solution B:

- Na$_2$CO$_3$: 15.0 g/L
- Na$_2$S•9H$_2$O: 1.0 g/L
- Bring to 1 L with nanopure water
Appendix 3: Colorimetric Phosphorus Determination


This method was used for determining P in saliva samples and was utilized during the in vitro experiment for filter bags and feed samples.

Reagents

Reducing reagent:

- 15 g Sodium bisulfate (NaHSO₃)
- 5 g p-methylaminophenol (C₇H₉NO•0.5 H₂SO₄)
- Dissolve components in 400 ml of nanopure water. Bring volume to 500 mL.
  Store in a brown glass bottle. Reagent is stable for 10 d at room temperature.

Acid molybdate reagent:

- 136 ml sulfuric acid (H₂SO₄)
- 25 g ammonium molybdate ((NH₄)₆Mo₇O₂₄•4H₂O)
- Add sulfuric acid to 400 ml of nanopure water in a 1 L volumetric flask and allow to cool. Dissolve ammonium molybdate in 400 ml of nanopure water and add to the sulfuric acid solution. Fill to volume with water.

Standards:

- Weigh 0.1379 g of sodium phosphate monobasic (NaH₂PO₄•H₂O) into 100 ml volumetric flask and fill to volume with nanopure water to create a working standard.
- Pipet 2, 4, 6, 8, and 10 ml of working standard into 100 ml volumetric flasks and fill to volume with nanopure water to create standards of 0.2, 0.4, 0.6, 0.8, and 1.0 mM phosphate, respectively.
- Store in refrigerator. Prepare fresh standards weekly.

Sample preparation

Saliva: Pipet raw saliva directly out of collection tube and into test tube for analysis.

Feed samples: Grind to pass through a 1mm screen. Weigh 2 g of feed into crucibles and ash at 500°C for 4 h. (The crucible should be previously acid washed, rinsed with nanopure water and dried). Remove crucible from oven and add 10 ml of 6N HCl to each crucible. Heat crucibles on hot plate in a fumehood for 20 min or until acid begins to boil. Remove crucibles and filter contents through #4 Whatman filter paper into a 100 ml volumetric flask. Rinse crucible with 0.2% K buffer into the flask and bring flask to volume using 0.2% K buffer. Use this solution for taking the 1 ml sample.

Filter bags: Ash whole bag with feed inside in a crucible at 500°C for 4 h. Follow same procedure as described above for feed samples.

Procedure

1. Pipet 1 ml of standard, sample, or water (blank) into test tubes in duplicate.
2. Add 2 ml of nanopure water to each tube.
3. Add 1 ml of acid molybdate reagent to each tube.
4. Add 1 ml of reducing reagent to each tube.
5. Add 5 ml of nanopure water to each tube and mix well by inverting tubes 3 times.
6. Allow 20 min for color development and then read absorbance at 660 nm.
**Calculations**

Calculate standard curve using concentrations (independent variable) and absorbances (dependent variable) of the standards. Plug the measured absorbance for the unknown sample into the standard curve equation to calculate concentration (mM P).
Appendix 4: Gravimetric Quimociac Total Phosphorus Method


Note: All glassware used in mineral analysis was washed in Contrex CA acidic liquid detergent (Decon Labs, King of Prussia, PA), rinsed with nanopure water and dried before use.

Quimociac Reagent Preparation – 1 L

Dissolve 70 g sodium molybdate dehydrate in 150 ml nanopure water. Dissolve 63.8 g citric acid dehydrate (or 53.9 g anhydrous citric acid) in 150 ml nanopure water and add 85 ml concentrated nitric acid with stirring. Add 5 ml synthetic quinoline to a mixture of 100 ml nanopure water and 35 ml concentrated nitric acid. Slowly add the quinoline mixture to the molybdate-citric-nitric solution mixing well during addition. Let solution stand overnight. Filter solution through #2V Whatman filter paper (or equivalent). Add 280 ml chemically pure (C.P.) acetone and dilute to 1 L with nanopure water. Mix well and store in polyethylene bottle.

Sample Preparation

Samples should be dried to a constant weight in a 55°C forced air oven and ground to pass through a 1 mm screen using a Wiley mill (Arthur H. Thomas CO., Philadelphia, PA). Weigh 2 – 3 g of ground feces or 3 g of ground feed sample into a quartz crucible and place in ash furnace at 550°C overnight. Remove and cool sample. Add 40 ml of 1:3 HCl (250 ml HCl: 750 ml nanopure water) and heat on hot plate on high for 20 minutes. Samples should boil, but take care that they do not boil over or “pop.” Transfer crucible
contents to a 250 ml volumetric flask, bring to volume with nanopure water and mix thoroughly. Let flask sit overnight.

**Analysis**

1. Place clean gooch crucibles with fiberglass filter paper circles in a 100ºC drying oven overnight. Remove the crucibles the next day, place them in a dessicator and allow to cool. Record the weights.

2. Transfer a 25 ml aliquot of the previously prepared sample solution to a 250 ml Erlenmeyer flask and add 25-50 ml nanopure water to ensure the sample does not boil dry.

3. Place Erlenmeyer flasks with the sample on a hot plate on high and bring to boil. Add 35 ml of quimociac reagent to each sample once it begins boiling. Allow to boil for 1 min or until a bright canary yellow color is achieved.

4. Quantitatively filter quimociac precipitate into previously weighed gooch crucibles with fiberglass paper circles, rinsing well with nanopure water to ensure all the precipitate is filtered.

5. Place gooch crucibles with precipitate in 100ºC drying oven and dry overnight (or 250ºC for 1 h).

6. Remove gooch crucibles from oven, place in dessicator and allow to cool. Weigh crucibles and record weight.

**Total P Calculation**

\[
\% P = \frac{\text{Weight of precipitate, g} \times 250 \text{ ml}}{25 \text{ ml aliquot}} \times \frac{0.013997 \times 100}{\text{sample weight, g}}
\]
Appendix 5: Phytate Phosphorus Determination

Phytate-P was determined according to Latta and Eskin (1980) and revised as follows.

Reagent Preparation

2.4% HCl: Add 24 ml of concentrated HCl to a 1 L volumetric flask and dilute to volume with nanopure water.

0.1 M NaCl: Dissolve 5.845 g NaCl in 1 L nanopure water.

0.7 M NaCl: Dissolved 40.915 g NaCl in 1 L nanopure water.

Wade’s reagent: Dissolve 0.15 g FeCl$_3$ and 1.5 g sulfosalicylic acid in 500 ml nanopure water.

Packing the columns

Weigh 0.5 g anion resin (200-400 Mesh AG 1-X8, chloride form; Biorad, Hercules, CA) into weight boat. Carefully add water to the weight boat to rinse resin into column (Econo-column chromatography, BioRad, Hercules, CA). Fill column with nanopure water and let it drip out into a beaker. Refill once or twice more until resin packs to the bottom. Use the same amount of water each time so the resin packs the same. Screw stopper in bottom and fill column with nanopure water when not in use. Resin can be used up to 3 times.

Phytic Acid Standards

Make stock standard by dissolving 0.5 g phytic acid (calcium salt) in 50 ml nanopure water. Create a set of standards of 0, 5, 10, 20, 40 µg phytic acid/ml. To make standards, pipette 10 ml of nanopure water into a test tube and remove 5 µl of water. Add 5 µl of the stock standard. To make the next standard, remove 10 µl of water and add 10 µl of stock standard, and so forth. Vortex all tubes for 5 seconds. Pipette 3 ml of each standard in
duplicate into test tubes and add 1 ml Wade’s reagent. Vortex for 5 seconds. Centrifuge for 10 min at 3,000 rpm. Read on spectrophotometer at 500 nm.

Phytate-P Analysis

1. Samples should be dried to a constant weight in a 55⁰ C forced air oven and ground to pass through a 1 mm screen using a Wiley mill (Arthur H. Thomas CO., Philadelphia, PA).

2. Weigh 1 g of the ground sample in duplicate into a 50 ml Erlenmeyer flask and record weight.

3. Add 20 ml of 2.4% HCl solution. Shake on low for 1 h on shaker.

4. Empty sample into a 50 ml centrifuge tube and centrifuge for 15 min at 2,500 rpm.

5. Pipette 1 ml of the supernatant into a clean 50 ml Erlenmeyer flask filled with 24 ml nanopure water if using feed. If using manure, pipet 6 ml of supernatant into 6 ml of nanopure water. Shake to mix.

6. Pipette 10 ml of the diluted sample solution (5 ml at a time) into anion columns. Discard the elute. Don’t let the resin go dry and add each solution slowly so as to not disturb the resin.

Note: When starting this procedure for the first time, ensure that there is minimal variation between columns by pipetting the diluted sample solution into the anion columns in duplicate. This will give 4 measurements per sample: 2 samples were weighed out and each of those samples were run through 2 columns.

7. Elute with 15 ml (5 ml at a time) of the 0.1 M NaCl solution. Discard the elute.

8. Elute with 10 ml (5 ml at a time) of the 0.7 M NaCl solution and collect in glass scintillation vials. Cap and shake.
9. Immediately (within 1 h) pipette 3 ml of the collected sample into a test tube. Add 1 ml of Wade’s reagent.

10. Vortex for 5 sec. Centrifuge for 10 min at 3,000 rpm.

11. Read on spectrophotometer at 500 nm.

Calculations

First, calculate concentration of phytate in the 10 ml elute by inserting the absorbance into the standard curve formula. Then, use the following equation to determine the percent phytate in the sample.

\[
\% \text{ Phytate} = \frac{\text{Phytate in elute, } \mu \text{g per 10 ml} \times 50}{\text{Sample weight, g} \times 1,000,000} \times 100
\]
Appendix 6: Ankom Fiber Analysis

From: Acid Detergent Fiber in Feeds – Filter Bag Technique, Ankom Technology and Neutral Detergent Fiber in Feeds – Filter Bag Technique, Ankom Technology

Reagents

Neutral Detergent Solution

- 30 g of sodium lauryl sulfate
- 18.61 g ethylenediaminetetraacetic disodium salt, dehydrate
- 6.81 g sodium tetraborate decahydrate
- 4.56 g sodium phosphate dibasic, anhydrous
- 10.0 ml triethylene glycol
- Dissolve in 1 L distilled water.

Acid Detergent Solution

- 20.0 g cetyl trimethlyammonium bromide
- 28 ml 1.00 N H₂SO₄
- Dissolve in 1 L distilled water.

Procedures

Neutral Detergent Fiber

Sample preparation

Samples should be dried and ground to pass through a 1mm screen. Soak Ankom F57 filter bags in acetone for 5 min and air dry prior to use. Label them with a black permanent marker and record their tare weight. Weigh 0.5 g samples into filter bags and
seal the bags closed with a heat sealer. Label, weigh and seal one empty bag per set to be used to calculate a correction factor.

*Analysis*

Place 3 bags per tray on the bag suspender. Make sure to include 1 blank bag per run. Stack trays on the center post with each tray rotated 90°. Up to 24 bags can be analyzed at a time. Place weight on top of bag suspender. Add 2000 ml of NDF solution to the Ankom Fiber Analyzer vessel and then lower the bag suspender into the vessel. Turn the agitator and heat on (100°C) and close the lid. Set timer for 75 min. After 75 min, turn off the heat and agitator, open the drain valve and let the solution all drain out before opening lid. Close valve, add 2000 ml of hot water and lower lid. Set heat at 95°C, turn agitate on and set timer for 7 min. After 7 min, drain hot water and rinse 2 more times or until water is a neutral pH. After final rinse, remove bags, gently press out excess water and soak them in acetone for 3 min. Remove bags and press out excess acetone. Let the acetone evaporate and then put bags in 55°C forced air oven overnight. Once dry, place bags in dessicator, allow them to cool and weigh.

*Calculations*

\[
\text{NDF, } \% = \frac{(W_3 - (W_1 - CF))/W_2}{100}
\]

Where:

- \( W_3 \) = empty bag weight
- \( W_2 \) = sample weight
- \( W_1 \) = final bag weight (NDF residue + bag)
- \( CF \) = final weight of blank bag/initial weight of blank bag

*Acid Detergent Fiber*

Follow the same method as shown above, substituting ADF solution for NDF solution.
Appendix 7: Total Calcium and Magnesium Analysis for Feed, Feces, and Blood


Reagents

Lanthanum diluent (5 mM): Add 1.63 g La₂O₃ and 15 ml concentrated HCl to a 1 L volumetric flask and dilute to volume.

Sample Preparation (Feed and Feces)

Feed samples should be dried to a constant weight in a 55°C forced air oven and ground to pass through a 1 mm screen using a Wiley mill (Arthur H. Thomas CO., Philadelphia, PA). Weigh 2 – 3 g of feces or 3 g of feed sample into a quartz crucible and place in ash furnace at 550⁰ C overnight. Remove and cool sample. Add 40 ml of 1:3 HCl (250 ml HCl: 750 ml nanopure water) and heat on hot plate on high for 20 minutes. Samples should boil, but take care that they do not boil over or “pop.” Transfer crucible contents to a 250 ml volumetric flask, bring to volume with nanopure water and mix thoroughly. Let flask sit overnight.

Analysis

Blood: Serum or plasma (oxalate or EDTA are not acceptable) samples must be diluted with lanthanum diluent (0.2 ml plasma + 10.0 ml diluent).

Feed and fecal samples: Take a sample aliquot from the 250 volumetric flasks prepared the day before and dilute 1:100 or 1:50 with a NaCl solution (1 g NaCl/1 L water), depending on the expected concentration of Ca or Mg in the sample.
Prepare a set of Ca and Mg standards from 0 to 5 ppm Ca or Mg starting from the 1000 ppm stock Ca or Mg solution. Dilute the stock to the appropriate concentrations using the same diluent used to dilute the samples. Read standards and samples on an atomic absorption spectrophotometer using the appropriate elemental lamp. The atomic absorption spectrophotometer reads out the concentration in ppm.
Appendix 8: Serum Insulin

*Insulin radioimmunoassay (Coat-A-Count® Insulin, Siemens Healthcare Diagnostics, Erlangen, Germany)*

1. Thaw serum samples at room temperature.

2. Label 6 uncoated 12x75 mm polypropylene tubes T (total counts) and NSB (non-specific binding) in triplicates. Label 24 insulin Ab-coated tubes for the 8 calibrators to be run in triplicate. Calibrator concentrations are 0, 4.15, 8.3, 18.7, 53, 105, 215, and 412 µIU/ml. Note that one additional calibrator was made by diluting the 15 calibrator with the 0 calibrator in a 50:50 ratio. Label insulin Ab-coated tubes in duplicate for the controls and samples that will be run.

3. Pipet 200 µL of 0 calibrator into the NSB and 0 calibrator tubes, and 200 µL of each remaining calibrator, controls, and samples into the labeled tubes. Pipet directly to the bottom.

4. Add 1 mL of $^{125}$I insulin to every tube and vortex. This step must be completed with 40 min of pipetting the samples into the tubes. T tubes may be set aside until counting (Step 7), as they need no additional processing.

5. Incubate for 18-24 h at room temperature.

6. Decant all tubes (except T tubes) thoroughly and allow them to drain for 2 to 3 min. Strike the tubes against absorbant paper to shake off any residual droplets.

7. Count for 1 min in a gamma counter.

*Calculations*

Net counts = (Average CPM) – (Average NSB CPM)

Percent bound = (Net counts/ Net MB counts) x 100
Using logit-log graph paper, plot Percent Bound on the vertical axis against Concentration on the horizontal axis for each of the non-zero calibrators. Draw a straight line through the points and calculate concentrations of unknowns using interpolation.
Appendix 9: Serum Bone Alkaline Phosphatase and Serum Osteocalcin

_Serum bone alkaline phosphatase (Microvue BAP EIA kit, Quidel Corporation, San Diego, CA)_

1. Thaw serum samples at room temperature.
2. Dilute the Wash buffer 1:10 with nano-pure water. Wash buffer is good for 21 d.
3. When samples have thawed, dilute long yearling and mature horse serum 1:3 with prepared Wash buffer.
4. Open package containing plate and make sure the wells are snapped into place.
4. Pipette 125 µL of Assay buffer into the wells to be used.
5. Pipette 20 µL of the standards, controls and diluted samples into the assay well. Gently swirl plate to mix buffer and sample. Complete this step within 30 min.
6. Incubate for 3 h ± 10 min at room temperature.
7. Prepare Substrate solution 30-60 min before use by adding one Substrate tablet per bottle of Substrate buffer and shake to mix. Use within 1 h of preparation.
8. After 3 h incubation, invert plate to empty contents. Add at least 250 µL of the wash buffer to each well and let sit for a minute or two before inverting again. Repeat 3 – 4 more times. Vigorously blot the plate on a dry paper towel between washes and after the last wash to ensure there are no bubbles in any well. Pop any remaining bubbles with a clean pipette tip after the last wash.
9. Pipette 150 µL Substrate solution into each well.
10. Incubated for 30 ± 5 min at room temperature.
11. Add 100 µL Stop solution to each well. Gently swirl to mix.
11. Read the optical density at 405 nm using the VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA). Read within 15 min of adding the Stop solution.

12. Analyze the results using a quadratic fit curve, \( y = A + Bx + Cx^2 \) in the SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

Osteocalcin

1. Thaw frozen serum samples in a water bath at 37ºC. When samples have just thawed, immediately put them on ice.

2. Dilute Wash buffer 1:10 using nano-pure water. Wash buffer is good for 21 d.

3. When samples are thawed, dilute long yearling serum 1:5 and mature horse serum 1:4 using the Wash buffer.

4. Reconstitute standards and controls using 0.5 ml of the Wash buffer. Allow 15 min for the pellet to dissolve and use within 1 h.

5. Open package containing plate and make sure the wells are snapped into place.

6. Pipette 25 µL of the standards, controls and diluted samples into the assay well. Complete this step within 30 min.

7. Pipette 125 µL of Anti-osteocalcin into the wells. Gently swirl to mix.

8. Incubate for 2 h ± 10 min at room temperature.

9. Prepare Enzyme conjugate by adding 10 ml of Wash buffer to each vial of Enzyme conjugate. Allow pellet to dissolve and use within 2 h.

10. After incubation, invert plate to empty contents. Add at least 300 µL of the Wash buffer to each well and let sit for 1 minute or two before inverting again. Repeat the wash step 3 – 4 more times. Vigorously blot the plate on a dry paper towel between washes and
after the last wash to ensure there are no bubbles in any well. Pop any remaining bubbles with a clean pipette tip after the last wash.

11. Add 150 µL of reconstituted Enzyme conjugate to each well.

12. Incubate for 60 ± 5 min at room temperature.

13. Prepare Working substrate solution by adding one Substrate tablet to each bottle of Substrate buffer. Allow 30-60 min for the tablet to dissolve and shake to mix. Use within 1 h.

14. After incubation, wash wells as described in step 10.

15. Add 150 µL Working substrate to each well.

16. Incubate for 35-40 min at room temperature.

17. After incubation, add 50 µL Stop solution to each well and swirl to mix.

18. Read the optical density at 405 nm using the VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA) within 15 min of adding the Stop solution.

19. Analyze samples using a 4-parameter calibration curve, \( y = \frac{(A-D)}{1+(x/C)^B}+D \) in the SoftMax Pro software (Molecular Devices, Sunnyvale, CA).
Appendix 10: Saliva Collection and Analysis Protocol

Procedure

1. Weigh the empty Salivette, tube and cap all together.

2. Thread the needle, put a knot in the end and push the needle through the Salivette as shown in Figure 1.

3. Lay the Salivette on the top of the chifney bit and wrap the thread around the bit and Salivette, securing the Salivette to the chifney. Tie the thread around the chifney at the end to keep it in place.

4. Put the chifney in the horse’s mouth for exactly 1 minute.

5. Remove the chifney and cut the thread. Remove the thread from the Salivette, being careful not to take any of the cotton out of the Salivette.

6. Place the Salivette back into its tube and cap. Get a weight on the Salivette, tube and cap again. Refrigerate immediately and analyze within 3 days.

7. Centrifuge at 1,000 rpm for 15 minutes.


NOTE: It is important not to touch the Salivette with your hands. Wear gloves when handling.
Figure 1. Threading of the Salivette
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VITA

Ashley Fowler is originally from Napa, California. She received her B.S. in Animal Science with an equine emphasis at the University of California, Davis in 2011. During her undergraduate career, she worked under the guidance of Dr. Frank Mitloehner on research projects involving animal welfare and the effects of animal agriculture on environmental quality. Ashley also worked with critically ill neonatal horses and alpacas at the Lucy G. Whittier Neonatal Unit at the UC Davis Veterinary Medical Teaching Hospital. While at UC Davis, Ashley received the Jack T. Pickett scholarship, the Henry A. Jastro scholarship, and studied in the Integrated Studies Honors Program.

Ashley moved to Lexington, KY to start her M.S. in equine nutrition under Dr. Laurie Lawrence in August 2011. Her main research focus has been on phosphorus digestibility in horses and developing an in vitro phosphorus digestibility technique for use in horses, but she has also done some work looking at mineral concentrations in cool-season grasses. In addition to research, she also was a teaching assistant for an undergraduate animal nutrition course, guest lectured at two local high schools, and served as the secretary and treasurer for the Animal and Food Science Graduate Student Association. While at the University of Kentucky, Ashley has received the University of Kentucky’s Graduate School Academic Year Fellowship and has also gained membership in the Honor Society of Agriculture Gamma Sigma Delta and the Honor Society of Phi Kappa Phi.
Publications and Abstracts


