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FACTORS INFLUENCING PHOSPHORUS EXCRETION BY HORSES

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FACTORS INFLUENCING PHOSPHORUS EXCRETION BY HORSES

Dissertation

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
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

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ABSTRACT OF DISSERTATION

FACTORS INFLUENCING PHOSPHORUS EXCRETION BY HORSES

Excessive phosphorus (P) excreted by animals can affect water quality and cause eutrophication. Better understanding of factors that influence P utilization and excretion in horses may reduce the environmental impact of P. Two animal experiments were conducted that examined P excretion by horses. The efficacy of titanium dioxide as an external marker to calculate digestibility was studied concurrently with both animal experiments. Additionally, pasture P concentrations were evaluated over the growing season using near-infrared spectroscopy (NIRS). Experiment 1 examined P excretion by post-lactational mares fed a low P diet immediately prior to weaning compared to non-lactating controls fed an adequate P diet. Post-lactational mares excreted more P compared to controls. Experiment 2 compared P excretion in horses fed to lose, maintain, or gain weight. Horses fed to lose weight tended to excrete more fecal P compared to horses fed to gain weight and had increased markers of bone turnover. The efficacy of titanium dioxide for estimating fecal output from limited fecal grab samples was variable. Titanium dioxide may be useful in situations where many fecal samples are collected over 5 d, but may not be as accurate if one fecal grab sample is expected to be representative of fecal output. Experiment 3 focused on examining the changes in pasture mineral concentration over the season using NIRS. A discussion of how these results may inform equine P supplementation programs is included. Overall, the work in this dissertation suggests that factors that influence P excretion in the horse include the dietary availability of P, physiological status, and active weight change. These variables can be incorporated into feeding programs to meet horses' needs more closely while minimizing P excretion in the environment.

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Date June 18, 2018

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LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
aFO	Actual fecal output
BAP	Bone alkaline phosphatase
BCS	Body condition score
BW	Body weight
cFO	Calculated fecal output
CP	Crude protein
CTX-1	Cross-linking C-terminal telopeptides of type I collagen
DE	Digestible energy
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
D_2O	Deuterium oxide
FECa	Fractional excretion of calcium
FEP	Fractional excretion of phosphorus
FFM	Fat-free mass
FGF23	Fibroblast growth factor-23
FO	Fecal output
GE	Gross energy
GI	Gastro-intestinal
NDF	Neutral detergent fiber
NEFA	Non-esterified fatty acid
NFC	Non-fiber carbohydrates
NIRS	Near infrared spectroscopy

NSH	Nutritional secondary hyperparathyroidism
OM	Organic matter
^{32}P	Phosphorus isotope
PTH	Parathyroid hormone
PUN	Plasma urea nitrogen
TBW	Total body water
TG	Triglycerides

CHAPTER 1: INTRODUCTION

Phosphorus is an essential nutrient for livestock. However, excess P excretion by animals affects water quality and causes eutrophication. Therefore, emphasis in livestock feeding has been placed on minimizing P excretion while maintaining animal performance.

There are two likely contributing causes of excess P excretion. The first is feeding large amounts of unavailable P that the animal cannot absorb so it passes out in the feces. The second reason for excessive P excretion is the provision of P in amounts above the animal's physiological need. Because organic P has been suggested to have low availability for monogastrics (including horses), inorganic P is often added to diets to ensure that enough P is available for optimal animal performance. However, if available P is underestimated, this practice results in overfeeding P and increasing P excretion.

In order to provide diets that meet but do not exceed P requirements, an understanding of P availability in all feed ingredients is necessary. Currently, true digestibility of P in diets fed to horses is estimated to be 35% in diets containing organic P and 45% in diets containing a combination of inorganic and organic P (NRC, 2007). However, these estimates of true P digestibility appear to be low in comparison to values applied in other species (NRC, 2005). The current true digestibility estimates for horse feeds appear to have originated from research conducted in the 1970's as the value of 35% first appeared in "The Nutrient Requirement of Horses" in 1978 (NRC, 1978) and the value of 45% appeared 11 years later (NRC, 1989). In previous editions of the publication, the true digestibility of P was estimated at 50% in mature animals and either 60% or 80% in growing horses (NRC, 1966; NRC, 1973). Furthermore, in the equine

literature, a wide range of true digestibility values have been reported for P (-10 to 79%)(Kichura et al., 1983; Lavin et al., 2009). There is little understanding of why such a broad range of values has been observed, but if true P digestibility is underestimated then the current dietary P recommendations will result in higher P intake and P excretion than necessary. The goal of this dissertation is to gain an understanding of the factors that might contribute to this large variability in P availability in order to ensure that rations fed to horses provide adequate but not excessive P.

CHAPTER 2: LITERATURE REVIEW

Phosphorus digestibility

Why digestibility is important

To calculate mineral requirements, one needs to know the digestibility of the mineral, the amount needed to replace endogenous losses, and the amount needed for production. The amount of nutrient needed to replace endogenous losses is usually the amount needed for maintenance. Products include milk during lactation, fetal tissue during pregnancy, and tissue accretion during growth. Dietary mineral requirements are calculated by dividing the amount of nutrient needed for maintenance and production by the true digestibility of the mineral in the diet. It has been suggested that the digestibility component has more influence on the calculated requirement than any other variable in the equation (NRC, 2001), emphasizing the importance of obtaining an accurate value for true digestibility.

Phosphorus digestibility in horses

Across the equine literature, true P digestibility ranges from -10 to 79%. Differences in physiological state, diet composition, environment, and methodology could all account for the wide range of values. Level of dietary P accounts for some of the differences seen in P digestibility. Pagan (1994) reported that the R^2 for the relationship between P intake and digestibility of 120 observations across a wide range of P intakes was 0.33, indicating that P intake accounted for 33% of the variability seen in P digestibility.

Source of P (organic or inorganic) has also been suggested as a factor that influences P digestibility. In order to calculate P requirements for horses, NRC (2007) used a true digestibility of 35% for mature, non-lactating horses and 45% for growing and lactating animals. Mature horses are assumed to only be consuming P from plant sources, while growing and lactating animals are typically supplemented with inorganic P, which is assumed to have a higher availability of P than organic (plant) sources of P (NRC, 2007). However, the P digestibility of a diet containing only organic P was estimated to be 42% (Fowler et al., 2015), indicating that organic P may be more available than previously believed.

Phosphorus need may also influence P digestibility. Cymbaluk (1990) reported greater P digestibilities by 8-mo old horses than by 12-mo old horses with lower P requirements. Additionally, pasture that provided sufficient P for requirements of lactating mares was reported to have an apparent digestibility of 43%, which would calculate to a true digestibility of 57% assuming endogenous losses of 10 mg/kg BW (Grace et al., 2002). Thus, lactating mares have the ability to efficiently digest plant sources of P without inorganic P supplementation. These results indicate the greater true digestibility values (45%) suggested by the NRC (2007) for lactating mares and growing horses can be achieved without inorganic P added to the diet.

Phosphorus metabolism in horses

Dietary P is absorbed mainly in the large intestine with some P absorbed in the small intestine (Schryver et al., 1972). Phosphorus can be absorbed by enterocytes via an active Na^+-dependent transporter or by paracellular passive diffusion (Muscher-Banse et

al., 2017). The majority of P absorbed in the small intestine is through passive diffusion, which is a relatively slow process and is dependent on P concentration in the gut lumen (Mushcer-Banse et al., 2017). Possibly diets that result in higher concentrations of soluble P in the small intestine will promote P absorption in this section of the GI tract. In the colon, P is actively transported, which implies that this is a process that can be regulated and saturated (Muscher-Banse et al., 2017). Phosphorus is also secreted into the GI tract of horses (Schryver et al., 1972; Cehak et al., 2012; Muscher-Banse et al., 2017). Active secretion of P from the equine jejunum was observed in an *in vitro* study, suggesting that P secretion into the gut is also able to be regulated (Muscher-Banse et al., 2017).

Secretion of P into the GI tract plays an influential role in maintaining P homeostasis, as urinary P in horses is relatively low when P is fed near the requirement (Schryver et al., 1971). While urinary P does somewhat increase when dietary P increases, the relationship is curvilinear, with significant amounts of P being excreted in the urine only when P intakes exceed 100 mg/kg BW (Figure 2-1), which is about 3.5 times the current maintenance requirement of mature horses. Combined with the knowledge that secretion into the GI tract in horses can be regulated, recycling of P into the GI tract is likely a major way that horses maintain homeostasis.

Hormones that regulate P homeostasis include parathyroid hormone (PTH), 1,25-hydroxyvitamin D, and fibroblast growth factor-23 (FGF23). Parathyroid hormone is released when serum Ca is low. Parathyroid hormone stimulates a release of Ca (and P) from bone mineral in an attempt to maintain Ca homeostasis. It also increases Ca reabsorption in the kidney and inhibits P reabsorption, resulting in an increase in urinary

P. Parathyroid hormone also acts in the kidney to increase production of the active form of vitamin D, 1,25-dihydroxycholecalciferol, which also influences P metabolism (discussed below) (Murayama et al., 1998). As demonstrated from Figure 2-2, at low dietary Ca:P ratios, urinary P excretion is greater than when Ca:P increases to 2:1 and above. Because the Ca:P ratio can influence Ca absorption in the small intestine, horses fed inverted Ca:P ratios have the potential to become Ca deficient, leading to stimulation of the PTH system and subsequent urinary excretion of P.

Nutritional secondary hyperparathyroidism (NSH) is a disease involving PTH that occurs in horses chronically fed a diet with an inverted Ca:P ratio (Krook and Lowe, 1964). Due to the lowered amounts of Ca in the blood over long periods of time, the parathyroid gland becomes hypertrophied, producing increased quantities of PTH. Parathyroid hormone increases bone resorption, particularly from the flat bones. The bone lesions are filled in with fibrous tissue, resulting in irregularly shaped bones and shifting lameness. Because of this symptom, NSH is also known as “bighead” disease, as horses suffering from this disease often develop soft, cartilage-like swellings on their facial bones.

Vitamin D can also influence P homeostasis. Unlike other species, the digestibility, renal excretion, and plasma concentration of Ca is not affected by vitamin D in horses (Harrington and Page, 1983; Bourdeau et al., 1986; Boass and Toverud, 1996). Additionally, circulating levels of Ca in horses are much higher than observed in other species and 1,25-dihydroxycholecalciferol levels are much lower (Breidenbach et al., 1998b). However, the digestibility, renal excretion, and plasma concentration of P is increased with large amounts of vitamin D in horses (Breidenbach et al., 1998a). Vitamin

D increases P absorption from the small intestine by acting on active P-transporters, as demonstrated in rodents (Katai et al., 1999). Thus, while vitamin D may not have the same effects on Ca metabolism as in other species, vitamin D status may influence both active absorption and excretion of P in the horse.

Fibroblast growth factor-23 is produced by osteoclasts and osteoblasts and is released in response to high circulating levels of P or active vitamin D (Jüppner, 2011). In other species, FGF23 has been shown to reduce blood P levels by decreasing reabsorption of P in the kidney (Bergwitz and Jüppner, 2010). It is unknown if the role of FGF23 is similar in horses, especially as urinary excretion of P is low.

Endogenous P losses

Endogenous P losses are composed of P contained in sloughed enterocytes, digestive secretions, and also P that is actively secreted into the GI tract by transporters. The NRC (2007) uses an estimate of 10 mg P/kg BW for endogenous fecal P losses for mature horses based on a study by Schryver et al. (1971). Schryver et al. (1971) used ^{32}P, an isotope of P, that was injected into the horse (intravenously or intramuscularly) and then measured in the feces. In this study, endogenous P losses were 9.4 mg/kg BW and were constant across P intakes ranging from 43 to 200 mg P/kg BW (16 total observations). However, fecal P increased with P intake, which may be due to absorbed dietary P being recycled back into the GI tract as this P would not be labeled, but still may be considered part of endogenous losses. Thus, the actual digestibility of dietary P may be higher than that observed in this study (40 to 47%). Conversely, Kichura et al. (1983) reported that endogenous P losses measured using ^{32}P were 3.3 mg P/kg BW in eight ponies averaging 12 yr old and that losses were not significantly affected by P or Ca

intake (whereas in yearling ponies Ca intake did influence endogenous P losses; discussed below). Using data from 18 studies using mature horses, P excretion was plotted against P intake (Figure 2-3). Using the equation for the relationship between intake and excretion, at a P intake of 0, fecal excretion would be 8.2 mg P/kg BW. This value represents the fecal endogenous P loss estimated from these studies, which is relatively close to the value obtained by Schryver et al. (1971).

For growing horses, the NRC (2007) uses an endogenous loss estimate of 18 mg P/kg BW, based on one study performed by Cymbaluk et al. (1989) using 18 growing horses. In that study, endogenous P loss was calculated as the y-intercept of an equation that included P intake and fecal Ca as variables to predict fecal P excretion. However, that study fed Ca in excess of requirements (range from 41 to 203% of Ca requirement) and Kichura et al. (1983) found that high amounts of dietary Ca (200% of requirement) increased endogenous fecal P losses in yearling horses. It is possible that the high levels of Ca fed by Cymbaluk et al. (1989) caused an increase in endogenous fecal P losses. In fact, other studies have suggested that endogenous P losses of growing horses may be more similar to that of mature horses (10 mg/kg BW). 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 P/kg BW. Furtado et al. (2000) estimated endogenous fecal losses to be 10.3 mg P/kg BW in growing horses. However, using data from 8 studies using growing horses, endogenous P losses were estimated to be 19 mg/kg BW (Figure 2-4), which is more similar to NRC (2007) estimates. However the relationship between P intake and fecal P

excretion is only moderate ($R^2 = 0.5$), suggesting that other factors influence fecal P excretion and potentially endogenous P losses in growing horses. If endogenous P losses are truly lower for growing horses than the value used by NRC (2007), then current P requirements for growing horses may be overestimated.

Recycled P

One reason for the discrepancy in measured endogenous fecal P losses and the broad range of true P digestibility estimates could be due to the P being recycled into the GI tract. In reviewing studies that have measured fecal and urinary P excretion it becomes apparent that P is almost entirely excreted in feces, rather than urine. If all of the fecal P is perceived to be from undigested P, it would lead to assumptions of very low P digestibility. As noted previously P can be secreted into the GI tract, or essentially recycled post-absorption. In other species, it is recognized that total endogenous fecal excretion is composed of a minimum endogenous fecal loss and a variable endogenous loss (Ammerman, 1995). The minimum endogenous loss is the inevitable loss that occurs as a part of digestion while the variable fraction consists of endogenous losses that differ depending on other dietary or physiological factors. The minimum endogenous fraction likely consists of P contained in sloughed intestinal cells, secretions involved in digestion (bile, pancreatic secretions, etc) as well as P contained in microbial cells that are excreted in the feces. The variable fraction would also include recycled P, or P that is absorbed and then secreted back into the GI tract. Increases in P intake cause increases in variable endogenous losses because P absorbed in excess of requirements is resecreted into the GI tract for excretion (NRC, 2001, 2012). Studies that measure endogenous losses via ^{32}P are only measuring endogenous P secretions originating from the body and do not include

microbial P (part of minimum endogenous losses) or P that is recycled (variable endogenous P fraction). Unfortunately, unless P is labelled, the minimum and variable fractions cannot be chemically separated and only exist in theory. However, the maintenance requirement of P is only the minimum endogenous P loss when dietary P meets the actual P requirement (NRC, 2001). By feeding P over the actual requirement, the variable fraction of endogenous P increases, and thus increases the amount of P excreted in the feces. By not accounting for microbial P or recycled P, estimates of P availability from feed will be underestimated due to an increase in fecal P.

Figure 2-5 demonstrates how the origins of excreted P can change with intake. As P intake increases, the minimum endogenous loss stays the same and the variable endogenous fraction increases, leading to an increase in total endogenous losses. The variable fraction is increasing with increasing P intake because at higher intakes more P is being absorbed than is required by the body and so excess P is resecreted into the GI tract. While this figure just demonstrates how P intake can affect composition of excreted P, other factors may affect variable endogenous losses. In dairy and swine, the minimum endogenous loss fraction has been shown to be dependent on DM intake (Conrad et al., 1956; Preston and Pfander, 1964; Almeida and Stein, 2010) and this is likely the case in other species as well. If we can better understand what the minimum endogenous losses in horses are, we can then begin to build our knowledge base of how the variable fraction is affected and therefore make better calculations of P requirements.

Calcium intake or the Ca:P ratio can affect endogenous losses. One study reported that high levels of dietary Ca increased endogenous fecal P in yearlings, but dietary Ca had no effect for weanlings or mature ponies (Kichura et al., 1983). Schryver et al. (1972)

fed ponies either an alfalfa-based or corn-based diet and measured the amount of P secreted into the GI tract. While a detailed diet composition was not given for this study, it is assumed that the alfalfa diet would have greater Ca and a larger Ca:P than the corn diet. These authors observed that ponies fed the alfalfa-based diet had greater amounts of P secreted into the GI tract compared to the corn-based diets. Therefore, it is possible that the higher Ca content in the alfalfa diets resulted in greater endogenous P secretions, although there are other confounding factors, such as dietary P intake and diet composition. In contrast, chickens appear to decrease endogenous losses in response to increasing Ca levels in the diet. Broiler chicks fed increasing levels of Ca with a constant P intake exhibited a decrease in endogenous P losses (Al-Masri, 1995). The reason for the different results seen in horses compared to chickens is unclear, but may be related to differences in GI anatomy and location of P absorption.

Physiological state may also impact endogenous P losses. Different physiological states may influence quantities of hormones produced (as discussed above), which can alter P excretion. During periods of increased Ca need (e.g. late pregnancy and early lactation), bone is resorbed to supply Ca to the animal as needed for fetal growth and lactation. However, for every 10 parts Ca released, 6 parts P are also released (NRC, 2001), indirectly increasing P in the blood. One study fed ewes the required amount of Ca and P during pregnancy and lactation and then measured endogenous P losses and resorption of P from bone during pregnancy and also at the onset of lactation when requirements increase (Braithwaite, 1983). At the onset of lactation, dietary Ca was observed to be inadequate to supply the demand for Ca during peak lactation and bone was mobilized to maintain milk Ca concentrations. As a result, the amount of P resorbed

from bones increased by 100% and endogenous losses of P also increased by 30%. Because P requirements for lactation were being met, the surplus P mobilized from bone was excreted as endogenous losses.

In addition to P secretions in the small and large intestine, salivary P could be involved in the secretion of P into the horse's GI tract. Ruminants rely on salivary P secretions to maintain P homeostasis by secreting excess P from the blood into the saliva (Horst, 1986). Horses do not secrete nearly the quantity of salivary P as ruminants (1 to 3 g P/d in horses compared to 30 to 40 g P/d in cattle), but it has been suggested that horses may secrete up to 24% of their daily P intake in their saliva (Fowler, 2013), which could influence homeostasis and estimates of subsequent P digestibility. Differences in salivary P secretion may cause differences in P availability in the gut and alter measured P digestibility values, as most salivary P is in the form of readily absorbable inorganic P.

By definition, the P requirement for an animal at maintenance should be calculated using minimum endogenous P losses (NRC, 2001, 2012) as well as the actual digestibility of dietary P. However, it is likely that the current maintenance requirement was calculated using minimum endogenous losses plus recycled P, which would result in maintenance P requirement that is actually greater than the actual P needed by the animal. By feeding horses more than their actual P requirement, P continues to be recycled, resulting in measurements of P digestibility that may be lower than they truly are. Figure 2-5 demonstrates the current model of how P is absorbed and excreted in a horse at maintenance, based on NRC (2007) estimates of endogenous losses at 10 mg P/kg BW and true P digestibility of 35%. In this scenario, the horse is being fed exactly the amount of P that is required by the body to replace endogenous losses and corrected for a P

digestibility of 35%. The resulting P requirement calculated from this scenario is 28 mg P/kg BW. However, Figure 2-6 shows an example of what could happen if P digestibility was actually 70% and how P would be recycled into the GI tract. In this scenario, more P is being absorbed than what is needed to replace endogenous losses of 10 mg P/ kg BW, so the additional P is recycled and excreted in the feces. The observed fecal excretion is the same in both scenarios, but in the second case, P is being overfed. In this situation where true P digestibility is really 70%, the P intake could be reduced to 14 mg P/kg BW to avoid recycling of P into the GI tract while still feeding to replace minimum endogenous P losses.

If P intakes were reduced from 28 to 14 mg P/kg BW for ten horses over a year, the difference in yearly fecal P excretion would be around 18 kg of P. Reducing fecal P excretion by reducing P intakes would not only reduce the P in runoff to surface waters, but would also reduce the feed cost for horse owners. Inorganic P is often added to commercial concentrates to increase P concentrations to ensure adequate P consumption by horses. The cost of adding inorganic forms of P to the diet could be substantial, considering that P is a limited resource and world reserves of P are declining (McGill, 2012). Unsupplemented feeds would likely easily meet the reduced P requirement of 14 mg P/kg BW. If a horse is consuming 10 kg of DM, it would only require 0.14% of P in the diet. This concentration is met or exceeded in most feeds fed to horses (NRC, 2007). Overall, reducing P intakes will reduce environmental impacts, is economical, is practical, and will likely not impact horse health, especially if true P digestibility is greater than 35%.

In vivo models to evaluate P endogenous recycling

One method to accurately elucidate minimum endogenous losses and estimate true digestibility would be to create a situation where P retention is high enough, and P intake low enough, that essentially all of the available P is retained by the animal. Young et al. (1966) performed a study with growing sheep that employed a period of P depletion. Lambs were either fed a P-depletion diet that was deficient in P but adequate in Ca, or a control diet adequate in both for 4.5 mo. A 21-d fecal collection period followed in which total endogenous P losses were measured using ^{32}P. Lambs fed the P-depletion diet had lower endogenous P losses than lambs fed a normal P diet. Because the depleted lambs had a greater need to retain P than control lambs, they were absorbing and retaining more P rather than resecreting absorbed P back into their GI tract. These results suggest that the physiological state of the animal has the potential to influence fecal endogenous P losses due to changes in P recycling within the gut.

In mature animals, another approach to understanding variation in P digestibility and excretion would be to create a weight change model. Weight gain and weight loss in horses during digestibility studies may account for variability in some estimates of P digestibility by possibly altering endogenous P losses. The NRC (2007) suggests that mature horses at maintenance should not be retaining P and should have a P balance of 0, but many studies have found that mature horses retain significant amounts of P (Schryver et al., 1971; Hintz et al., 1973; Schryver et al., 1987; Morris-Stoker et al., 2001; Patterson et al., 2002; van Doorn et al., 2004a). The location of the retained P has not been evaluated, but it is possible horses in these studies were gaining weight and that the positive P balance reflects P in accreted tissue.

Weight gain occurs when calorie intake exceeds calorie use. In digestibility studies when feces are being collected, horses are typically confined. Confinement and subsequent lack of voluntary exercise may reduce caloric expenditure. Also, diets that are higher in concentrates have increased caloric density. For example, Hintz et al. (1973) reported that mature ponies retained significant amounts of P (up to 24 mg P/kg BW/d), however the ponies were confined to metabolism crates and were fed a low forage/high concentrate diet. It is possible that the ponies were gaining weight during the period of their confinement and high concentrate intake, leading to retention of P in the weight that was being gained. Conversely, some horses could lose weight during a study, particularly as it is difficult to feed mature horses at maintenance a diet that does not exceed P requirements but meets DE needs. Phosphorus could be secreted into the gut during periods of weight loss, contributing to endogenous P losses. Weight gain and loss are not typically reported in digestibility studies in horses but these gains and losses may be important when investigating P balance and digestibility.

Weight change and nutrient balance: Could it affect endogenous P losses?

As discussed above, weight change is one potential model to study P recycling in horses. However, an understanding of the composition of the horses' body and what the composition of the weight change might be is important for evaluating this question. Additionally, knowledge of the P content of body components is also essential.

Body composition of horses

In other species, body composition varies among breeds within a particular species (Lohman, 1971), and it appears horses are no exception (Kearns et al., 2002a).

Differences in body composition are probably due to genetic differences based on the desired purpose of the horse. For example, Arabians were bred for endurance, Quarter Horses for speed, and draft horses for power and strength. In addition to genetics, differences in nutrition, physical conditioning, age, gender, and physiological class also contribute to differences in body composition.

Of all the body components, body fat is the most variable and fat-free mass (FFM) is relatively more constant (Lohman, 1971). As such, there are many methods described in the literature for measuring or estimating body fat, including body condition score (BCS), morphometric measurements, ultrasound assessment of fat depots, measurement of total body water (TBW), and carcass analysis. Table 2-1 shows body fat of horses as measured by a variety of techniques. Body fat was quite variable among studies, ranging from 1 to 24%. Body fat seemed to be influenced by apparent body condition of the animals, with thin or lean animals having less body fat than animals described as fleshy. Differences in breeds used among the studies also suggests that body fat is influenced by breed as well. Additionally, the method used to calculate body fat could also impact the differences seen among studies, as Ferjak et al. (2017) reported that body fat measured by deuterium oxide dilution was consistently lower than body fat measured by ultrasound. Thus, method of estimating body fat is important to consider. Each method has advantages and disadvantages, and will be discussed below.

Body condition scoring system

The BCS system was first introduced in the 1980's and was adapted from a similar scoring system used in cattle (Henneke et al., 1983). This system assigns a score

(1 to 9) to animals based on palpable fat deposits on the neck, shoulder, ribs, loin, and tailhead. A score of 1 is considered poor where the animal is emaciated, while a score of 9 is extremely fat. Typically, a range of 4 to 6 is desired, depending on the horse's purpose. This system requires minimal training to use and no equipment or supplies are needed. Consequently, it is widely used in the equine community, and allows for accurate and consistent descriptions across research and management situations when describing the body condition of horses. However, the BCS system is only qualitative and does not provide quantitative data regarding fat content of the animals. It is also relatively subjective and even experienced observers may score the same animal differently (Mottet et al., 2009). Additionally, small changes in body fatness are not detected by the BCS system as well as they would be using more objective techniques (Mottet et al., 2009).

Morphometric measurements

Morphometric measurements may provide a more objective assessment of body fat, although results differ among studies. Morphometric measurements include circumference of the neck at various locations, heart girth and belly girth circumference, and height. One study reported that neck and heart girth circumference were not related to measurement of body fat by deuterium dilution or ultrasonic techniques (Ferjak et al., 2017), suggesting that morphometric measurements may not be useful in predicting actual body fat percentages. Conversely, another study reported that heart girth circumference, normalized for height, was strongly correlated ($r^2 = 0.91$) with total chemically-extracted lipid of the body (Dugdale et al., 2011a).

Frank et al. (2006) reported that neck circumference was greater in horses with BCS equal to or above 7 compared to horses with BCS between 4 and 6, while heart girth circumference was not different between the groups. Another study found that individual morphometric measurements were not useful, but heart girth:height ratio had the strongest correlation to BCS in horses (r^2=0.64) and in ponies (r^2=0.83) (Carter et al., 2009).

Morphometric measures may be useful for tracking changes in body fat. Fat horses (BCS 7) subjected to regular exercise exhibited no changes in heart girth or neck circumferences, but did show a decrease in abdominal circumference compared to control horses (Carter et al., 2010). Horses gaining weight and increasing BCS exhibited a concurrent increase in heart girth, belly, and neck circumferences, while horses not changing BCS showed no changes in morphometric measurements (Dugdale et al., 2011c). Fat ponies (BCS 7.8) losing weight exhibited a decrease in heart girth and belly girth circumference in a linear or curvilinear manner, respectively (Argo et al., 2012).

While morphometric measurements are inexpensive and easy to obtain, lack of consistent results among studies suggest that other factors, such as how fat or thin the animal is, breed, or person performing the measurements, may affect the usefulness of this tool.

Ultrasonic measurement of subcutaneous fat depots

Westervelt et al. (1976) reported that ultrasonic measurements of subcutaneous fat depth at the rump (defined as 5 cm lateral from the midline at the center of the pelvic bone) could predict actual body fat percentage (as determined by chemical composition

of the ground carcasses). Actual rump fat thickness, as measured after slaughter, was relatively similar to values obtained using ultrasound (1.30 cm using ultrasound compared to 1.53 cm actual depth in *ad libitum* fed ponies; 0.44 cm using ultrasound compared to 0.46 cm actual depth in limit fed ponies). Ultrasonic measurement of rump fat could also track changes in body fat relatively well as horses gained BW. However, another study demonstrated that the equation to predict body fat differed depending on the location along the rump that was measured (Kane et al., 1987), indicating that consistency in anatomical location is important for an accurate prediction of body fat.

Other sites of subcutaneous fat deposition have been evaluated using ultrasound, including the neck, withers, shoulder, ribs, and tailhead (Westervelt et al., 1976; Gentry et al., 2004; Quaresma et al., 2013; Martin-Gimenez et al., 2016). These studies have found fat depth is greatest at the rump and tailhead compared to other sites. Furthermore, fat thickness increases from the croup proceeding down towards the tailhead, with actual and ultrasonic measurements being more similar in sites closer to the tailhead, where fat was thickest (Kane et al., 1987). Thus, some researchers have chosen to use the area in which fat is thickest along the rump for estimating body fat (Kearns et al., 2006). The tailhead, or area in which fat depth is greatest, may very well be more useful than measurements taken in other locations. In mares undergoing drastic changes in BCS (7 to 3), fat thickness at the tailhead changed more than thickness at the crown of the rump (Gentry et al., 2004). Additionally, fat thickness at the tailhead better predicted BCS than fat thickness at the rump in Andalusians (Martin-Gimenez et al., 2016). Tailhead fat thickness had a better relationship with body fat percentage as calculated TBW than rump fat thickness in ponies (Dugdale et al., 2011c).

The use of ultrasound to estimate body fat through measurements of subcutaneous fat depth is a relatively quick procedure and is non-invasive. While the average horse owner does not own an ultrasound machine, the portability of the machine means that many veterinarians would have one and could monitor changes in subcutaneous fat depth at the tailhead for horses undergoing weight loss treatments. However, in order to be useful as a tool to assess and monitor body fat, the ultrasound technique requires a trained technician, consistency in anatomical locations used, and accurate analysis of the ultrasound image (Quaresma et al., 2013).

Use of deuterium oxide to calculate body fat

Calculation of body fat by measuring total body water (TBW) has become more common in equine research in recent years (Carter et al., 2010; Dugdale et al., 2011b; Dugdale et al., 2011c; Dugdale et al., 2012). Total body water is measured by dilution of deuterium oxide, tritiated water, alcohol, or urea in the body, however deuterium oxide has been used most frequently in the literature. Fat-free mass is calculated assuming that fat is anhydrous and lean tissue is 73.2% water. Fat mass can then be calculated by subtracting fat-free mass from total body mass. This procedure has the potential to provide a more quantitative measure of total body fat than any of the previous methods, however, some issues have been raised as to the accuracy of this method. Only one study has validated the use of deuterium oxide for calculating body fat in horses by comparing it to body fat measured by chemical analysis of the carcass (Dugdale et al., 2011b). This study reported a high correlation between deuterium-derived estimates of body fat and fat measured by chemical analysis of the carcass ($R^2 = 0.99$). In the studies from the same lab that validated the technique, deuterium-derived calculations of body fat have been

reported to be correlated with BCS (Dugdale et al., 2012), although the strength of the relationship decreases above a BCS of 6 (Dugdale et al., 2011c; Argo et al., 2012).

Measuring TBW is still only an indirect way to estimate fat content and relies on a number of assumptions. First, the hydration status of the horse can impact TBW content (Kearns et al., 2002a), thus a similar hydration status of all horses is assumed. Second, differences in gut water content can influence measurements of TBW. Horses with more gut fill and gut water (e.g. horses on a high-forage diet) will have greater TBW content, and thus body fat can be underestimated. In the study that validated deuterium oxide for use in horses, digesta water varied widely among horses (6.7 to 17.8% of body weight) (Dugdale et al., 2011b). One thin pony had a negative value for body fat measured by TBW and this pony also had a relatively large amount of water in the digesta, which was suggested to be the source of error in calculating fat based on TBW. Third, the equation to calculate fat from TBW assumes that fat is anhydrous and lean tissue contains 73% water based on work in guinea pigs (Pace and Rathbun, 1945). In reality, the water content of fat-free body varies among species (Reid et al., 1968), ranging from 73.8% in goats to 77% in sheep (Sheng and Huggins, 1979). These values are greater than the assumed 73% lean hydration factor, and underestimation of this value could result in inaccurate estimates of body fat. Fourth, this procedure assumes that the tracer is being equilibrated throughout the body and no tracer is lost outside the body. Deuterium losses in feces and urine have been suggested to influence estimates of TBW (Houseman et al., 1973). Respiratory water may also contain some deuterium, and has been considered to influence estimation of TBW (Burkholder and Thatcher, 1998). Additionally, the calculation for estimating body fat using deuterium contains a correction factor of 4% to

account for isotopic exchange with readily exchangeable non-water hydrogens, such as those associated with proteins or carbohydrates (Dugdale et al., 2011b). However, this correction factor comes from work in humans, which may or may not be accurate for use in horses as body composition may differ (Racette et al., 1994).

Chemical analysis of carcass

Finally, chemical analysis of the carcass provides the most accurate and quantitative measurement of total body fat. Most of the knowledge regarding actual body composition of the horse has been derived using carcass analysis (Robb et al., 1972; Westervelt et al., 1976; Webb and Weaver, 1979; Gunn, 1987; Kane et al., 1987). Additionally, techniques described above, such as ultrasound and TBW, have been validated using carcass analysis as the gold standard (Westervelt et al., 1976; Dugdale et al., 2011b). However, there are several limitations in applying this method. First, longitudinal studies on the same animals cannot be performed. Second, horses are large and the methods needed to grind a carcass to obtain a whole body sample for chemical analysis are time consuming and intensive. If dissection methods to separate body components such as muscle, fat, and bone are used, considerable expertise is necessary. In all cases, there is potential for the carcass to lose moisture prior to the chemical analysis. Third, slaughter of horses is objectionable to some individuals as horses are viewed as companion animals compared with other animals that are viewed as food.

Achieving weight change in horses

There are two ways to achieve weight change in horses. One method is to change the amount of DE the horse is consuming, either by changing feed intake or changing diet

composition. The other method is to change the energy expenditure. For the purpose of this review, the following section will focus on changing DE intake.

Feeding DE below a horse's need will result in weight loss, while feeding DE above a horse's need will result in weight gain. To simplify the following section, the discussion will focus on describing weight gain, however the opposite effects are expected to be seen during weight loss. The NRC (2007) suggests that a total of 16 to 24 Mcal of DE above maintenance are required to achieve a weight gain of 1 kg in mature horses. The amount of DE required to achieve a kg of weight gain depends on dietary composition as well as composition of the gain and the individual animal's voluntary activity, as demonstrated by the range in DE values required for 1 kg of weight gain. For example, the net energy derived from 5 Mcal of DE from vegetable oil will be much higher than the net energy from 5 Mcal of DE provided by grass hay. Consequently, horses fed a high fat diet may require less DE to achieve weight gain than horses fed a high-forage diet (NRC, 2007). For a mature horse to change one unit of BCS (within the middle of the BCS range), it has been suggested that 16 to 20 kg of weight needs to be gained (Lawrence, 2000). This estimate is based on a study where mature horses gained 33 to 45 kg and increased two condition scores (4 to 6) (Heusner, 1993). Therefore, using the assumptions that 1 kg of gain requires 20 Mcal of DE above maintenance and 16 to 20 kg results in a change of one condition score, 5.3 to 6.7 Mcal DE over maintenance (32 to 41% over maintenance) needs to be fed each day in order for the change to be achieved in 60 d (NRC, 2007).

The composition of gain is also important. The caloric density of fat is higher than the caloric density of lean tissue. Thus, if weight gain is predominantly lean, it may cost fewer calories than if weight gain is primarily fat.

Phosphorus content of the body

As P is contained in every cell of the body, P would be contained in the weight gained or lost. However, the amount of P in fat differs from the amount of P in bone or muscle. Most of the P in the body is found in the bones (~80%) and the rest is distributed among the muscle (~16%), adipose, organs (2.5%), blood (0.2%), and skin (0.2%) (Grace et al., 1999). Mature horses that are gaining weight may be increasing their body stores of adipose tissue, but are also likely increasing their muscle mass and bone density to support the increased weight load.

There are limited data evaluating P concentration in dissected equine tissues, but there are plentiful data in animals raised for meat. In Holstein-Friesan bulls, muscle P concentration was about 0.22% of fresh weight (around 0.63% P if tissue DM assumed to be 35%) (Czerwonka and Szterk, 2015). Muscle P concentration in beef cattle fed different levels of P ranged from 0.44 to 0.50% (DM basis) (Williams et al., 1991). Camel meat contained almost twice the concentration of P compared to cattle meat (P was 1.2% of DM) (Ebadi, 2015). There is a lack of research examining P muscle content in mature animals; all of the above studies were conducted in young animals (< 2 yr old) and these animals are probably still growing. However, the concentration of P in the dissected soft tissue of growing pigs (18 to 54 kg) remained relatively constant as pigs grew (0.17% P of fresh weight) (Pettey, 2004). A constant P concentration in growing

tissue indicates P concentration remains relatively stable throughout growth when fed a P-adequate diet and will likely stay constant in mature animals that are gaining or losing weight.

However, there is some evidence that muscle P concentration can change in certain circumstances. In dogs fed a diet deficient in P, muscle P concentration decreased from 28 to 22 mmol P/100 g (Fuller et al., 1976). The decrease in muscle P concentration was reversed upon P repletion. Additionally chronic, severe alcoholism in humans is associated with an approximately 50% decrease in muscle P concentration (Knochel et al., 1975; Knochel, 1980). Similar results have been observed in dogs fed ethanol (Ferguson et al., 1984). While there is evidence that tissue P can decrease, there may also be opportunities for tissue P concentration to increase. In humans, phosphate loading (high intakes of P) has been shown to improve performance (Horswill, 1995). The proposed mechanism is that by increasing the intake of phosphate salts, muscle concentrations of phosphocreatine and ATP will go up and the buffering capacity of intracellular phosphate in the muscle will reduce muscle acidosis during exercise (Horswill, 1995). However, no studies have examined muscle P concentration in phosphate-loaded humans. One study did report that creatine loading increased the concentration of phosphocreatine in the muscle of sedentary men and the authors suggested that muscle phosphate uptake occurs in conjunction with creatine uptake (Hultman et al., 1996). Other studies have shown than insulin administration decreases serum P concentrations (Perlzweig et al., 1923), while at the same time increasing muscle P content (Harrop and Benedict, 1924).

Bone contains the most P out of all the tissues in the body. In young, growing horses (≤ 2 yr old), P concentration in the ash of limb bones was 16% (Schryver et al., 1974). In foals (150 d of age), concentrations of P in limb bones averaged 8.6% on a DM basis, while rib P concentration was 7.0% (Grace et al., 1999). In lactating dairy cattle, concentration of P in rib bone was around 10% of dry bone (Ferris et al., 2010), while in beef cattle, rib bone P concentration was 16.5 to 17.5% of bone ash (Williams et al., 1991). In growing pigs, concentration of P in whole body bone ranged from 7 to 8% of dry bone on a fat-free basis (Pettey, 2004). Differences in the basis in which bone P content was reported (ash, dry, fat-free dry) as well as the type of bone studied (limb, rib, whole body) makes it difficult to compare bone P concentration among species.

Relationship between weight change and P balance

Because P is contained in every tissue in the body, weight change can influence P retention. In growing yellowtail fish, increasing lipid content of the diet while maintaining P content resulted in an increase in feed-conversion-ratio, a numerically greater weight gain, and reduced P excretion (Satoh et al., 2004). In growing rainbow trout, diets differing in protein content and quality but containing similar P, resulted in different weight gains and fish with the greatest weight gain also had the lowest soluble-P excretion (Cheng et al., 2003). In preterm human infants, P retention was significantly correlated with daily weight gain (Trotter and Pohlandt, 2002). Many of these studies are in growing animals and the composition of the gain is likely different compared to mature animals gaining weight. However, these studies do demonstrate that P does accumulate in the body during periods of weight gain, which may be similar in mature animals.

A 500-kg horse gaining 0.5 kg/d would be retaining about 1 g P/d if the assumption is that the 0.5 kg of gain is 100% muscle and that muscle P concentration is 0.2%. The P concentration of adipose tissue in horses is not well studied, but may be less than 0.2%, as adipose tissue is primarily composed of triglycerides (> 80%) with less than 1% of the tissue composed of phospholipids (Chartrin et al., 2006). In an adult human, P concentration in adipose tissue was measured at 0.048% (Mitchell et al., 1945). If the 0.5 kg of weight gained is assumed to be all adipose tissue with a P concentration of 0.048%, then horses will be expected to retain 0.25 g P/d. Additionally, increased bone density due to greater weight placed on the skeleton is a possibility (Holbrook and Barrett-Connor, 1993), further increasing P retention in the skeleton. If a horse increased bone weight by 0.5 kg over time, then an additional 43 g of P would be stored in bone mineral (assuming bone P concentration to be 8.6% based on Grace et al., 1999). The growing horse deposits 8 g P/kg BW gain (Schryver et al., 1974), which suggests that P accretion in gained tissue of the growing animal is a mix of bone and soft tissue in the growing animal. This is also likely the case for mature animals gaining weight, although the ratio of bone to soft tissue in the gained tissue is likely lower than that seen in growing animals.

A retention of at least 1 g P/d has been observed in many studies using mature sedentary horses (Kapusniak et al., 1988; Cymbaluk et al., 1989; Cymbaluk, 1990; Patterson et al., 2002; van Doorn et al., 2004a; Lavin et al., 2009). The location of the retained P has not been elucidated to date, however it is possible that these horses were gaining weight and the retained P accumulated in the gained tissue. Realistically, composition of gain may not be uniform and increases in muscle, adipose, and bone mass

during weight gain could contribute to P retention. Thus, P retention may be different for a horse gaining mostly adipose or mostly muscle or mostly bone. Altogether, weight gain (or loss) has the potential to influence P retention in horses.

Summary and conclusions

Based on the literature, factors that may influence endogenous P losses and estimates of true P digestibility include P intake, P required by the body due to physiological state, as well as dietary Ca. Probably the most important relationship that influences P recycled into the GI tract is the relationship between dietary P and P required by the body. The variation in estimates of true P digestibility in horses likely arises from experimental conditions rather than the form of P in the diet. Thus, if recycled P into the GI tract can be minimized, actual true P digestibility of feeds can be measured.

When the estimates of true P digestibility in the Nutrient Requirements of Horses were first reduced to less than 50% in the 1970's and 1980's, the main focus was ensuring that horses received adequate P for physiological needs. Little attention was given to the potential effect of underestimating true digestibility on the environment. Today, the detrimental effects of P on water quality are better understood. In addition, world P reserves are diminishing and P is not a renewable resource. Thus, good environmental stewardship includes reducing inorganic P use in animal agriculture.

After reviewing the literature, it appears that the current true digestibility estimates for P in horse feeds may be low. However, obtaining a better estimate is problematic because of P recycling. Therefore, the goal of this dissertation is to better understand factors that may influence P recycling and fecal P excretion by horses.

Tables and Figures

Table 2-1. Review of average percent body fat in the horse (Adapted from Kearns et al. (2002a) and updated to include recent publications)

Author (year)	*n*	Breed	Body condition	Method	% Fat, average
Julian et al. (1956)	6 4	Hot blood[a] Percheron	N/A	Calculated from body water	12.8 24.5
Robb et al. (1972)	11	Pony	N/A	C.C.	6.6 – 18.9
Westervelt et al. (1976)	8 12	N/A	N/A	C.C./ultrasound	15.9 10.7
Webb and Weaver (1979)	17	Thoroughbred Pony	Emaciated-thin; Poor-lean	Dissection	5.1
Elser et al. (1983)	10	Pony	N/A	Urea dilution	8.1[b]
Lawrence et al. (1986)	10	N/A	N/A	Urea dilution	15.4
Gunn (1987)	9 5	Thoroughbred Other[c]	Lean	Dissection	1.1 2.1
Kane et al. (1987)	6	N/A	N/A	C.C./ultrasound	13.0
Webb et al. (1989)	6	N/A	Fleshy-moderate	Ultrasound	10.1 7.4
Lawrence et al. (1992)	38	Arab & Arab-cross	Moderate	Ultrasound	7.8
Kearns et al. (2001)	23	Standardbred	Moderately fleshy	Ultrasound	22.3
Kearns et al. (2002b)	19	Standardbred	Lean	Ultrasound	Male: 6.9 Female:10.3
(Znamirowska, 2005)	107	N/A	N/A	Dissection	7.85
Carter et al. (2010)	12	Arab & Arab-cross	BCS 7	D_2O	19.4
Dugdale et al. (2011b)	7	Welsh pony	BCS 1.25-9	C.C. D_2O	12.6 12.1
Dugdale et al. (2012)	77	Varied[d]	BCS 3-9	D_2O	19.6
Brinkmann et al. (2013)	10	Shetland	BCS 3-4	D_2O	16.1[b]
De Palo et al. (2013)	18	Italian heavy draught	N/A	Dissection	16.3

Table 2-1 (continued)

Ferjak et al. (2017)	18	Stock-type	BCS 4-6	D_2O Ultrasound	6.62 10.0

[a]Hot-blooded horses included Thoroughbred, Quarter Horse, Arabian, and American Saddlebred
[b]Values calculated from author's data
[c]Other breeds included Welsh Mountain Pony, Shetland, Clydesdale, and Thoroughbred-cross
[d]Breeds included: 5 Shetland, 1 Dartmoor, 45 Welsh Pony, 5 mixed ponies, 17 cob, 2 Warmbloods, 2 Thoroughbred-cross
C.C.: chemical composition of carcass
D_2O: calculated from body water using deuterium oxide

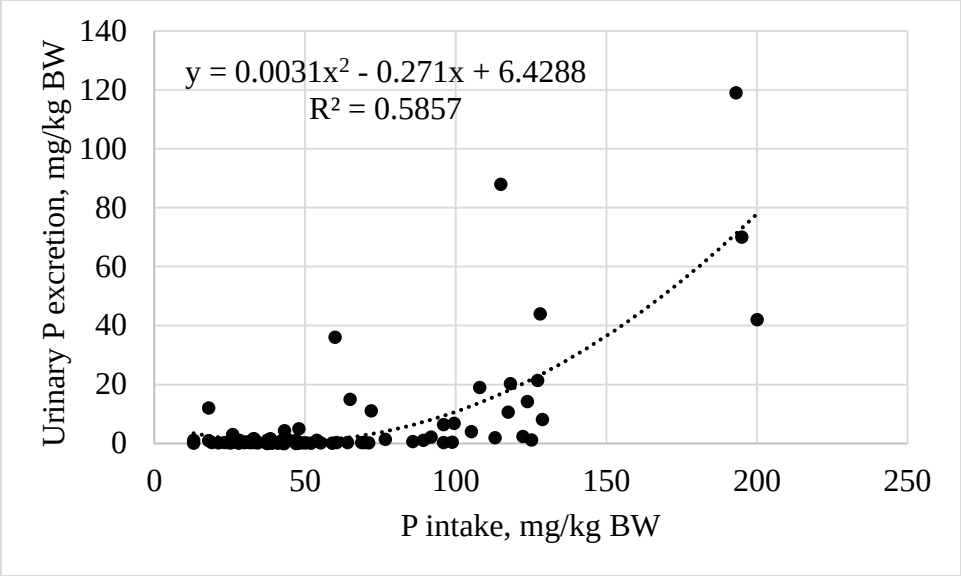


Figure 2-1. Relationship between P intake and urinary P excretion from 16 studies using growing and mature horses. Data from: Schryver et al. (1971); Hintz and Schryver (1972); Kichura et al. (1983); Hoyt et al. (1995); Nielsen et al (1998a, 1998b); Buchholz-Bryant et al. (2001); Morris-Stoker et al. (2001); Patterson et al. (2002); van Doorn et al. (2004a, 2004b); Oliveira et al. (2008); Weir (2012); Lavin et al. (2013); Ögren et al. (2013); van Doorn et al. (2014).

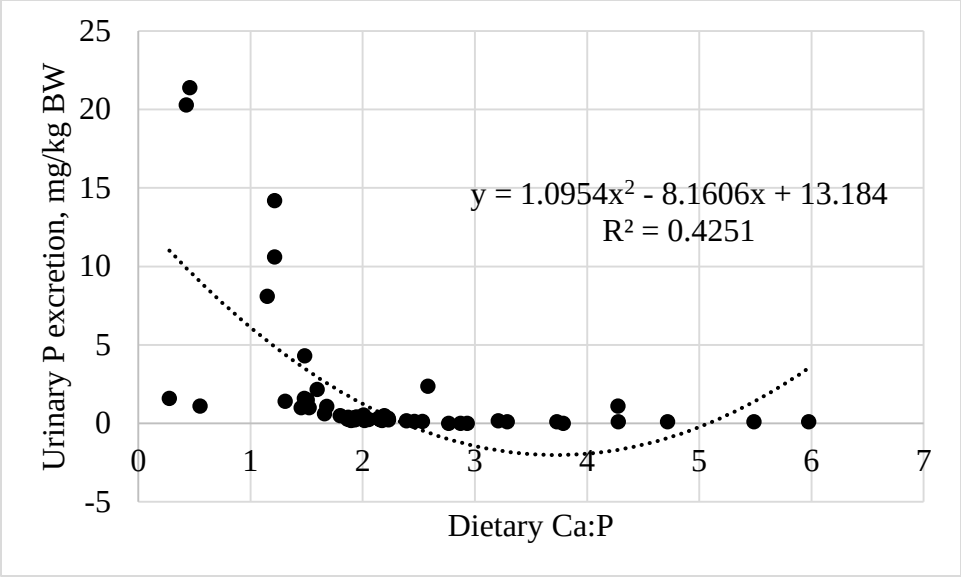


Figure 2-2. Urinary P excretion as a function of dietary Ca:P ratio from 12 studies using growing and mature horses. Data from: Schryver et al. (1971); Hintz and Schryver (1972); Kichura et al. (1983); Hoyt et al. (1995); Nielsen et al (1998a, 1998b); Buchholz-Bryant et al. (2001); Morris-Stoker et al. (2001); Patterson et al. (2002); van Doorn et al. (2004a, 2004b); Oliveira et al. (2008); Weir (2012); Lavin et al. (2013); Ögren et al. (2013); van Doorn et al. (2014).

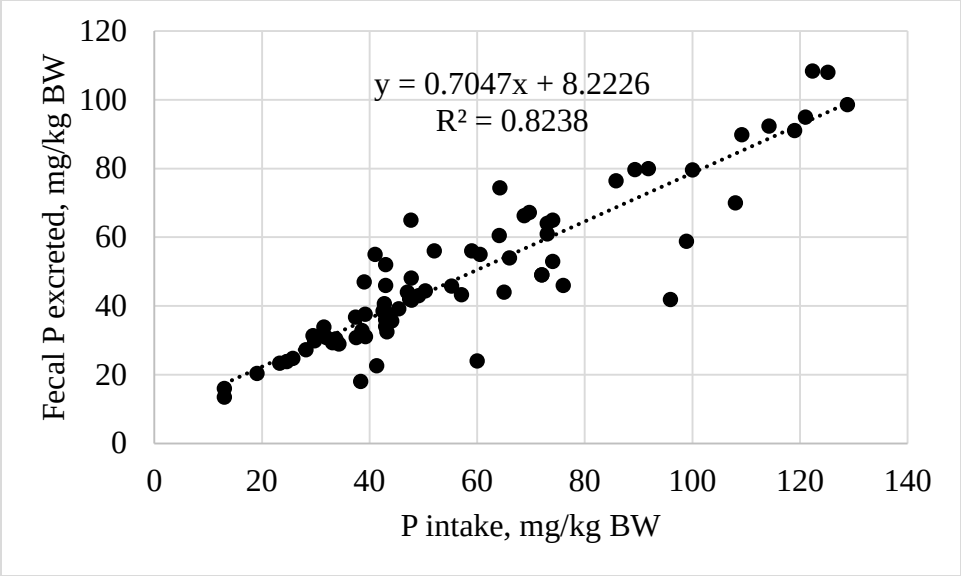


Figure 2-3. Intake and fecal excretion of P from 18 studies using mature horses (age > 2 yr). Data from: Schryver et al. (1971); Hintz and Schryver (1972); Hintz et al. (1973); Kichura et al. (1983); Schryver et al. (1987); Kapsuniak et al. (1988); Cymbaluk et al. (1989); Buchholz-Bryant et al. (2001); Morris-Stoker et al. (2001); Patterson et al. (2002); van Doorn et al. (2004a, 2004b); Lavin et al.. (2009); van Doorn et al. (2011); Weir (2012); Lavin et al. (2013); Fowler et al. (2015); Skurupey et al. (2015).

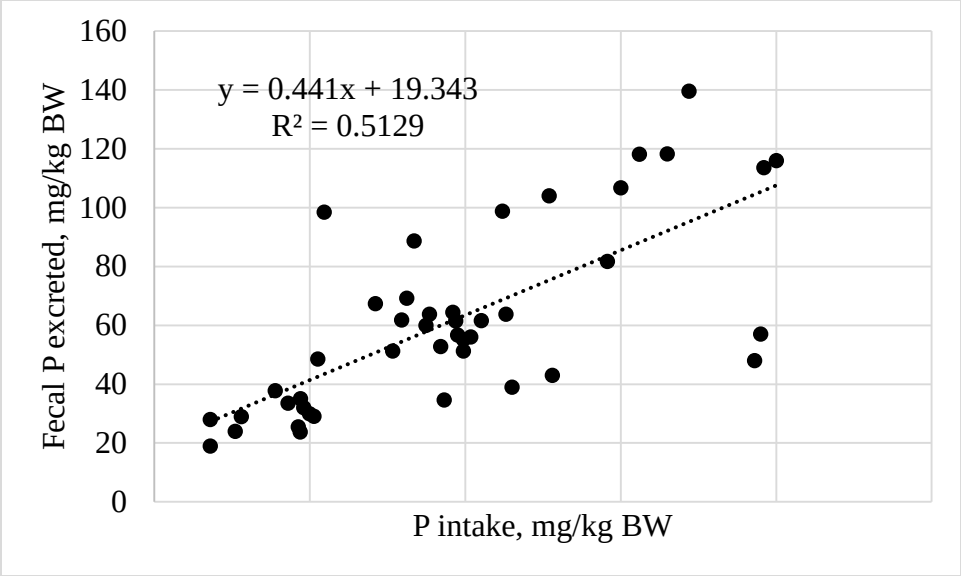


Figure 2-4. Intake and fecal excretion of P from 8 studies using growing horses (age ≤ 2 yr). Data from: Schryver et al. (1971); Kichura et al. (1983); Cymbaluk et al. (1989); Cymbaluk (1990); Hainze et al. (2004); Oliveira et al. (2008); Ögren et al. (2013); Fowler et al. (2015).

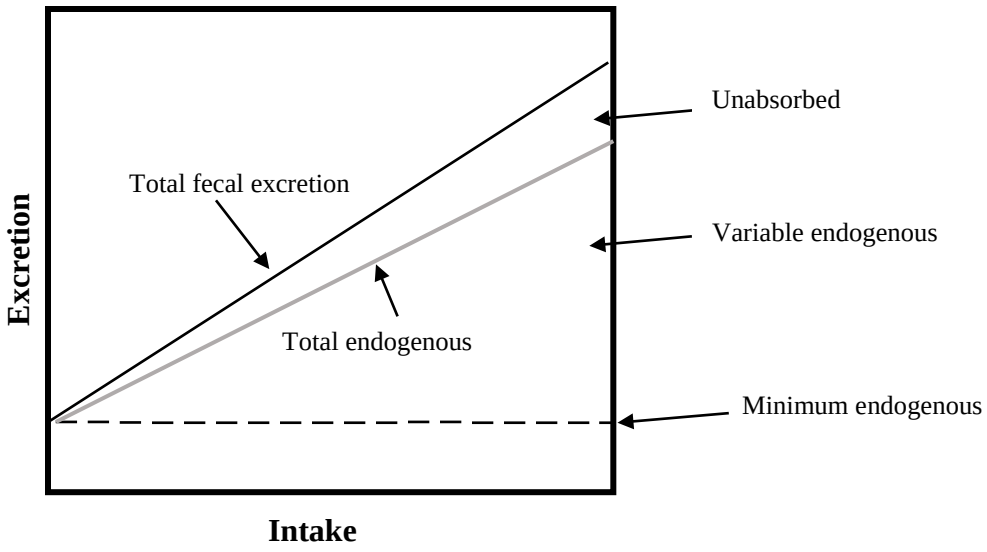


Figure 2-5. Total fecal P excretion and its components. As intake increases, so does excretion, but the origin of the excreted P changes (minimum endogenous, variable endogenous and unabsorbed). Adapted from Ammerman (1995).

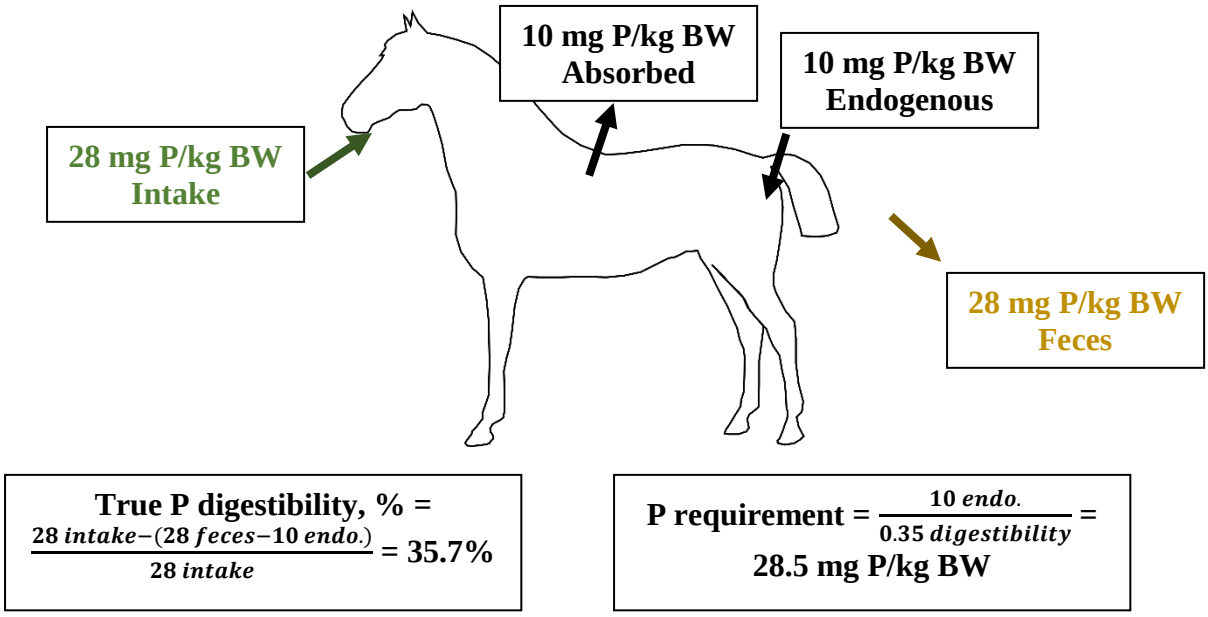
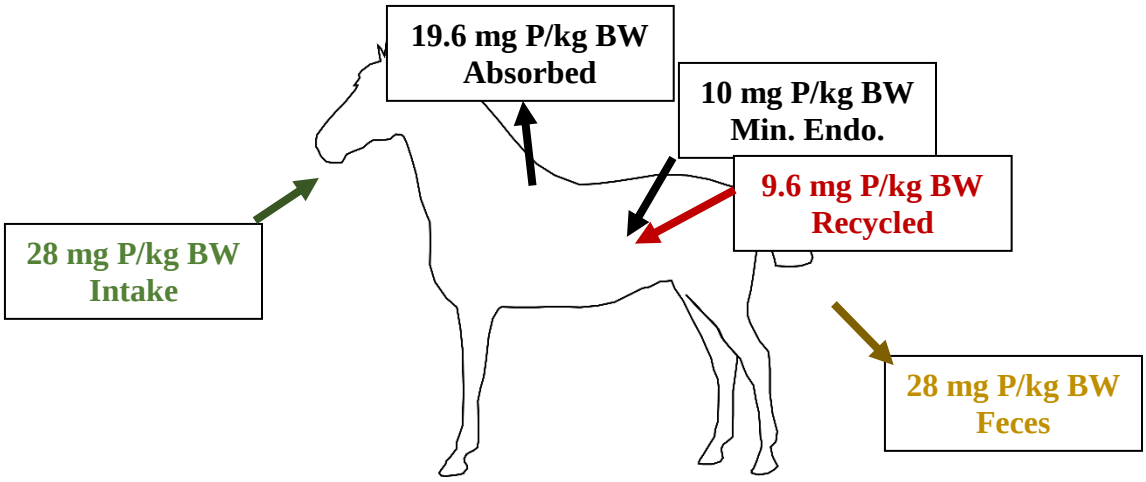


Figure 2-6. Demonstration of the current accepted model for estimation of true P digestibility and P requirement based on a fixed value of endogenous P losses of 10 mg/kg BW in a horse at maintenance and true P digestibility of 35% (NRC, 2007).



$$\textbf{P requirement} = \frac{10\ endo.}{0.70\ digestibility} = \textbf{14 mg P/kg BW}$$

Figure 2-7. Demonstration of how recycled P may influence measurement of true P digestibility and P requirement in a horse at maintenance, assuming 10 mg P/kg BW is the minimum endogenous loss but dietary P digestibility is actually 70%. Measured true P digestibility is still 35% due to recycled P. However, P requirement calculated using a true digestibility of 70% is 14 mg/kg BW. Feeding at this amount would eliminate recycled P.

CHAPTER 3: PHOSPHORUS DIGESTIBILITY IN POST-LACTATING MARES

Introduction

Dietary phosphorus requirements for horses are calculated using estimates of true digestibility (NRC, 2007). However, in the equine literature true P digestibility values have been reported to range from -10 to 79% (Kichura et al., 1983; Lavin et al., 2013). The NRC (2007) uses estimates of true P digestibility of 35 and 45% depending on the form of P in the diet. However, if true P digestibility is higher than these estimates, then horses are receiving more P than necessary. The excretion of P by animals can affect water quality. One way to reduce P excretion is to meet but not exceed P requirements. By better understanding the factors that have caused such large variation in reported P digestibility among studies, more accurate P requirements can be calculated and diets can be more precisely formulated to avoid excess P intake and excretion.

In the horse, P excreted in the feces consists of undigested P as well as endogenous P and recycled P. Undigested P travels through the GI tract without being digested and absorbed. In many other species, the form of P influences digestibility, with phytate-P being relatively unavailable (Adedokun and Adeola, 2013). However in horses, phytate-P is 95% degraded by the time it is excreted in the feces, suggesting that phytate-P is relatively available to the horse (Lavin et al., 2013; Fowler et al., 2015).

In addition to undigested P, feces also contain P that is recycled into the GI tract. If P is absorbed, but not retained in the body, it is recycled into the small intestine and cecum to be excreted in the feces. In mature horses that are retaining minimal amounts of P, essentially all absorbed P will be excreted in the feces. As a result, when P digestibility

is calculated, very low estimates are likely to result, which may not accurately represent the actual amount of digested and absorbed P by the animal. Horses secrete different amounts of P into their digestive tract depending on the diet they are fed (Schryver et al., 1971). These researchers also reported that P excretion in urine is very low under most circumstances. We hypothesized that when fed similar amounts of P, horses that are retaining more P in the body will secrete (recycle) less P in their GI tract.

In sheep, a depletion model was used to evaluate endogenous P secretions. Researchers observed an increase in P retention and a reduction in the amount of endogenous P secreted into the digestive tract in P-depleted animals (Young et al., 1966). Similarly, we hypothesized that feeding mares a low P diet at the end of lactation would deplete body P and reduce P recycling.

The objective of the study was to develop a model in horses that minimizes P recycling using post-lactating mares fed a low P diet prior to weaning and an adequate P diet post-weaning compared to control (non-lactating) mares. The hypothesis was that when fed similar amounts of P, depleted mares would recycle less P, have reduced fecal P excretion and thus have increased estimates of P digestibility.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Animals

Four mares in the fifth month of their lactation (12 ± 5.7 yr; 576 ± 26 kg) and four non-lactating mares (11 ± 4 yr; 570 ± 35 kg) were used. Prior to the study, horses were

kept in pastures containing cool-season grasses and were fed commercially available concentrate to meet their requirements (lactating mares fed 2.5 kg of concentrate and control mares fed 1 kg of concentrate). The experiment was conducted in two blocks with two lactating mares and two control mares in each block. Each block consisted of a 22-d adaptation followed by a 4-d total fecal collection.

Diets and Feeding Management

All mares were fed timothy cubes, a pelleted concentrate and soybean oil (Table 3-1). The amounts of the feeds were adjusted during the experiment to alter P intake as described below. On d 1 of adaptation, mares were moved to small paddocks with minimal available forage and the transition to the experimental diet was initiated twice a d when mares were fed in individual stalls. On d 3 of adaptation, mares were muzzled during the day to encourage complete consumption of their meals. By d 8 of adaptation, all horses were receiving the same diet, which met the requirements for all nutrients for mares in the fifth month of lactation (NRC, 2007). Foals were fed their meal in a feed bag while the mares ate so that foals would not consume the experimental diets. On d 9 of adaptation, mares and foals were brought into stalls overnight and turned out during the day. From d 9 to d 12 of adaptation, feed amounts were adjusted to provide 26 mg P/kg BW, which was 55% of the lactating mares' P requirement and 100% of the non-lactating mares' P requirement. The forage to concentrate ratio was 85:15. All other nutrients met or exceeded requirements (NRC, 2007). All horses received this diet from d 12 to 22 of adaptation as well as during total fecal collections. Lactating mares were weaned on d 14 of adaptation; thus they were fed P in amounts below their requirement from d 9 through d 14 in order to create a P depletion period. Once mares were weaned they continued on

the same diet which provided adequate P for mature mares at maintenance (NRC, 2007). Total fecal collections were conducted from d 23 through d 26. Two d before total fecal collections began, mares were kept in stalls for 24 h/d and hand-walked for 15 min twice daily. Also starting 2 d before fecal collections began, they were fed half of their daily ration every 12 h. Horses had *ad libitum* access to water at all times. Horses were weighed three times per wk throughout the adaptation period and were also weighed at the beginning and end of the fecal collection period.

Sample collection

Horses were fitted with fecal collection harnesses (Bun-bag, Sagle, ID) on d 1 of the collection period and monitored closely during the 4-d period to ensure that all feces were collected in the bags. To ensure that urine did not enter the fecal collection bag, a plastic shield was attached under the tail of the mare that blocked urine from entering the fecal collection bag, but allowed feces to fall into the bag.

During the fecal collection period, daily output of feces was compiled for each horse and then thoroughly mixed at the end of each 24 h period. A subsample, 10% of the total fecal weight per d, was collected and frozen for later analysis to measure and calculate digestibility of DM, P, Ca, and Mg.

Horses were given *ad libitum* access to water and consumption from the water bucket was measured daily. Water buckets were weighed at least every 12 h to record the weight of the water consumed. Water was added as needed throughout the collection period and the weight was always recorded. Water volume was estimated assuming that 1 kg water = 1 L water.

Blood was collected from the jugular vein into plain tubes and tubes containing sodium heparin on d 13 (pre-weaning), and d 22 of adaptation (post-weaning). Tubes containing sodium heparin were centrifuged within 1 h of collection at 1,500 x *g* for 20 min at 4 ^{0}C for collection of plasma. Plain tubes were centrifuged at 1,500 x *g* for 20 min at 16^{0}C after a clot had formed to obtain serum. The serum or plasma supernatant was pipetted into 1.5 ml microcentrifuge tubes and frozen at -20 ^{0}C within 2 h of collection. Long term storage of plasma and serum occurred at -80 ^{0}C.

Sample analysis

Feed and daily composite feces were analyzed for total P using a gravimetric method (Shaver, 2008), and Ca and Mg using atomic absorption spectroscopy (Bowers Jr and Rains, 1988). Samples were re-analyzed if the CV between duplicates exceeded 10%.

To determine plasma P concentrations, plasma samples were first deproteinated by adding 1 ml of serum to 5 ml of 6% trichloroacetic acid (TCA), centrifuged at 1,700 x *g* for 10 min and then the supernatant was used to determine P concentrations using a colorimetric assay (Fiske and Subbarow, 1925). Plasma Ca was determined by diluting plasma samples with a La_2O_3 and HCl solution and then using atomic absorption to determine Ca concentration (Bowers Jr and Rains, 1988). No samples were re-run as CVs between duplicates were below 6% for both P and Ca.

Serum samples were analyzed for bone alkaline phosphatase (BAP) and cross-linking C-terminal telopeptides of type I collagen (CTX-1) using immunoassays (Microvue BAP EIA Kit, San Diego, CA; Immunodiagnostic Systems Serum Crosslaps® [CTX-1] ELISA, Tyne & Wear, United Kingdom). All samples were analyzed in

duplicate. Values for BAP and CTX-1 were within the standard curve limits and all CV's between duplicates were less than 15%. No samples were re-run.

Calculations & statistical analysis

Apparent absorption of Ca, P, and Mg was calculated as intake – fecal excretion. Apparent digestibility was calculated as [(nutrient intake – fecal nutrient excretion)/nutrient intake] × 100%. True digestibility of P, Ca and Mg was calculated as [(nutrient intake – [fecal nutrient excretion – fecal nutrient endogenous loss])/nutrient intake] × 100%. Estimates of fecal endogenous losses were obtained from NRC (2007). Because of the logistical difficulty in collecting urine from mares, urine was not collected. Urinary P is very low (< 2 mg P/kg BW) when P intake is less than 100 mg/kg BW (Figure 2-1; Schryver et al., 1971). Nutrient intake and excretion are expressed on an mg/kg BW basis. The BW from d 11 of adaptation was used to make these calculations, to formulate diets, and calculate requirements.

The effect of physiological status on nutrient balance data was determined using ANOVA with repeated measures (SAS 9.4; SAS Institute, Inc, Cary, NC). Each block of four horses (two control and two lactating) that were collected at the same time was treated as a block. Physiological status (control or lactating) was the main effect, block was the random effect, and day of fecal collection was the repeated measure. Day was not a significant variable in any of the models. Pre-weaning and post-weaning blood values were analyzed using ANOVA with repeated measures with physiological status as the main effect and block as the random effect. When main effects or interactions were significant, means were separated using an LSD test. Data are presented as least squares

means. Results were considered significant when $P < 0.05$ and were considered a trend when $0.05 < P < 0.10$.

Results and Discussion

There were no feed refusals during the fecal collection periods. Total fecal output for the first day of fecal collections was lost for 1 control mare, but the fecal output from the remaining days of fecal collection for this mare were collected and the data was included in the analysis.

Phosphorus intake was 23.36 mg P/kg BW for both groups and was not different between groups ($P > 0.10$; Table 3-2). Despite similar P intakes, fecal P excretion tended to be greater in post-lactational mares compared to control ($P = 0.0821$), which was contrary to the hypothesis. Apparent amount of P absorbed as well as apparent P digestibility was negative for both groups but tended to be more negative for post-lactational mares ($P = 0.0863$). True P digestibility, which accounts for endogenous P losses as estimated by NRC (2007), was positive for both groups and tended to be greater in the control mares (35.12%; $P = 0.0849$) that in post-lactating mares (22.45%). These data indicate that P metabolism within post-lactational mares was different than that within control mares.

There are two possible explanations for the increased fecal P in post-lactational mares. First, these mares may have had decreased P absorption compared to control mares. However, other measures of digestive capacity were not affected by treatment, as DMD, Ca and Mg digestibility were not different between groups (Table 3-2 and 3-3). Additionally, there is no reason to believe that the two main factors that influence P

absorption at the transporter level (dietary P intake and vitamin D status) were different among treatments. Phosphorus is actively transported from the large intestine, the main site of P absorption, into the blood via a Na^+-dependent mechanism (Bai et al., 2000; Cehak et al., 2012; Muscher-Banse et al., 2017), with small amounts of P being passively absorbed in the small intestine. Increases in Na^+-dependent P transporter activity and subsequent increases in P absorption are observed when low P diets are fed and when 1,25-dihydroxyvitamin D_3 is administered to vitamin D-deficient animals (Katai et al., 1999). Both control and post-lactating horses were fed the same diet and housed in similar conditions with the same access to sunlight, so these two factors should have been the same for both groups of mares and thus should not have contributed to the differences in P excretion observed in the current study. Overall, it seems unlikely that the increase in fecal P excretion was due to a decrease in P absorption in post-lactational mares.

Alternatively, it is possible that the higher fecal P in post-lactating mares was not dietary in origin, but rather part of variable endogenous losses, or P secreted into the GI tract. Regulated secretion of P in the GI tract would cause elevated fecal P. According to Schryver et al. (1972), P is both absorbed and secreted into the equine GI tract. Factors that influence the amount of P secreted into the GI tract are not well studied. However, in pigs, regulated secretion of P in the gut occurs once requirements for P retention in the body are met (Rodehutscord et al., 1999). Thus, if an animal has a low requirement for retention, more of the absorbed P would be secreted into the gut. Because the lactating mares were fed a diet below their P requirement during late lactation and were expected to be depleted, our hypothesis was that requirements for retention would be greater in post-lactational mares, resulting in less regulated P secretion into the gut. However,

because post-lactational mares had greater fecal P than control mares, our model did not increase retention and decrease recycling in post-lactational mares.

Bone represents the largest store of Ca and P in the body (NRC, 2007) and could be a source of P secreted into the gut. Parathyroid hormone acts to regulate Ca and P homeostasis in the body. Parathyroid hormone is secreted in response to low serum Ca and acts to increase serum Ca via a few mechanisms, one of which is bone resorption. However, as bone mineral is released in order to increase blood Ca, P is also released from bone, increasing blood P levels. In most species, PTH acts in the kidney to increase P excretion, as most species rely on renal P excretion to maintain homeostasis (Agus et al., 1973). However in the horse, urinary P concentrations are relatively low and the majority of P is excreted via the feces (Schryver et al., 1971). According to the equation shown in Figure 2-1, for horses consuming 23 mg P/kg BW, urinary P excretion would be 1.78 mg P/kg BW, or about 1 g P/d for these mares (570 kg BW). Transport of P into the intestine appears to be the primary way that horses excrete unneeded P instead of maintaining homeostasis through the kidney. In fact, active transport of P into the equine intestine has been recently documented (Muscher-Banse et al., 2017), suggesting that P secretion into the intestine can be regulated. Therefore, horses that are resorbing bone to maintain blood Ca levels, may be excreting unneeded P from the bone into the intestine, rather than the urine, for excretion.

When diets were altered to decrease P intake for lactating mares, Ca was also decreased in order to keep the Ca:P ratio close to 2:1 and to ensure similar Ca intakes for the control mares. As a result, lactating mares were also consuming less Ca than required for mares in late lactation (requirement = 74.8 mg Ca/kg BW; intake in current study =

55.8 mg Ca/kg BW). This low Ca intake may have stimulated PTH release in post-lactational mares. Serum Ca in post-lactating mares tended to increase from pre- to post-weaning (Table 3-4; P = 0.0950) and CTX-1, a marker of bone resorption, also numerically increased from pre- to post-weaning (Table 3-4). Together, these data could indicate that post-lactating mares were increasing bone resorption after weaning to improve blood Ca levels due to low Ca intakes during late lactation. The excess P released from the bone may have been secreted into the intestine for excretion, causing post-lactating mares to have greater fecal P excretion compared to controls.

Another potential source of recycled P could be resorbed and recycled milk components. Total fecal collections began only 9 d after weaning and full mammary gland involution can take close to 30 d (Holst et al., 1987). During involution of the mammary gland, components of the milk, including Ca and P, are resorbed into the body, causing increases in blood concentrations of these milk components (Hurley, 1989). These milk components can either be used by the body or excreted. In support of this hypothesis, there was a trend for an interaction between physiological state and time for blood Ca levels, with post-lactational mares increasing blood Ca while control mares stayed the same (Table 3-4; P = 0.0950). Unlike Ca, there were no differences in blood P concentrations between the groups, but it is possible that the resorbed milk P was secreted into the gut for excretion. A mare in the fifth month of lactation is expected to produce 0.02 kg milk/kg BW containing 0.5 g P/kg milk (NRC, 2007). This level of milk production equates to approximately 5.5 g of P in the milk per day for a 550-kg mare. The difference in fecal P excretion between control and post-lactational mares was 1.56 g

P/d, suggesting that resorbed milk P could provide more than enough P to account for the greater fecal P excretion in post-lactational mares.

Another factor that could contribute to P secretion into the gut would be P from mobilized tissue when horses are in a state of weight loss. Because P is present in all cells, mobilization of fat, protein, and bone during weight loss could contribute to an increase in fecal P excretion. Although the horses consumed diets that contained recommended amounts of DE (NRC, 2007), they all exhibited some weight loss. Figure 3-1 shows the relationship between horses' weight change over the dietary treatment period and fecal P excretion. When all 8 horses are included, the negative relationship between weight loss and P excretion is not significant and the R^2-value is only 0.3550. However, if one horse is removed (weight change of -46.25 kg and 14.28 g P excreted), then the relationship becomes significant ($P = 0.0153$) and the R^2-value improves to 0.7229 (Figure 3-2). Therefore, weight change has the potential to influence P excretion due to differences in endogenous P losses.

The y-intercepts of Figures 3-1 and 3-2 indicate P excretion when weight is being maintained (weight change = 0 kg). According to the graphs, these mares would excrete between 12.8 and 13.6 g P/d if their weights were being maintained and they were fed the same diet. The NRC (2007) estimates that mares of this weight (570 kg) at maintenance would excrete approximately 16 g P if endogenous losses of 10 mg P/kg BW and a true digestibility of 35% are used. These mares would be excreting less P than predicted by the NRC (2007), suggesting that endogenous losses in these mares are less than previously predicted. Figure 2-1 suggests that endogenous losses of mature horses are 8.2 mg P/kg BW. If this lower estimate of endogenous losses is used instead of 10 mg P/kg

BW as suggested by the NRC (2007), these mares would be expected to excrete 13.3 g P/d, which is very similar to the expected P excretion for mares maintaining weight based on Figures 3-1 and 3-2.

In conclusion, even though the current study was unable to elucidate true P digestibility by minimizing P secretion into the gut, this study supports the hypothesis that fecal P excretion and presumably endogenous losses in horses vary depending on physiological status. The assumption that all mature horses have the same endogenous losses may be inaccurate, leading to incorrect calculation of daily P requirements. Another interesting finding from this study was that weight loss has the potential to influence P excretion due to P mobilized from tissue being secreted into the gut for excretion. It would be of interest to further explore the impact that weight loss as well as weight gain has on P excretion. Horses gaining weight would theoretically be retaining more P than horses losing weight, and weight gain might be another useful model with which to minimize P secretion into the gut. Overall, it is possible that some of the low P digestibility values in the literature have occurred because of P recycling in combination with increased endogenous losses with weight loss, while higher estimates result in studies where P retention is increased by weight gain in mature horses or growth in immature horses.

Tables and Figures

Table 3-1. Nutrient composition of the forage and concentrate components of the diet (DM basis)[1]

Nutrient[2]	Timothy cubes[3]	Pelleted concentrate[4]
DE, Mcal/kg	2.20	2.97
CP, %	11.7	14.4
NDF, %	57.6	35.4
ADF, %	38.0	23.6
Ca, %	0.58	0.66
P, %	0.24	0.32
Mg, %	0.21	0.25
K, %	1.95	1.30
Na, %	0.024	0.287
Fe, ppm	495	300
Zn, ppm	31	91
Cu, ppm	8	29
Mn, ppm	57	77
Mo, ppm	0.9	0.8

[1]Forage to concentrate ratio was 85:15. Horses in the first period were fed 0.33 kg soybean oil per day and horses in the second period were fed 0.54 kg soybean oil per day.
[2]Nutrient analysis and DE calculation performed by DairyOne Forage Lab (Ithaca, NY). These values were used to formulate diets
[3]Premium Timothy Cubes (Ontario Dehy Inc., Ontario, Canada)
[4]Custom mixed at McCauley Bros., Versailles, KY. The concentrate was formulated without any added inorganic P sources.

Table 3-2. Phosphorus, calcium, and magnesium balance data for control and post-lactating mares[1]

Item	Control mares[2]	Post-lactating mares[2]	SEM	*P*-value
Phosphorus				
Intake, mg/kg BW	23.36	23.36	0.02	0.8669
Fecal excretion, mg/kg BW	25.15	28.11	3.06	0.0821
Apparent absorbed, mg/kg BW[3]	-1.79	-4.75	3.09	0.0852
Excreted as % of intake, %	107.67	120.36	13.2	0.0863
Apparent digestibility, %	-7.68	-20.36	13.2	0.0863
True digestibility, %[4]	35.12	22.45	13.2	0.0849
Calcium				
Intake, mg/kg BW	55.79	55.79	0.065	0.9123
Fecal excretion, mg/kg BW	32.80	34.80	4.2	0.2508
Apparent absorbed, mg/kg BW[3]	23.01	20.99	4.2	0.2539
Excreted as % of intake, %	58.79	62.39	7.6	0.2525
Apparent digestibility, %	41.21	37.61	7.6	0.2525
True digestibility, %[5]	77.05	73.46	7.5	0.2515
Magnesium				
Intake, mg/kg BW	15.97	15.97	0.019	0.9123
Fecal excretion, mg/kg BW	10.39	10.45	1.18	0.8824
Apparent absorbed, mg/kg BW[3]	5.59	5.52	1.2	0.8667
Excreted as % of intake, %	65.05	65.45	7.4	0.8731
Apparent digestibility, %	34.95	34.55	7.5	0.8731
True digestibility, %[6]	72.50	72.12	7.4	0.8789

[1]Mineral concentrations in the diet were from in-house lab analysis and used to calculate mineral intakes
[2]n=4
[3]Amount apparently absorbed was calculated as nutrient intake minus fecal excretion
[4]Calculated using estimates of endogenous fecal P losses of 10 mg P/kg BW (NRC, 2007)
[5]Calculated using estimates of endogenous fecal Ca losses of 20 mg Ca/kg BW (NRC, 2007)
[6]Calculated using estimates of endogenous fecal Mg losses of 6 mg Mg/kg BW (NRC, 2007)

Table 3-3. DM intake, water intake, fecal excretion and DM digestibilities for control and post-lactating mares

Item	**Control mares**	**Post-lactational mares**	**SEM**	**P-value**
DM intake, kg/d	5.81	5.76	0.15	0.6904
DM intake, g/kg BW	10.00	10.00	0.011	0.9622
Water intake, L/d	16.41	18.28	2.35	0.7599
Water intake, ml/kg BW	28.12	31.65	3.42	0.5767
Fecal DM excretion, kg/d	2.79	2.62	0.15	0.5423
Fecal DM excretion, g/kg BW	4.86	4.56	0.36	0.7451
DM digestibility, %	51.46	54.43	3.69	0.7464

Table 3-4. Concentrations of Ca, P, Ca;P, BAP[1], CTX-1[2], and BAP:CTX-1 in control and post-lactating mares pre-weaning and post-weaning

Item	Control		Post-lactation		Pooled	P-value		
	Pre	Post	Pre	Post	SE	Treatment	Time	Interaction
Ca, mg/dl	11.35	11.08	10.81	11.18	0.23	0.4673	0.7740	0.0950
P, mg/dl	3.87	3.67	4.19	4.05	0.23	0.2181	0.4392	0.8775
Ca:P	2.96	3.03	2.66	2.76	0.19	0.1756	0.6422	0.9147
BAP, ng/ml	53.43[a]	42.05[b]	52.89	52.32	3.41	0.3054	0.0286	0.0409
CTX-1, ng/ml	0.269	0.281	0.185	0.271	0.039	0.3614	0.1326	0.2445
BAP:CTX-1	208.06	155.34	386.72	211.90	77.71	0.2347	0.1277	0.3798

[ab]Means within a treatment differ between timepoints ($P < 0.05$)

[1]BAP; bone alkaline phosphatase

[2]CTX-1; cross-linking C-terminal telopeptides of type I collagen

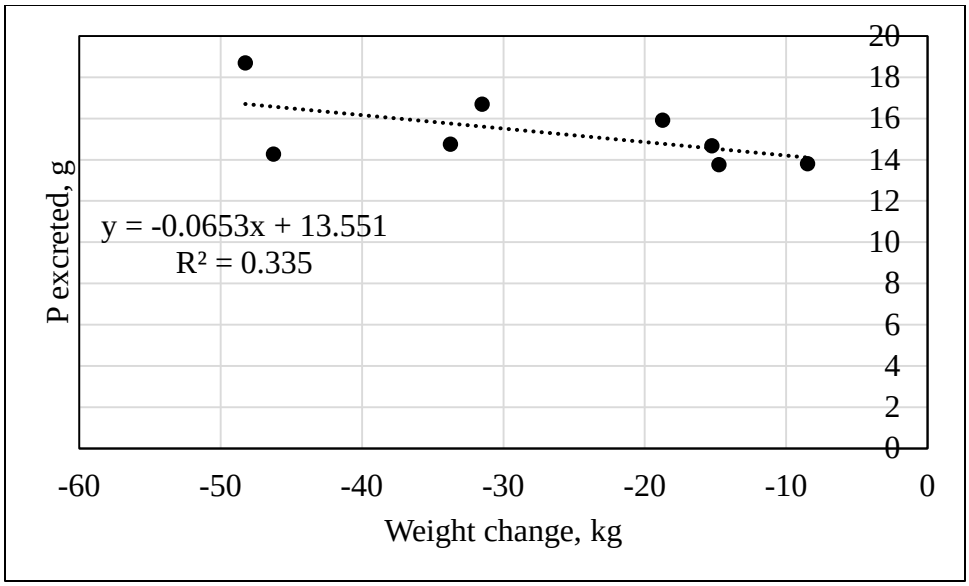


Figure 3-1. Relationship between weight change (kg) and P excretion (g). Weight change is measured as the difference between the average of 2 d of consecutive BW (d 11 and d 13) when dietary treatments were imposed and the average BW over the total fecal collection period. Excreted P is the average fecal P excreted over the 4 d of fecal collections.

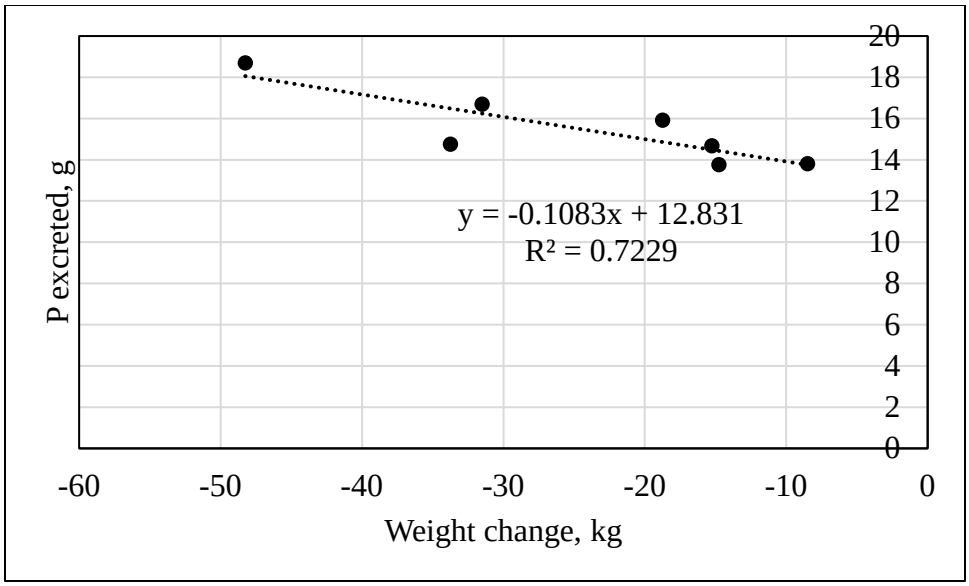


Figure 3-2. Relationship between weight change (kg) and P excretion (g) with one horse removed. Weight change is measured as the difference between the average of 2 d of consecutive BW (d 11 and d 13) when dietary treatments were imposed and the average BW over the total fecal collection period. Excreted P is the average fecal P excreted over the 4 d of fecal collections.

CHAPTER 4: CHANGE IN BODY WEIGHT INFLUENCES MINERAL DIGESTIBILITY AND MARKERS OF BONE METABOLISM

Introduction

With the increased prevalence of obesity in the equine population, weight loss diets are being prescribed more frequently for many horses. While the main goal of weight loss programs is to reduce the amount of adipose tissue, other tissues such as muscle and bone may also be impacted inadvertently. Macrominerals are widely distributed in many body tissues, and the tissue that is lost or gained during weight change contains some amount of these minerals. Thus, during weight loss or gain, mineral balance may be impacted.

Phosphorus is found in every cell of the body, with the majority of P located in bone (75 to 80%) and the remaining P located in soft tissues and blood (NRC, 2007). If an animal is gaining or losing weight, P contained in the soft tissue will be retained or excreted, respectively. Therefore, weight change may affect P balance. Many studies that have examined P digestibility have observed positive P retention in mature horses at maintenance, suggesting that these horses may have been gaining weight during the study and thus retaining P in the new tissue. Conversely, horses losing weight may be secreting P from lost tissue into their GI tract for excretion, leading to an increase in fecal excretion and low apparent and true P digestibilities. A lower observed value for P digestibility would cause an overestimation of the P requirement.

As discussed above, during weight change, adipose tissue as well as muscle and bone are impacted. Therefore, measuring changes in total BW is not informative about changes in body composition. Changes in body composition would be more helpful in

evaluating differences in mineral balance as concentrations of minerals vary among tissue types. There are many methods to evaluate adiposity in horses, with the body condition scoring system (BCS) being the most prevalent and easiest to use (Henneke et al., 1983). However, this method is subjective and variable even among trained scorers and the ability of this system to detect modest changes in adiposity over time is poor (Mottet et al., 2009). Changes in morphometric measurements, such as the circumferences of heart girth, belly girth, and mid-neck, have also been used to track changes in body fat (Dugdale et al., 2011c). However, morphometric measures do not distinguish between adipose and muscle tissue, nor do they account for differences in body size. Subcutaneous fat depth has been successful at estimating overall adiposity as well as tracking changes in adiposity (Westervelt et al., 1976; Gentry et al., 2004; Dugdale et al., 2011c; Martin-Gimenez et al., 2016). Additionally, measurement of total body water (TBW) and subsequent calculation of body fat has also been validated in horses (Dugdale et al., 2011b), and may allow for the most accurate calculation of overall adiposity in horses.

The objectives of this study were to (1) evaluate digestibility of Ca, P, and Mg in horses fed to lose, maintain or gain weight, (2) examine markers of bone, muscle, and fat metabolism, and (3) evaluate relationships between different measures of adiposity.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Animals and housing

Fifteen mature horses (9.4 ± 3.9 yr; 554.9 ± 8.3 kg) were used; nine geldings and six mares, 14 of which were Thoroughbreds and one Thoroughbred-cross. Horses were housed in 3.6 x 3.6 m stalls overnight and turned out in paddocks in groups of three (one horse per treatment) during the day (approximately 0900h to 1530h). They were muzzled to prevent ingestion of pasture during turnout. Horses were fed individually in their stalls and had access to water at all times.

Experimental design

Horses were blocked by age and sex and then randomly assigned to one of three treatments: weight gain (GA), weight maintenance (MA), and weight loss (LO). The study was divided into two phases. During Phase 1, horses were fed a diet to achieve a steady body weight. Horses were weighed three times per week during this phase. This phase lasted for at least 3 wk while diets were adjusted and a stable weight was achieved. During Phase 2, horses were fed their assigned treatment diets designed to cause weight gain, weight maintenance or weight loss. The goal was to achieve approximately 0.5 kg of weight change per day for the GA and LO treatments. Horses were weighed daily during this phase and diets were adjusted as needed in order to achieve a steady rate of weight gain or weight loss in the GA and LO horses, respectively. Phase 2 lasted 3 to 4 wk, depending on the rate of weight change.

Diets

During Phase 1, the diet was formulated to closely meet DE and P requirements and to meet or exceed all other requirements based on initial BW (NRC, 2007).

Digestible energy for dietary formulation was calculated based on DE values provided from a laboratory analysis (DairyOne, Ithaca, NY). Diets were adjusted as needed to achieve a stable weight for each horse while maintaining similar intakes. The diet for weight maintenance consisted of 35.1% timothy cubes, 37.7% chopped timothy hay, 14.2% beet pulp, 5.7% oats, 2.2% soybean oil, and 4.9% of a balancer pellet to meet protein and mineral requirements. Sodium chloride was added to meet Na and Cl requirements. Table 4-1 shows the nutrient composition of the feeds used to formulate diets.

Once a stable weight was achieved, Phase 2 began and weight change treatments were imposed. The GA and LO diets were formulated to contain 145 and 75%, respectively, of each horse's maintenance DE requirement determined during Phase 1. Horses assigned to the MA treatment continued to receive the maintenance diet fed during Phase 1. Table 4-2 shows the composition of the diets for each treatment. The GA diet consisted of 44.6% forage (timothy hay and cubes), 24.9% beet pulp, 16.3% oats, 9.3% soybean oil, and 4.6% balancer pellet. The LO diet was mainly forage-based, consisting of 94.7% forage (timothy cubes and hay), and a balancer pellet. Sodium chloride was added as needed to meet Na and Cl requirements for each horse. Although it would have been desirable to keep the ingredient profile consistent across treatment diets and manipulate DE by changing total DM intake, that method would have resulted in different P intakes. Consequently, ingredient profiles of each diet were manipulated to achieve the desired DE intake while keeping P intake and DMI similar among treatments. Table 4-3 shows nutrient intakes for each treatment compared to nutrient requirements for a horse at maintenance.

The horses' daily rations were split into two equal meals fed at 0700 h and 1530 h during Phase 1 and the beginning of Phase 2. All feed ingredients for each meal were combined into one bucket and thoroughly mixed together, resembling a total mixed ration. At least 12 d prior to the beginning of total fecal collections, the daily ration was split into three equal meals fed at 0700 h, 1500 h, and 2300 h.

Sample collection

Whole blood was collected via jugular venipuncture into separate tubes containing no additive (serum) and tubes containing sodium heparin additive (plasma). Blood samples were collected at the end of Phase 1 after horses achieved a stable body weight but before weight change treatments were imposed and a second sample was collected at the end of Phase 2 (weight change period). Plasma was used to measure non-esterified fatty acids (NEFA), triglycerides (TG), and urea-N (PUN). Serum was used to measure creatinine, Ca, P, bone alkaline phosphatase (BAP), and C-terminal telopeptides of type I collagen (CTX-1).

Saliva samples were collected on three separate days for each horse in the week preceding the fecal collection period to measure salivary P concentration. Salivary P was averaged across the three sample days for each horse. Prior to the 1500 h meal, horse's mouths were rinsed with water to wash out any residual feed. A cotton swab containing citric acid (Salivette, Sarstedt, Germany) was attached to a bit using zip ties and placed in the horse's mouth for 5 minutes to ensure saturation of the swab. Swabs were then centrifuged to collect saliva and the saliva was frozen until analysis.

Morphometric measurements were taken weekly. These measurements included BCS (Henneke et al., 1983), wither height, and circumferences of the heart girth, belly girth, mid-neck, and lower neck as described previously (Carter et al., 2009). Measurements were taken by the same person blinded to previous measurements. Morphometric measurements were made with a plastic measuring tape reinforced with nylon to resist stretching. Horses were measured while standing square and efforts were made to position the horse the same way for each measurement. Heart girth circumference was modified from the method described by Carter et al. (2009) in that the circumference was measured around the tallest point of the withers with the measuring tape perpendicular to the ground instead of behind the slope of the withers. Differences in wither slope may affect location of the measurement, whereas wither height is relatively consistent among horses. Belly girth was measured around the widest part of the belly with the measuring tape perpendicular to the ground. Mid-neck circumference was measured at a point half-way between the poll and the tallest point of the withers. Lower neck circumference was measured at the widest point of the neck, directly in front of the shoulder.

Ultrasound was used to measure subcutaneous fat depth at specific locations at the end of Phase 1 and just prior to total fecal collections at the end of Phase 2. Ultrasound measurements were taken on two consecutive days and measurements were averaged. The settings on the ultrasound machine (Aloka, Hitachi Medical Systems, Switzerland) were near gain set to 28, far gain set to 4.6 and overall gain set to 86. Horses were brushed to remove any dirt on the skin before measurement and vegetable oil was used as a conductor. Measurements were taken with the horses standing square with all four

hooves flat on the ground. Subcutaneous fat depth (cm) was measured on the neck midway between the poll and the tallest point of the withers perpendicular to the top of the neck, at the shoulder behind the scapula, between the 12^{th} and 13^{th} rib, on the rump midway between the tuber coxae and the tuber ischii, and at a point located 10 cm cranial from the tail-head root. Unless otherwise specified, measurements were made with the ultrasound probe perpendicular to the ground and depth of fat was measured 5 cm from the midline.

Total body water (TBW) was measured at the end of Phase 1 and just prior to total fecal collections in Phase 2. A deuterium oxide (D_2O) dilution technique was used that has been previously validated in ponies (Dugdale et al., 2011b). A catheter was placed in the left jugular vein of each horse on the morning of the procedure. Horses were weighed and a pre-dose blood sample was collected in a heparin tube. The BW was used to calculate the dose of D_2O to be given (0.12 mg D_2O/kg BW). To calculate the exact amount of D_2O given, a syringe and needle were weighed to obtain a tare weight. Sterile D_2O was then drawn from a bottle and the syringe, needle, and D_2O were re-weighed to obtain the exact weight of D_2O to be administered to each horse. The isotope was administered via the catheter over approximately 60 sec and then was allowed to equilibrate into all body water compartments for 4 h. During this time period, food and water were withheld from the animals and attempts were made to keep horses cool with fans, as needed, to reduce any sweat losses. After the 4-h equilibration period, a post-dose blood sample was collected from the right jugular vein into a tube containing heparin. Horses were weighed again. Pre-dose and post-dose plasma samples were used to determine TBW.

A 5-d total fecal collection was performed at the end of Phase 2. Geldings were fitted with collection harnesses (The Horse Diaper, Equisan Marketing Pty Ltd., Victoria, Australia; Nappy, Stablemaid Horse Hygeine and Waste Management, Australia) that allowed for the capture of all feces and urine. Mares were fitted with fecal collection harnesses (Bun-Bag, Sagle, ID) to allow for capture of all feces. A voluntarily voided spot sample of urine was collected on d 5 for mares, but complete collection of urine was not performed for mares. Urine from one mare on the MA diet was not collected.

Horses were adapted to the collection harnesses for at least 2 wk prior to the collection period. During the collection period, horses remained in stalls and were hand-walked for 15 min twice daily. Horses were monitored throughout the collection period. Feces were composited for each horse over each 24-h period. At the end of a 24-h period, feces were thoroughly mixed, weighed, and a 10% subsample was saved for analysis. Spot samples of feces (approximately 250 g each) were taken every 4 h for indirect determination of digestibility by measuring TiO_2, which will be discussed in Chapter 5. Total daily fecal output was calculated by adding the weight of composite feces and the weights of all the spot samples of feces in the 24-h period.

Urine from the geldings for each 24-h period was thoroughly mixed using a paint mixer, weighed, and a 10% subsample was acidified according to O'Connor and Nielsen (2006) and frozen at -20°C. Any spilled urine was absorbed by pre-weighed absorbent pads to obtain weight, but this urine was not combined with uncontaminated urine. Volume of urine was calculated by dividing the weight of the urine by the density. Density was obtained by weighing 100 ml of urine.

Sample analysis

Digestibility of DM, OM, NDF, ADF, energy, Ca, P, and Mg were determined by analyzing samples of feed, refusals, and feces. Dry matter was determined by drying 1 g of wet sample in a 55 °C forced-air oven for 24 h and reweighing to obtain the dry weight. Organic matter was determined by ashing samples in pre-weighed crucibles at 600°C overnight and reweighing to determine ash content. Organic matter was calculated as dry feed weight minus ash weight. Samples were analyzed for NDF and ADF using the Ankom 200 Fiber Analyzer (Ankom Technology, Macedon, NY). Gross energy (GE) was measured using a bomb calorimeter and used to calculate actual DE intake. Ether extract of the feeds was analyzed by boiling feeds in petroleum ether and weighing extracted fat (AOAC method 920.39).

Phosphorus in feed, composite feces, and refusals was determined by a gravimetric quimociac technique (Shaver, 2008). Serum, salivary, and urinary P were determined using a colorimetric assay (Fiske and Subbarow, 1925). Calcium in serum, urine, feed, feces, and refusals was determined via atomic absorption (Bowers Jr and Rains, 1988). Magnesium in urine, feed, feces, and refusals was also determined via atomic absorption (Bowers Jr and Rains, 1988).

Bone alkaline phosphatase (BAP) in serum was measured using an EIA (MicroVue BAP EIA; Quidel, San Diego, CA). Concentration of cross-linking C-terminal telopeptides of type I collagen (CTX-1) was measured in serum using an ELISA previously validated in horses (Carstanjen et al., 2004; Serum CrossLaps CTX-1 ELISA; Immunodiagnostic Systems Limited, Tyne & Wear, UK). Plasma non-esterified fatty acid

(NEFA) concentration was determined colorimetrically (Free Fatty Acid Quantitation Kit; Sigma-Aldrich, St. Louis, MO). Plasma triglyceride (TG) concentration was determined enzymatically (Serum Triglyceride Determination Kit; Sigma-Aldrich, St. Louis, MO). Serum creatinine was determined colorimetrically (Creatinine Assay Kit; Sigma-Aldrich, St. Louis, MO). Plasma urea-N (PUN) was analyzed using a colorimetric assay as previously described (Urschel et al., 2007). Urinary creatinine was analyzed using a chemistry analyzer (Konelab; Thermo-Scientific, United States). All samples were analyzed in duplicate and concentrations were calculated using appropriate standards included in the kit or described in the procedure. Samples were diluted as needed to ensure results were in the middle of the standard curve. For BAP and CTX-1, quality control samples provided in the kit were analyzed on the same plate as the samples and concentrations fell within the acceptable range of expected concentrations. If more than one plate was needed to analyze samples for any assay, control samples from a donor horse were included on each plate for quality control. Samples were rerun if the CV for duplicates was greater than 10%.

Plasma samples for determination of D_2O were analyzed by a commercial laboratory (Metabolic Solutions Inc., Nashua, NH) as follows. Plasma was deproteinated using zinc sulfate and then analyzed for D_2O using wavelength-scanned cavity ring-down spectroscopy. Plasma D_2O abundance was used to calculate total body fat as described by Dugdale et al. (2011b). Briefly, the difference in D_2O concentration pre- and post-dosing was used to calculate TBW in kg and was then corrected using a 4% correction factor for isotopic exchange between D_2O and non-water body components. Total body water was converted to a percentage using BW (average of pre-dose BW and post-dose BW) and

then fat-free mass (FFM) was calculated using a lean tissue hydration factor of 0.732 (Pace and Rathbun, 1945). Body fat was calculated as: body fat, % = 100 – FFM (%).

Calculations and statistics

Apparent absorption of minerals was calculated by subtracting fecal losses from dietary intake. Retention of Ca, P, and Mg was calculated by subtracting fecal and urinary losses (geldings only) from dietary intake. Apparent digestibility of DM, NDF, ADF, Ca, P, and Mg were calculated as follows: (intake – feces)/intake x 100. In the case of feed refusals, the nutrient concentration of the feed refusal was analyzed and the amount of nutrient in the feed refusal was subtracted from the amount of nutrient offered per day in order to calculate actual intake. True digestibilities of Ca, P, and Mg were calculated by correcting for fecal endogenous losses; 10 mg P/kg BW, 20 mg Ca/kg BW, and 6 mg Mg/kg BW (NRC, 2007). Fractional urinary excretions of Ca, P, and Mg were calculated using the following equation: $\frac{\text{urine mineral conc} \times \text{serum creatinine conc}}{\text{serum mineral conc} \times \text{urine mineral conc}}$. Serum for fractional excretion calculations was taken 2 d prior to the collection period and urine was collected on d 5 of collection for the mares and on all days of collection for the geldings.

Actual DE intake was measured by subtracting fecal GE from feed GE. Digestible energy intake estimated from values obtained from commercial analysis (DairyOne, Ithaca, NY) and from two other equations were compared to actual DE intakes. The first equation is from Pagan (1998) and is as follows: DE, kcal/kg DM = 2,118 + (12.18 x CP%) – (9.37 x ADF%) – (3.83 x hemicellulose%) + (47.18 x fat%) + (20.35 x NFC%) – (26.3 x ash%); where hemicellulose = NDF – ADF and where non-fiber carbohydrate

(NFC) = 100 – NDF% - fat% - ash% - CP%. The second set of equations is from NRC (2007) and they are as follows. For forages and roughages, DE, Mcal/kg DM = 4.22 – (0.11 x ADF%) + (0.0332 x CP%) + (ADF%^{2}); for energy feeds and protein supplements, DE, Mcal/kg DM = 4.07 – 0.055 x (ADF%). The difference between calculated DE from all three sources (commercial laboratory, Pagan (1998) and NRC (2007)) and actual measured DE for each treatment were evaluated using paired t-tests.

Digestibility and balance data were analyzed using an ANOVA with repeated measures with treatment as the main effect, block as the random effect, and day of collection as the repeated measure (SAS 9.4; SAS Institute, Inc, Cary, NC). Day of collection was not significant in any of the models.

Blood constituents were analyzed in two ways. First concentrations in the final blood samples (end of Phase 2) were analyzed with an ANOVA using baseline values (end of Phase 1 samples) as co-variants to examine differences in final concentrations. Then they were analyzed using repeated measures ANOVA to ensure there were no differences in pre-treatment (end of Phase 1) concentrations. Treatment was the main effect and block was the random effect. For salivary P, values for each horse were averaged across the three days of saliva collection to obtain one value per animal and then data were analyzed using an ANOVA with treatment as the main effect and block as the random effect.

Body weight, BCS, body fat, morphometric and ultrasonic measurements were analyzed using an ANOVA with repeated measures. Treatment was the main effect,

block was the random effect, and time (beginning or end of Phase 2) was the repeated measure.

When effects were found to be significant, means were separated using an LSD test. Significance was considered when $P < 0.05$ and a trend when $0.05 < P < 0.10$. All data are presented as LS means.

Relationships between all BW, BCS, morphometric, ultrasonic, and TBW measurements were evaluated using simple linear regression. Simple linear regression was also used to evaluate the relationships between P excretion and blood variables.

Results and Discussion

All horses completed all phases of the study including the total fecal collection period. The results will be divided into three sections: (1) mineral digestibility and balance; (2) energy intake, BW change, and changes in measures of body composition; and (3) the effect of energy restriction and BW loss on mineral metabolism.

Mineral digestibility and balance

As planned, P intake was not different among treatments (Table 4-4; $P > 0.10$), however, fecal excretion of P tended to be greatest in LO horses and lowest in GA on a BW basis ($P = 0.0853$). As a result, apparent P absorption tended to be greater in GA horses compared with LO horses, with MA being intermediate ($P = 0.0662$). Additionally, apparent and true P digestibilities tended to be greatest in GA, lowest in LO and intermediate in MA ($P = 0.0672$ for apparent digestibility and $P = 0.069$ for true digestibility). True P digestibilities calculated using estimates of endogenous losses (NRC, 2007) ranged from 25.4% in LO to 35.6% in GA horses.

In this study, the GA horses were close to P balance. When endogenous P losses of 10 mg P/kg BW are assumed (NRC, 2007) true digestibility was 35% for GA horses, which is the same as the 35% true P digestibility used by the NRC (2007) to calculate daily P requirements. This result is not surprising, as the NRC (2007) calculated the P requirement using estimated endogenous losses of 10 mg P/kg BW. Horses were fed the NRC (2007) requirement and used NRC (2007) estimated amounts of endogenous losses to calculate true P digestibility. By these calculations, a 500-kg horse at maintenance would be excreting approximately 10 g of "undigested" P (10 mg P/kg BW = 5 g P of endogenous origin; 15 g P excreted – 5 g P endogenous = 10 g P "undigested"). However, if some of the assumed "undigested" P is actually P that was recycled post-absorption, then horses that are retaining more P would exhibit greater P digestibilities due to reduced P recycling. The GA horses did tend to absorb more P and excrete less P and as a result tended to have a greater true P digestibility of 35%. On the other end, horses losing weight had lower true P digestibilities (25%), potentially due to increased P recycling caused by P being mobilized from tissue during weight loss. These results supported the hypothesis that horses losing weight would excrete more P in their feces than horses gaining weight, due to P secretion into the GI tract.

On a BW basis, MA horses consumed more Ca than GA horses and LO consumed an intermediate amount (Table 4-5; P = 0.0427), however fecal excretion was not different among treatments ($P > 0.10$). As a result, apparent Ca absorption was lower in GA horses compared with MA (P = 0.0210), but was not different than LO (P > 0.10). In addition, there was a tendency for GA to have a lower apparent Ca digestibility compared to LO and MA (P = 0.0683). True Ca digestibility ranged from a low of 76.8% for GA

horses and a high of 83.0% for MA horses and was not different among treatments (P > 0.10). The NRC (2007) estimates true Ca digestibility to be 50%, but Pagan (1994) has reported true Ca digestibilities of 75% by mature horses over many trials, which would be closer to the values observed in this study (77 to 83%; Table 4-5). Additionally, previous research in this lab have reported true Ca digestibility to be 70% for mature horses fed close to their Ca requirement (Fowler et al., 2015). The NRC (2007) does contend that a conservative estimate of true Ca digestibility was used to calculate requirements for all horses in order to ensure appropriate absorption for bone health, particularly in growing animals. Thus, the large values observed here for true Ca digestibility are not unusual.

Simple linear regression was used to examine the relationship between dietary Ca:P ratio and fecal Ca and P excretion, as there were slight differences in Ca intake but not in P intake among groups. However, there were no relationships between dietary Ca:P ratio and fecal P excretion or fecal Ca excretion (P > 0.10; data not shown).

Magnesium intake was greater in MA and GA horses compared to LO horses (Table 4-6; P =0.0044), but fecal excretion was not different among treatments (P > 0.10). As a result, MA and GA horses apparently absorbed more Mg compared to LO (P =0.0226). However, apparent and true digestibilities of Mg were not different among treatments (P > 0.10). True Mg digestibilities ranged from 60.1% in LO to 62.5% in MA. The NRC (2007) estimates true Mg digestibility to be 40%, although values in the literature range from 40 up to 67% (Schryver et al., 1987; Fowler et al., 2015).

For the geldings (n = 3 per diet), mineral balance could be calculated due to the collection and analysis of urine. Tables 4-7, 4-8, and 4-9 show mineral balance data for the geldings only, with the inclusion of urinary mineral concentration and overall mineral retention. There were no differences in P intake, excretion, digestibility or retention (Table 4-7) among dietary treatments. Daily urinary P excretion was low for all treatments (< 2 mg P/kg BW), further supporting the notion that the primary route of P excretion is in the feces. Urinary P concentrations ranged from a low of 0.000054% in the LO treatment to 0.000163% in the GA treatment ($P = 0.0169$). Urinary P concentrations were intermediate in MA horses, but were not significantly different from the concentrations in the LO treatment. Horses receiving all treatments were in negative P balance, but the LO treatment was the only group of horses that had P retention that was different from zero ($P < 0.05$). Fecal P excretion as a percentage of intake ranged from 100% in GA to 110% in LO, but was not different among treatments ($P > 0.10$). Urinary P excretion was very low as a percentage of intake and did not exceed 0.058% of intake. Despite no statistical differences for P excretion, digestibility, or retention, numerically the geldings P data followed a similar pattern to P data with all horses. A power test reveals that 5 observations per treatment are needed to observe differences in P digestibility among treatments, so it is likely that the reduced number of horses (n=3) in the gelding-only dataset decreased the ability to detect significant differences.

On a BW basis, Ca intake by geldings was lowest for GA compared to LO and MA (Table 4-8; $P = 0.0081$). In addition, MA horses apparently absorbed more Ca than GA, and LO was intermediate to MA and GA ($P = 0.0216$). Urinary Ca concentrations ranged from 0.074 to 0.14%, with the lowest concentrations observed in the LO treatment

and the largest concentrations observed in the GA and MA treatments ($P < 0.05$). However, urinary Ca excretion (mg Ca/kg BW) and overall Ca retention were not different among treatments ($P > 0.10$). All treatment groups were in positive Ca balance and Ca retention was greater than zero for all treatments ($P < 0.05$). Apparent Ca digestibility tended to be greatest in MA and lowest in GA ($P = 0.0688$), but true digestibility was not different among treatments ($P > 0.10$). Fecal Ca excretion as a percentage of intake tended to be greatest in GA and LO and lower in MA ($P = 0.0688$) and ranged from 50% to 64%. Urinary Ca excretion as a percentage of intake ranged from 29% to 35% and was not different among treatments ($P > 0.10$). For the geldings only, Ca balance data appeared similar to data when all 15 animals are included.

For the geldings only, Mg intake and fecal excretion were not different among treatments (Table 4-9; $P > 0.10$). Apparent Mg absorption was less for horses receiving the LO diet than horses receiving MA or GA ($P = 0.0498$). However LO horses excreted less Mg in their urine than MA and GA horses ($P = 0.0536$), leading to no differences in overall Mg retention among treatments ($P = 0.4081$). All horses were close to Mg balance and Mg retention was not different from zero for any treatment ($P > 0.10$). Neither apparent nor true Mg digestibility were different among treatments ($P > 0.10$). Fecal Mg excretion ranged from 61% to 69% of intake but was not different among treatments ($P > 0.10$) and urinary Mg excretion ranged from 32% to 41% of intake, but again was not different among treatments ($P > 0.10$).

Table 4-10 shows fractional urinary excretion of Ca (FECa) and P (FEP) calculated using a spot sample from the mares ($n = 5$) as well as daily urine samples collected from the geldings ($n = 9$). Fractional Ca excretion followed a similar pattern

among treatments for mares and geldings, with GA having numerically lower FECa and LO having numerically higher values, although there were no statistical differences. Fractional Ca excretion ranged from a low of 10.9% to 23.2%. There were no statistical differences for FEP among treatments for mares or geldings and FEP was below 1% for all horses. However, patterns of FEP were different for mares and geldings among treatments. Both mares and geldings in LO had numerically greater FEP, but GA mares had numerically lower FEP, while MA geldings had the numerical lowest FEP. This difference between mares and geldings, while not significant, may have been due to differences in the method of urine collection between mares and geldings.

Dry matter digestibility, energy intake, changes in BW and measures of body composition

Dry matter intake was similar between GA and MA horses, but lower in LO horses (Table 4-11). Despite similar DMI, DMD was greater in the GA horses compared to MA and was lowest in the LO horses (Table 4-11; $P < 0.0001$), consistent with differences in diet composition and expected digestibility of ingredients. There was a significant positive relationship between actual DE intake (measured using feed and fecal GE values) and DMD ($P < 0.001$) and DMD explained approximately 86% of the variation in actual DE intakes ($R^2 = 0.8561$; Figure 4-1), indicating that horses consuming more digestible diets were able to digest more energy from the diets. This relationship may be useful as a tool to estimate DE of feeds based on their DMD, either *in vitro* or *in vivo*.

Diets were formulated using DE values calculated from a feed analysis provided by a commercial laboratory and actual DE intake was measured using collected fecal

samples. Table 4-11 shows the DE intakes estimated from commercial feed analysis as well as those calculated from intake and fecal excretion. Additionally, DE values calculated from equations reported by Pagan (1998) and NRC (2007) are also included in this table. For all calculated and measured DE values, the LO treatment consumed the least amount of DE, the MA treatment consumed an intermediate amounts, and the GA treatment consumed the most DE ($P < 0.0001$).

Because there were differences in DE intake among treatments, changes in BW, BCS, TBW, body fat, morphometric and ultrasonic measurements in each treatment from the beginning (Pre) to the end (Post) of the weight change phase (Phase 2) of the experiment were examined (Table 4-12). The time period in which the horses were expected to gain, maintain or lose weight was relatively short (3 to 4 wk) and the goal was to have GA and LO horses in a state of weight change during the total fecal collection period. From the beginning to the end of Phase 2, LO horses went from a BW of 566.10 to 555.05 kg ($P < 0.05$), MA horses went from 558.30 to 548.95 kg ($P < 0.05$), and GA horses went from 550.55 to 556.80 kg ($P < 0.05$).

During Phase 2, horses fed the GA diet had the smallest weight change of 6.25 kg, but this weight change was significantly different from horses fed MA and LO ($P < 0.05$). Despite being fed diets with greater DE, horses fed MA lost a similar amount of weight as horses fed LO (-9.35 kg and -11.05 kg, respectively; $P > 0.10$). The reason that MA horses lost weight is not known. Figure 4-2 shows the comparison of actual DE intake versus calculated DE intake using equations based on commercial laboratory analysis, NRC (1989) and Pagan (1998). The actual DE in the MA diet was slightly lower than estimated, which could account for the unexpected weight loss. However, the MA horses

received the same diet in Phase 2 as in Phase 1, when weight was maintained. Actual DE was lower than all calculated DE values for the GA treatment ($P < 0.05$). For the LO treatment, actual DE was accurately calculated by some equations, but tended to be overestimated by the NRC (2007) equation ($P < 0.10$).

There was a significant relationship between DMD and weight change ($P < 0.05$; Figure 4-3), with horses losing weight having lower DMD values compared with horses gaining weight, although this relationship may be confounded with DE intake. Accordingly, actual DE intake is positively related to weight change ($P < 0.05$; Figure 4-4) with horses consuming more DE showing greater weight gain than horses consuming less DE. Using the relationship between actual DE and weight change, the amount of actual DE needed to maintain weight (weight change = 0) is 39.71 kcal/kg BW. While the elevated estimate of DE required for weight maintenance as suggested by the NRC (2007) is 36.3 kcal/kg BW, Barth et al. (1977) reported that 39.5 kcal/kg BW was required to maintain the BW of pony stallions, which is similar to the value generated from the equation in this study. Greater DE values needed for maintenance may be a result of individual variation or may be affected by breed.

Subjective measures of body fatness (i.e. BCS) as well as objective measures (i.e.morphometric measurements) are being used by horse owners to estimate body composition of horses, so they were included here (Table 4-12). Despite significant weight changes, only LO horses had a significant change in BCS from 5.20 to 4.85 on a 9-point scale ($P < 0.05$). Numerically, heart girth circumference and belly girth circumference values changed in the expected directions for each treatment based on BW changes, but the only significant change was for MA horses' heart girth circumference to

decrease over time. Mid-neck and low-neck circumferences did not change in the expected directions and the only significant difference was for MA horses to have an increase in mid-neck circumference over time.

It was of interest to understand the tissue changes that occur with weight gain and weight loss; so measurements to estimate body fat, such as subcutaneous fat depth and body fat as measured by TBW, were employed. There were no significant differences in subcutaneous fat depth at the neck, shoulder, ribs, or rump sites (Table 4-12). However, subcutaneous tail-head fat depth was decreased in LO and MA horses during Phase 2 ($P < 0.05$). Body fat percentage was calculated based on subcutaneous rump fat depth as described by (Westervelt et al, 1976). Body fat percentages within treatments did not change during Phase 2 and calculated values ranged from 11.1% in LO to a high of 13.3% in GA at the end of Phase 2.

Subcutaneous fat depth at the tailhead was the ultrasonic measurement that was most related to BCS ($P < 0.05$), although the R^2 was very low ($R^2 = 0.143$; data not shown). Other studies have found similar results. In 5-mo old Thoroughbred foals, tailhead fat depth measured 10 cm cranial to the tailhead and 4 cm from midline explained 71% of the variation in body fat mass measured by chemical analysis post-euthansia (Gee et al., 2003). Tailhead fat depth was the strongest predictor of BCS in donkeys in moderate condition (Quaresma et al., 2013) as well as in horses that had low BCS (3 to 3.5) and high BCS (8 to 8.5) (Gentry et al., 2004). Rump fat thickness measured in these studies was not as good at predicting BCS as tailhead fat thickness and in horses losing weight rump fat thickness changed less than tailhead fat thickness (Gentry et al., 2004).

Other studies have argued that rump fat thickness is a good predictor of body fat. Westervelt et al. (1976) demonstrated that ultrasonic rump fat thickness measured at the center of the pelvic bone and 5 cm from midline explained 86% of the variation in ether extractable body fat in horses and 64% of the variation in Shetland ponies. In Andalusian horses, tailhead fat thickness and rump fat thickness were highly correlated to obesity status, but tailhead fat thickness was dependent on sex and age, while rump fat thickness remained highly correlated independent of sex and age effects (Martin-Gimenez et al., 2016).

Differences in average subcutaneous fat depth was evaluated between sexes. Subcutaneous fat depth at each measured site was averaged for each horse and geldings were compared to mares using a t-test. Despite having similar BCS (5.2 and 5.4 for mares and geldings, respectively), mares tended to have greater fat depth at the neck than geldings (1.16 versus 0.85 cm, respectively; $P = 0.0529$) and had greater fat depth at the ribs than geldings (0.78 versus 0.40 cm, respectively; $P < 0.05$). Differences in fat deposition sites between sexes is well documented in human literature (Karastergiou et al., 2012) and some information is also available for horses. Racing Standardbred geldings have been reported to have less total body fat than mares of similar BW and athletic capability (Kearns et al., 2002b). In Andalusians, mares had greater fat thickness at the ribs compared to stallions, but in contrast with the current study, stallions had greater fat deposition along the neck (Martin-Gimenez et al., 2016). However, the current study used geldings and differences in fat deposition between intact and castrated males have yet to be investigated. Because BCS is a holistic scoring system which accounts for fat at multiple locations on the animal (including the neck and ribs), it is possible that

BCS may not be different among sexes, but individual fat depots might differ. However, due to differences in how the two sexes carry fat, care may be necessary when using scoring systems that focus on one anatomical location of body fat, such as the cresty neck scoring system (Carter et al., 2009).

Table 4-13 shows relationships between BCS and morphometric measurements. Body weight, belly girth:height, heart girth:height, heart girth:BW, and belly girth:BW were all significantly related to BCS. However, all R^2 values were low, with the highest significant R^2-value being 0.3885 for heart girth:height. Another study measuring relationships between BCS and similar morphometric measurements also found that the significant relationships were weak to moderate, with R^2-values ranging from 0.35 for BW:height to 0.68 for belly girth:height (Carter et al., 2009). Carter et al. (2009) found the relationship between BCS and heart girth:height ratio was also significant and the authors stated that this measurement was the most useful measure to predict body condition, as belly girth circumference may be impacted by gut fill or pregnancy. However, based on observations from the current study, heart girth circumference measurements may have its limitations as well. Heart girth circumference may be useful for horses with a BCS greater than a 5, when fat is being deposited across the ribs, but at BCS below a 5 when fat disappears from the ribs, the circumference of the rib cage limits further changes in heart girth circumference and thus may become less useful as a measurement of body condition. Overall, morphometric measures in the current study were not strongly related to measures of TBW or BCS and were also not sensitive enough to detect changes in body composition during the treatment period.

Measurements of TBW were performed on all horses before and after the weight change phase. However, TBW was not able to be accurately measured in 1 gelding in GA before the weight change phase due to a malfunction of the syringe used to dose the D_2O. Therefore, only 29 observations of TBW were collected.

Overall, there was a positive linear relationship between TBW (kg) and BW, with heavier horses having more TBW (Figure 4-5; $P < 0.05$; $R^2 = 0.8075$). Total body water percentage was not related to any of the individual morphometric measurements (neck circumference, heart girth, or belly girth; $P > 0.05$), but there were some relationships between TBW (%) and ratios of morphometric measurements (Table 4-14). The largest R^2-value was observed for the relationship was between heart girth:BW and TBW ($P < 0.05$; R^2-value = 0.242), although the R^2-value was low.

Additionally, TBW (%) tended to be related to subcutaneous fat depth at the tailhead ($P = 0.0749$), although the R^2-value was low ($R^2 = 0.113$; data not shown). However, if only horses with a BCS ≥ 5 are included in the regression, the relationship between tailhead fat depth and TBW becomes significant ($P < 0.05$) and the R^2-value increases ($R^2 = 0.5475$; Figure 4-6). No other relationships existed between TBW (%) and subcutaneous fat depth. The non-linear relationship between tailhead fat depth and TBW demonstrates that tailhead fat depth loses accuracy in predicting TBW when horses have BCS < 5. Exponential relationships have also been described between measures of fat and BCS, with measurements being less useful in horses with higher BCS (fleshy to extremely fat) (Dugdale et al., 2011a; Dugdale et al., 2012; Quaresma et al., 2013; Martin-Gimenez et al., 2016).

It appears that above a BCS of 5, horses begin to deposit more fat subcutaneously at the tailhead compared to horses that are thinner. In growing sheep, subcutaneous fat is one of the last fat depots to develop, suggesting that development of other internal fat depots are prioritized over subcutaneous fat (Butler-Hogg, 1984). While the horses in the current study were not growing, it is likely that in thinner horses, internal fat is also prioritized over subcutaneous fat and thus, the depth of the subcutaneous fat at the tailhead only increases when horses reach a certain body condition where internal fat depots are sufficient.

There was a significant curvilinear relationship between TBW (%) and BCS ($P < 0.05$; $R^2 = 0.5104$; Figure 4-7), which is similar to what has been observed in other studies (Martin-Rosset et al., 2008; Dugdale et al., 2011a). When only horses between a BCS of 4 and 5.5 were included in the regression, the relationship was linear and significant (Figure 4-8). There was no relationship between TBW (%) and BCS for horses with BCS above 5.5 ($P > 0.10$; data not shown). Others have also suggested that the sensitivity of BCS to detect changes in body fat declines above BCS of 5 to 6 (Dugdale et al., 2011a). Therefore, other measurements of body fatness, such as subcutaneous tailhead fat depth, may be more useful as BCS increases above 5.5.

Total body water (kg) was not affected by treatment or time of sampling (Table 4-12; $P > 0.10$). Measurements of TBW were used to calculate body fat for each horse. Individual calculated values for body fat ranged from -3.2 to 10.7%. Body fat percentage decreased from pre- to post-treatment for LO and MA, while remaining unchanged for GA (Table 4-12; $P < 0.05$). Two LO horses had negative values for body fat at both timepoints. Other studies that have used D_2O to calculate body fat in horses have also

reported some negative values for percent body fat in individual horses (Dugdale et al., 2011b; Ferjak et al., 2017). When horses with negative values for body fat were removed from the data set, average body fat for LO pre-treatment was 7.95% and significantly decreased to 5.82% post-treatment. One of the LO horses that had negative values for body fat also drank twice as much water (84 ml water/kg BW) and excreted three times more urine (61 ml urine/kg BW) than the other horses in the study. Water intake for this gelding was more than 8 SD away from the overall mean of water intake and was treated as an outlier. It is possible that the large water intake of this horse affected measurement of TBW and thus influenced calculation of body fat.

Body fat percentages calculated from measurements of TBW were low compared to estimates calculated from rump fat (Table 4-12). In other equine studies, actual total body fat measured by dissection ranged from 1% in Thoroughbreds of lean and emaciated condition (Gunn, 1987) to 20% in 18-mo old draft horses (De Palo et al., 2013). In the study that validated the TBW technique in horses, dissected adipose tissue ranged from a low of 1.35% of BW in a pony with BCS of 1.25 up to a high of 26.34% in a pony with a BCS of 7 (Dugdale et al., 2011b). In this study, 10% was the largest body fat percentage estimated by TBW was in an animal that had a BCS of 6.5.

Possible explanations for the low body fat percentages seen here include D_2O losses associated with fecal and urinary excretion during D_2O equilibration, variation in gut water content, or differences in lean hydration. All horses voided feces and almost all horses urinated (21 out of 30) during the 4 h equilibration periods. During the total fecal collection period, urine output and water intake was measured for the geldings. Geldings fed LO tended to have greater urinary volumes than GA, and MA was not different from

either treatment (Table 4-15; P = 0.1060). Water intake was greatest for MA and lowest for GA (P < 0.05), but LO was not different from either treatment group. Water retention was calculated by subtracting fecal and urine water from feed and free water intake. There were no differences in water retention (data not shown; P > 0.10). It is possible that these differences among treatments also existed on the day of the D_2O test at the end of Phase 2 when horses had been on their treatment diets for at least 3 wk. Differences in urinary output and fecal water may have influenced measurement of TBW.

Additionally, differences in diet composition among treatments may have resulted in differences in gut water. Horses fed a high fiber diet have more fluid in their GI tract than horses fed a low fiber diet (Warren, 2001). Water in the GI tract is exchangeable with D_2O and can affect estimation of body water and of body fat. In this study, horses fed LO would be consuming the most amount of fiber (Appendix A) and these horses also have the lowest estimates of body fat. Another potential source of error is in the calculation of body fat. Body fat is calculated based on an assumption that lean hydration is 73.2%, although this value comes from a study done in guinea pigs (Pace and Rathbun, 1945) and others have reported lean hydration to range from 73.8% in goats to 77% in sheep (Sheng and Huggins, 1979). Underestimation of lean hydration could result in underestimation of body fat. For example, using a hydration factor of 73.2% for a horse with 67% body water would result in an estimate of 8.5% body fat. If a hydration factor of 77% is used, the estimate of body fat is 13%.

Effect of energy restriction and BW loss on mineral metabolism

Because there was no significant difference in weight change between LO and MA treatments, these treatments were combined into one 'Loss' treatment group and compared to the original GA treatment group ('Gain'). Table 4-16 shows mineral digestibility data for this treatment grouping. Phosphorus intake was not different between Gain horses and Loss horses ($P > 0.10$), but Gain horses excreted less fecal P than Loss horses ($P = 0.0434$). Gain horses tended to have a greater P absorption, apparent and true P digestibility ($P \leq 0.10$). Gain horses consumed less Ca than Loss horses ($P = 0.0312$), but there was no difference in fecal Ca excretion ($P > 0.10$). Therefore, Gain horses absorbed less Ca and had a lower apparent Ca digestibility than Loss horses ($P < 0.05$), although true Ca digestibility was not different between treatments ($P > 0.10$). There were no statistical differences between treatments for Mg balance data.

Horses were also grouped by actual weight lost, gained or maintained and mineral digestibility was compared (Table 4-17). There were nine horses that lost weight (weight loss > 2 kg; 'Loss'), two horses that maintained weight (weight change between -2 and +2 kg; 'Main'), and four horses that gained weight (weight gain > 2 kg; 'Gain'). When grouped this way, Ca intake was not different among groups ($P > 0.10$), but fecal Ca excretion tended to be greatest in Main horses and lowest in Loss horses ($P = 0.1034$). Thus, Loss horses had greater Ca absorption and apparent Ca digestibility compared with Main horses and Gain horses ($P < 0.05$). There were no differences among groups for P or Mg balance data. However, fecal P excretion numerically followed a similar pattern to the original treatment groups, with Loss horses excreting the most P (32.23 mg P/kg BW)

and Gain horses excreting the least P (29.91 mg P/kg BW) and Main horses in the middle (30.55 mg P/kg BW).

While minimal changes in BCS, ultrasonic measurements of fat depth and morphometric measures occurred as a result of the dietary treatment, it was of interest to determine if there were changes in markers of bone, protein, and fat metabolism. Table 4-18 shows concentrations of variables measured in the blood at the end of Phase 2, using values measured at the end of Phase 1 as covariates for testing differences among treatments. There were no differences among treatments in concentrations of Ca, P, or the Ca:P in the blood. However BAP, a marker of bone formation activity, was lower in horses in LO and MA compared to horses in GA ($P = 0.0481$). Conversely CTX-1, a marker of bone resorption, was greater in LO horses than GA horses ($P = 0.0143$). Using these two markers of bone turnover, the ratio of BAP:CTX-1 would indicate bone formation in relation to bone resorption. The BAP:CTX-1 was greatest in GA compared with LO and MA horses ($P = 0.0279$). Creatinine and PUN, markers of protein turnover, were greatest in LO horses and lowest in GA horses ($P < 0.05$). Non-esterified fatty acids and TG concentrations were not different among treatments.

Table 4-19 shows concentrations of blood variables for each treatment at the end of Phase 1 and the end of Phase 2. There were no differences in any of the blood variables measured among treatments at the end of Phase 1 before dietary treatments were imposed. Horses in GA had increases in BAP and BAP:CTX-1 ($P < 0.05$) and decreases in CTX-1 and creatinine from the end of Phase 1 to the end of Phase 2 ($P < 0.05$). Horses in MA had a significant decrease in CTX-1 from the end of Phase 1 to the end of Phase 2. These data indicate that GA horses were increasing bone activity, as

indicated by larger BAP values and a larger BAP:CTX-1 ratio. Horses in LO were undergoing bone resorption, as indicated by greater levels of CTX-1. Additionally, horses in LO also had greater markers of protein metabolism, altogether suggesting that LO horses were mobilizing both protein and bone. Phosphorus contained in these mobilized tissues may have resulted in the increased fecal P excretion observed in these horses.

Salivary P concentration was of interest to determine if recycling of P into the GI tract occurred in the saliva, as it does in ruminants (Horst, 1986). However salivary P concentration was not different among treatments and averaged 31.8 ± 1.6 mg P/L saliva. It is estimated that ponies weighing 150 kg secrete 10 to 12 L/d of saliva, or 66 to 80 ml/kg BW per day (Alexander and Hickson, 1970). Using these salivary flow rates, horses in this study secreted approximately 2.10 to 2.54 mg P/kg BW in their saliva, which equates to 7 to 8.4% of their daily P intake. This estimate of salivary P is slightly lower than values previously measured in our lab (Fowler, 2013), but greater than the concentrations of 6.2 to 8.6 mg P/L saliva observed in other studies (Alexander, 1966; van Doorn et al., 2011). Differences in method of saliva collection, processing, and analysis may have caused these differences among studies.

Fecal P excretion was plotted against weight change to determine if a relationship exists (Figure 4-9). There was no relationship between fecal P excretion and weight change over the study period ($P = 0.1281$), indicating that overall weight change does not explain much of the variation in P absorption. While this study did not observe a relationship between weight change and P excretion, the differences seen among horses could be due to differences in the composition of the weight gained or lost. For example, horses that were gaining mostly fat would have a lower P retention compared to horses

gaining mostly bone or muscle, due to the low concentration of P in adipose tissue. To investigate this theory further, samples of muscle, fat, hide, and salivary glands were taken from one pony that was euthanized as part of another study. Phosphorus was analyzed in these samples as previously described (D'Angelo et al., 2001). Table 4-20 shows the results from this one pony. As expected, adipose tissue had a relatively low P concentration compared to muscle (about 0.0175 and 0.585%, respectively). Therefore, if a horse lost 1 kg of adipose tissue, only 0.175 g of P would be lost with that tissue. However, if a horse lost 1 kg of muscle (DM basis), 5.85 g of P would be lost. However, if muscle is assumed to contain 27% DM (based on 73% lean hydration as estimated by Pace and Rathburn (1945)), then the amount of P in 1 kg of wet muscle would be 1.58 g. Therefore, relationships between fecal P excretion and markers of bone, adipose, and protein were evaluated to investigate if metabolism of these various body components would help to explain differences in fecal P excretion.

There were significant relationships between fecal P excretion and CTX-1 as well as P excretion and creatinine (Table 4-21; $P < 0.05$) and a tendency for a relationship between P excretion and PUN ($P = 0.1098$). The strongest relationship was between P excretion and CTX-1, with an R^2-value of 0.5477. As CTX-1 is a marker of bone resorption, horses with greater concentrations of CTX-1 would be undergoing more bone resorption, releasing Ca and P into the body. Unneeded P would be secreted into the GI tract for excretion, leading to greater fecal P excretion, thus supporting the hypothesis that tissue loss influences secretion of P into the GI tract. Bone loss during weight loss has been thoroughly examined in humans (Compston et al., 1992; Pritchard et al., 1996; Ensrud et al., 2003), but this is the first study in horses that has documented changes in

bone metabolism in response to weight change. Human studies have also demonstrated that an increase in bone loss is associated with increased excretion of P, although the P excretion in humans was mostly urinary (Hulley et al., 1971; Sebastian et al., 1994).

No relationships existed between fecal P excretion and any other variables measured in the blood. While there were no differences in blood markers of fat metabolism, there were some changes in measures of adiposity, such as TBW, BCS, and subcutaneous fat depth at the tailhead. Despite the inability of weight change alone to explain differences in P excretion, amount of bone resorption as well as protein turnover do assist in explaining differences in P excretion.

Conclusion

Weight loss affects markers of muscle and bone metabolism, and increases excretion of P. As a result, there tended to be differences in fecal P excretion and digestibility in horses gaining weight and horses losing weight, despite being fed the same amount of P and similar amounts of DM. These data suggest that endogenous secretions of P into the GI tract are not constant and change depending on physiological state. Digestibility of P may be greater than previously believed if endogenous losses have been underestimated due to recycling of P into the GI tract.

Tables and Figures

Table 4-1. Nutrient composition of feeds used to formulate diets (DM basis)

Item	Balancer pellet[1]	Oats	Beet pulp	Timothy cubes	Timothy hay	Soybean oil
DM, %[2]	90.8	89.2	91.8	91.1	91.9	100
CP, %[2]	29.5	11.9	9.03	11.0	11.7	--
ADF, %[2]	19.6	16.7	24.6	41.0	39.5	--
aNDF, %[2]	31.3	26.2	39.9	64.4	63.3	--
Ether extract, %[3]	5.40	2.25	0.18	1.23	1.17	100
NFC, %[4]	17.5	62.7	39.9	15.6	14.5	--
Ca, %[2]	0.49	0.08	0.70	0.57	0.36	--
P, %[2]	0.68	0.43	0.10	0.20	0.26	--
Mg, %[2]	0.55	0.15	0.30	0.24	0.16	--
K, %[2]	1.67	0.47	0.46	1.54	2.03	--
Na, %[2]	0.495	0.012	0.090	0.040	0.010	--
Fe, ppm[2]	565	136	176	718	66	--
Zn, ppm[2]	420	27	15	24	26	--
Cu, ppm[2]	251	4	7	8	8	--
Mn, ppm[2]	251	44	41	53	65	--
DE, Mcal/kg[2]	2.97	3.28	2.76	1.64	2.07	9.19

[1]Balancer pellet formulated and provided by Buckeye™ Nutrition, Dalton, OH
[2]As analyzed by Dairy One Forage Laboratory (Ithaca, NY) except for soybean oil
[3]As analyzed in-house using Sohxlet petroleum ether extraction
[4]NFC (non-fiber carbohydrates) = 100 - %NDF - %ether extract - %ash - %CP

Table 4-2. Composition of diets (%) for loss (LO), maintain (MA), and gain (GA)

treatments

Feed	LO	MA	GA
Balancer pellet[1]	5.0	4.9	4.6
Oats	0.0	5.7	16.3
Beet pulp	0.0	14.2	24.9
Timothy cubes	63.1	35.1	22.0
Timothy hay	31.6	37.7	22.6
Soybean oil	0.0	2.2	9.3
Sodium chloride	0.3	0.2	0.2

[1]Balancer pellet formulated and provided by Buckeye™ Nutrition, Dalton, OH

Table 4-3. Nutrient intakes of horses fed loss (LO), maintenance (MA), and gain (GA) diets compared to nutrient requirements for an average horse at maintenance[1]

Nutrient	**LO**	**MA**	**GA**	**Requirement for horse at maintenance[1]**
DE, kcal/kg BW[2]	24.09	34.34	44.68	33.4
NDF, g/kg BW	7.67	8.06	6.50	--
ADF, g/kg BW	3.58	4.53	3.58	--
Ether extract, %	1.52	3.44	10.75	--
NFC, %[3]	13.80	22.59	28.67	--
CP, g/kg BW	1.53	1.56	1.48	1.26
Ca, mg/kg BW	60.37	67.03	61.16	40
P, mg/kg BW	30.55	31.52	30.37	28
Mg, mg/kg BW	27.77	32.42	31.33	15

[1]Average maintenance requirements as described by NRC (2007)
[2]Calculated DE based on commercial lab analysis (DairyOne, Ithaca, NY)
[3]NFC (non-fiber carbohydrates) = 100 - %NDF - %ether extract - %ash - %CP

Table 4-4. Phosphorus digestibility over the fecal collection period for all horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means; n=15)

Item	**LO**	**MA**	**GA**	**SEM**	***P*-value**
Intake, g	16.74	17.23	16.80	0.61	0.8178
Intake, mg/kg BW	30.55	31.52	30.37	0.46	0.2024
Fecal excretion, g	18.13	17.36	16.35	1.27	0.2569
Fecal excretion, mg/kg BW	32.90	31.64	29.62	1.80	0.0853
Apparent absorbed, g[1]	-1.39[b]	-0.13[ab]	0.45[a]	0.87	0.0500
Apparent absorbed, mg/kg BW[1]	-2.35	-0.12	0.76	1.57	0.0662
Apparent P digestibility, %	-7.38	-0.44	2.59	5.02	0.0672
True P digestibility, %[2]	25.41	31.29	35.62	5.25	0.0690

[abc] Means within a row lacking a common superscript differ ($P < 0.05$)
[1]Absorbed = Intake – fecal output
[2]True digestibility calculated using fecal endogenous P losses of 10 mg/kg BW (NRC, 2007)

Table 4-5. Calcium digestibility over the fecal collection period for all horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means; n=15)

Item	LO	MA	GA	SEM	*P*-value
Intake, g	31.11	32.69	29.70	1.46	0.2843
Intake, mg/kg BW	56.84[ab]	59.80[a]	53.67[b]	1.84	0.0427
Fecal excretion, g	16.89	16.59	17.76	1.43	0.7987
Fecal excretion, mg/kg BW	30.50	30.30	32.24	1.84	0.7101
Apparent absorbed, g[1]	14.22[ab]	16.11[a]	11.94[b]	1.20	0.0233
Apparent absorbed, mg/kg BW[1]	26.34[ab]	29.50[a]	21.43[b]	2.22	0.0210
Apparent digestibility, %	46.59	49.42	39.09	3.22	0.0683
True digestibility, %[2]	81.81	82.96	76.79	3.08	0.3273

[abc] Means within a row lacking a common superscript differ

[1]Absorbed = Intake – fecal output

[2]True digestibility calculated using fecal endogenous Ca losses of 20 mg/kg BW (NRC, 2007)

Table 4-6. Magnesium digestibility over the fecal collection period for all horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means; n=15)

Item	LO	MA	GA	SEM	*P*-value
Intake, g	12.69[b]	14.36[a]	14.10[a]	0.41	0.0286
Intake, mg/kg BW	23.25[b]	26.33[a]	25.51[a]	0.73	0.0044
Fecal excretion, g	8.38	8.68	8.58	0.47	0.8988
Fecal excretion, mg/kg BW	15.21	15.89	15.57	0.68	0.7810
Apparent absorbed, g[1]	4.31[b]	5.69[a]	5.52[a]	0.49	0.0129
Apparent absorbed, mg/kg BW[1]	8.04[b]	10.44[a]	9.94[a]	1.00	0.0226
Apparent digestibility, %	34.18	39.54	38.87	3.32	0.2446
True digestibility, %[2]	60.05	62.50	62.43	2.97	0.7248

[abc] Means within a row lacking a common superscript differ

[1]Absorbed = Intake – fecal output

[2]True digestibility calculated using fecal endogenous Mg losses of 6 mg/kg BW (NRC, 2007)

Table 4-7. Phosphorus balance for geldings only fed loss (LO), maintenance (MA) and gain (GA) diets (LS means; n=9)

Item	**LO**	**MA**	**GA**	**SEM**	***P*-value**
Intake, g	17.55	17.43	16.54	0.89	0.6798
Intake, mg/kg BW	31.24	31.58	29.93	0.51	0.1298
Fecal excretion, g	19.49	18.45	16.53	1.75	0.2007
Fecal excretion, mg/kg BW	34.44	33.38	30.05	2.42	0.1118
Absorbed, g[1]	-1.94	-1.01	0.0047[2]	1.21	0.2277
Absorbed, mg/kg BW[1]	-3.20	-1.81	-0.12[2]	2.13	0.2687
Urinary concentration, %	0.000054[b]	0.000081[b]	0.000163[a]	0.000023	0.0195
Urinary excretion, g	0.0082	0.0067	0.0093	0.0013	0.4417
Urinary excretion, mg/kg BW	0.015	0.012	0.017	0.0025	0.4382
Retention, g[3]	-1.95	-1.02	-0.005	1.22	0.2285
Retention, mg/kg BW[3]	-3.21	-1.82	-0.13	2.13	0.2696
Apparent digestibility, %	-9.87	-5.75	-0.33	6.85	0.2886
True digestibility, %[4]	22.19	25.91	33.08	7.13	0.2117
Urinary P, % of intake	0.046	0.039	0.057	0.0082	0.3555
Fecal P, % of intake	109.87	105.75	100.33	6.85	0.2886
Total P excreted, % of intake[5]	109.92	105.79	100.39	6.85	0.2897

[1]Absorbed = Intake – fecal output
[2]Values for absorbed P (g) and absorbed P (mg/kg BW) are not different from 0.
[3]Retention = Intake – fecal losses – urinary losses
[4]True digestibility calculated using fecal endogenous P losses of 10 mg/kg BW (NRC, 2007)
[5]Total excreted as percent of intake = (urinary excretion + fecal excretion)/intake*100
[abc]Means lacking a common letter differ ($P < 0.05$)

Table 4-8. Calcium balance for geldings only fed loss (LO), maintenance (MA) and gain (GA) diets (LS means; n=9)

Item	LO	MA	GA	SEM	*P*-value
Intake, g	32.43	31.97	28.64	1.37	0.1650
Intake, mg/kg BW	57.81[a]	57.92[a]	51.90[b]	1.01	0.0081
Fecal excretion, g	19.36	16.03	18.22	1.83	0.4022
Fecal excretion, mg/kg BW	34.12	29.02	33.10	2.40	0.2277
Absorbed, g[1]	13.07[b]	15.94[a]	10.42[c]	0.74	0.0044
Absorbed, mg/kg BW[1]	23.69[ab]	28.90[a]	18.80[b]	1.86	0.0216
Urinary concentration, %	0.074[b]	0.13[a]	0.14[a]	0.014	0.0169
Urinary excretion, g	10.93	11.03	8.42	1.12	0.2005
Urinary excretion, mg/kg BW	19.60	19.94	15.25	1.98	0.2315
Retention, g[2]	2.13	4.90	2.00	1.53	0.1399
Retention, mg/kg BW[2]	4.09	8.96	3.55	2.86	0.1372
Apparent digestibility, %	41.18	49.87	36.30	3.59	0.0688
True digestibility, %[3]	75.81	84.41	74.88	3.97	0.1704
Urinary Ca, % of intake	33.96	34.53	29.30	3.64	0.5606
Fecal Ca, % of intake	58.82[α]	50.13[β]	63.70[α]	3.59	0.0688
Total Ca excreted, % of intake[4]	92.77	84.65	93.00	5.09	0.1606

[abc] Means within a row lacking a common superscript differ

[αβ] Means lacking common Greek letter tend to differ ($0.05 < P < 0.10$)

[1]Absorbed = Intake – fecal output

[2]Retention = Intake – fecal losses – urinary losses

[3]True digestibility calculated using fecal endogenous Ca losses of 20 mg/kg BW (NRC, 2007)

[4]Total excreted as percent of intake = (urinary excretion + fecal excretion)/intake*100

Table 4-9. Magnesium balance for geldings only fed loss (LO), maintenance (MA) and gain (GA) diets (LS means; n=9)

Item	LO	MA	GA	SEM	*P*-value
Intake, g	13.11	14.03	13.94	0.62	0.5468
Intake, mg/kg BW	23.45	25.45	25.21	1.08	0.2176
Fecal excretion, g	9.08	8.58	8.74	0.71	0.8836
Fecal excretion, mg/kg BW	16.05	15.59	15.87	1.00	0.9467
Absorbed, g[1]	4.03[b]	5.45[a]	5.20[a]	0.62	0.0233
Absorbed, mg/kg BW[1]	7.40[b]	9.87[a]	9.34[a]	1.22	0.0498
Urinary concentration, %	0.031[b]	0.066[a]	0.10[a]	0.012	0.0100
Urinary excretion, g	4.21[b]	5.26[a]	5.71[a]	0.41	0.0536
Urinary excretion, mg/kg BW	7.66	9.52	10.35	0.91	0.1474
Retention, g[2]	-0.18	0.19	-0.51	0.43	0.4446
Retention, mg/kg BW[2]	-0.26	0.35	-1.00	0.78	0.4081
Apparent digestibility, %	31.26	38.88	36.98	4.18	0.2851
True digestibility, %[3]	56.87	62.66	60.80	3.83	0.5446
Urinary Mg, % of intake	32.40	37.67	41.04	3.51	0.2840
Fecal Mg, % of intake	68.74	61.12	63.02	4.18	0.2851
Total Mg excreted, % of intake[4]	101.14	98.78	104.06	3.09	0.4270

[abc] Means within a row lacking a common superscript differ

[1]Absorbed = Intake – fecal output

[2]Retention = Intake – fecal losses – urinary losses

[3]True digestibility calculated using fecal endogenous Mg losses of 6 mg/kg BW (NRC, 2007)

[4]Total excreted as percent of intake = (urinary excretion + fecal excretion)/intake*100

Table 4-10. Fractional urinary excretion (%) of Ca (FECa), and P (FEP) in mares (n = 5) and geldings (n= 9) fed loss (LO), maintenance (MA), and gain (GA) diets (LS means)

Item	**LO**	**MA**	**GA**	**SEM**	***P* - value**
Mares					
FECa	18.25	16.09	11.75	2.78	0.5156
FEP	0.036	0.026	0.022	0.017	0.8618
Geldings					
FECa	23.22	16.36	10.85	5.86	0.4115
FEP	0.076	0.035	0.061	0.029	0.6198
All horses					
FECa	21.23	15.99	11.21	3.59	0.2119
FEP	0.060	0.027	0.045	0.018	0.4993

Table 4-11. Dry matter digestibility and DE intake over the fecal collection period over the fecal collection period for horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means)

Item	**LO**	**MA**	**GA**	**SEM**	***P*-value**
Intake, kg	6.63[b]	7.81[a]	7.95[a]	0.17	0.0003
Intake, g/kg BW	12.13[b]	14.30[a]	14.39[a]	0.17	<0.0001
Fecal excretion, kg	3.29[b]	3.59[a]	3.05[b]	0.11	0.0045
Fecal excretion, g/kg BW	6.02[b]	6.56[a]	5.52[c]	0.13	<0.0001
DMD, %	50.38[c]	54.07[b]	61.57[a]	0.95	<0.0001
Calculated DE intake[1], kcal/kg BW	24.09[c]	34.34[b]	44.68[a]	0.53	<0.0001
DE intake[2], kcal/kg BW	24.05 [c]	32.62 [b]	41.26 [a]	1.13	<0.0001
DE intake[2], Mcal/kg DM	1.99 [c]	2.25 [b]	2.90 [a]	0.07	<0.0001
DE intake[2], Mcal/d	13.15[c]	17.58[b]	23.09[a]	0.65	<0.0001
DE intake[3], Mcal/d	13.38[c]	18.88[b]	25.06[a]	0.03	<0.0001
DE intake[4], Mcal/d	14.09[c]	18.62[b]	24.72[a]	0.37	<0.0001

[abc] Means within a row lacking a common superscript differ

[1]DE calculated using values provided by commercial laboratory (DairyOne, Ithaca, NY). These values were used to formulate diets.

[2]Digestible energy calculated by subtracting GE of feces from GE of feed

[3]Digestibile energy calculated using equation from Pagan (1998) as follows: DE, kcal/kg DM = 2,118 + (12.18 x CP%) – (9.37 x ADF%) – (3.83 x hemicellulose%) + (47.18 x fat%) + (20.35 x NFC%) – (26.3 x ash%); where hemicellulose = ADF – NDF and non-fiber carbohydrate (NFC) = 100 - NDF% - fat% - ash% - CP%.

[4]Digestible energy calculated using equations from NRC (2007) as follows: for roughages and forages, DE, Mcal/kg DM = 4.22 – (0.11 x ADF%) + (0.0332 x CP%) + (ADF%^{2}); for energy feeds and protein supplement, DE, Mcal/kg DM = 4.07 – 0.055 x (ADF%).

Table 4-12. Values for BW, BCS, TBW, body fat, morphometric, and ultrasonic measurements at the end of Phase 1 (Pre) and the end of Phase 2 (Post) for horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means)

Measurement	LO		MA		GA		SEM	*P*-value		
	Pre	Post	Pre	Post	Pre	Post		Trt	Time	Trt*Time
BW, kg	566.10[a]	555.05[b]	558.30[a]	548.95[b]	550.55[b]	556.80[a]	15.31	0.9148	0.0027	0.0002
BCS	5.20[a]	4.85[b]	5.50	5.40	5.50	5.70	0.33	0.4063	0.3200	0.0490
Heart girth circumference, cm	198.80	197.40	197.60[a]	195.80[b]	194.80	196.20	1.97	0.6122	0.2173	0.0299
Belly girth circumference, cm	214.80	211.40	211.40	208.80	213.00	215.20	2.33	0.4271	0.2036	0.0657
Mid-neck circumference, cm	92.20	94.00	92.60[a]	96.40[b]	96.40	97.20	1.87	0.3436	0.0237	0.3504
Low-neck circumference, cm	122.80	122.60	119.00	123.20	121.80	121.00	2.08	0.8146	0.3393	0.1578
TBW, kg[1]	395.35	393.58	379.83	379.88	375.81	373.44	14.50	0.5576	0.6831	0.9515
Body fat, %[1,2]	4.18[a]	2.38[b]	6.51[a]	4.97[b]	5.99	7.41	1.54	0.2203	0.1345	0.0144
Body fat, %[3]	11.59	11.10	13.29	13.10	12.37	13.32	0.74	0.1551	0.8184	0.3106
SubQ neck fat, cm	0.847	1.146	1.058	1.054	0.807	0.933	0.199	0.6309	0.1626	0.4460
SubQ shoulder fat, cm	0.309	0.345	0.363	0.323	0.382	0.378	0.049	0.6415	0.9264	0.5629
SubQ rib fat, cm	0.627	0.418	0.699	0.416	0.471	0.694	0.24	0.9119	0.5193	0.2923
SubQ rump fat, cm	0.628	0.524	0.989	0.948	0.7940	0.997	0.158	0.1551	0.8184	0.3106
SubQ tail-head fat, cm	1.378[a]	0.872[b]	1.253[a]	0.963[b]	1.407	1.386	0.1823	0.4459	0.0009	0.0252

[ab]Means lacking a common letter within treatment differ between timepoints ($P < 0.05$)

[1]Data lost for one GA horse at the pre timepoint. N=4

[2]Body fat percentage calculated based on TBW measured using deuterium oxide corrected for a lean hydration factor of 0.732, and divided by BW

[3]Body fat percentage calculated based on subcutaneous rump fat thickness using the equation: Body fat, % = 8.64 + 4.70*rump fat, cm; as described by Westervelt et al. (1976)

Table 4-13. Associations of BCS with morphometric measurements

Measurement	**R^2**	***P*-value**
BW	0.1392	0.0423
Heart girth	0.0186	0.4724
Belly girth	0.0140	0.2449
BMI (BW/height)	0.0191	0.4660
Belly girth:Height	0.2663	0.0035
Heart girth:Height	0.3885	0.0001
Heart girth:BW	0.2464	0.0031
Belly girth:BW	0.1954	0.0145
Belly girth:Heart girth	0.0120	0.5467

Table 4-14. Relationships between total body water (TBW, %) and morphometric measurement ratios

Ratio	**R^2-value**	***P*-value**
Belly girth:heart girth	0.017	0.4999
BMI (BW/height)	0.074	0.1528
BCI[1]	0.118	0.0677
Belly girth:height	0.129	0.0559
Heart girth:height	0.150	0.0378
Belly girth:BW	0.183	0.0205
Heart girth:BW	0.242	0.0067

[1]Body condition index (BCI) = $\left\{\frac{HG^{0.5}+BG+NC^{1.2}}{height^{1.05}}\right\}^{2.2}$; where HG = heart girth, BG = belly girth, NC = mid-neck circumference; (Potter et al., 2013)

Table 4-15. Urine output, density, and water intake for geldings only (n=8; LS means)

Item	**LO[1]**	**MA**	**GA**	**SEM**	***P*-value**
Water intake, ml/kg BW	41.86[ab]	44.94[a]	35.47[b]	2.79	0.0438
Urine output, ml/kg BW	20.73	16.35	11.18	2.38	0.1060
Urine density, g/100 ml	101.03	101.88	102.33	0.65	0.4925

[1]Outlier removed

[ab]Means lacking a common letter differ ($P < 0.05$)

Table 4-16. Mineral digestibility (LS means ± SE) for all horses divided into two treatment groups: Loss (consists of all horses originally assigned to LO and MA) and Gain (consists of all horses originally assigned to GA)

Item	**Loss[1]**	**Gain[2]**	***P*-value**
Calcium			
Intake, mg/kg BW	58.32 ± 1.55	53.67 ± 1.88	0.0312
Fecal excretion, mg/kg BW	30.40 ± 1.32	32.24 ± 1.79	0.3961
Absorption, mg/kg BW	27.92 ± 1.87	21.43 ± 2.24	0.0109
Apparent digestibility, %	48.00 ± 2.50	39.09 ± 3.17	0.0226
True digestibility, %	82.39 ± 2.26	76.79 ± 3.00	0.1296
Phosphorus			
Intake, mg/kg BW	31.04 ± 0.34	30.37 ± 0.48	0.2746
Fecal excretion, mg/kg BW	32.27 ± 1.66	29.62 ± 1.80	0.0434
Absorption, mg/kg BW	-1.23 ± 1.44	0.76 ± 1.59	0.1097
Apparent digestibility, %	-3.91 ± 4.60	2.59 ± 5.10	0.1019
True digestibility, %	28.35 ± 4.84	35.62 ± 5.30	0.0667
Magnesium			
Intake, mg/kg BW	24.79 ± 0.71	25.51 ± 0.93	0.5035
Fecal excretion, mg/kg BW	15.55 ± 0.47	15.57 ± 0.67	0.9802
Absorption, mg/kg BW	9.24 ± 0.93	9.94 ± 1.05	0.4297
Apparent digestibility, %	36.86 ± 2.90	38.87 ± 3.39	0.5187
True digestibility, %	61.27 ± 2.42	62.43 ± 2.97	0.7024

[1]n=10
[2]n=5

Table 4-17. Mineral digestibility for all horses grouped by actual weight change. Gain contains horses with a positive weight change greater than 2 kg. Main contains horses with a weight change between -2 and +2 kg. Loss contains horses with a weight change less than -2 kg. Results presented as LS means ± SE.

Item	Loss[1]	Main[2]	Gain[3]	*P*-value
Calcium				
Intake, mg/kg BW	57.79 ± 1.79	57.12 ± 3.42	54.30 ± 2.40	0.4132
Fecal excretion, mg/kg BW	29.59 ± 1.63	35.80 ± 2.80	31.81 ± 2.04	0.1034
Absorption, mg/kg BW	28.44 ± 2.42[a]	20.26 ± 3.55[b]	22.46 ± 2.81[b]	0.0180
Apparent digestibility, %	49.22 ± 3.36[a]	35.40 ± 4.97[b]	40.44 ± 3.91[b]	0.0081
True digestibility, %	83.66 ± 2.90[a]	71.96 ± 4.68[b]	77.72 ± 3.52[ab]	0.0358
Phosphorus				
Intake, mg/kg BW	31.02 ± 0.38	30.56 ± 0.80	30.49 ± 0.57	0.7043
Fecal excretion, mg/kg BW	32.23 ± 1.73	30.55 ± 2.42	29.91 ± 1.96	0.2711
Absorption, mg/kg BW	-1.15 ± 1.49	0.059 ± 2.21	0.43 ± 1.74	0.4990
Apparent digestibility, %	-3.66 ± 4.78	0.56 ± 7.05	1.44 ± 5.56	0.4805
True digestibility, %	28.56 ± 5.03	33.20 ± 7.20	34.55 ± 5.76	0.3734
Magnesium				
Intake, mg/kg BW	24.57 ± 0.76	26.17 ± 1.56	25.50 ± 1.07	0.5397
Fecal excretion, mg/kg BW	15.26 ± 0.50	16.40 ± 1.07	15.79 ± 0.76	0.5983
Absorption, mg/kg BW	9.24 ± 0.98	9.60 ± 1.54	9.94 ± 1.18	0.7912
Apparent digestibility, %	37.16 ± 3.09	36.99 ± 5.22	38.62 ± 3.85	0.9182
True digestibility, %	61.87 ± 2.59	60.11 ± 4.81	61.94 ± 3.41	0.9365

[1]n=9
[2]n=2
[3]n=4
[abc]Means lacking a common letter differ (P < 0.05)

Table 4-18. Concentrations of blood variables in horses at the end of Phase 2 for horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means[1])

Item	**LO**	**MA**	**GA**	**SEM**	***P*-value**
Ca, mg/dl	12.03	12.07	12.46	0.22	0.3831
P, mg/dl	3.26	3.22	2.72	0.18	0.1808
Ca:P	3.77	3.81	4.58	0.35	0.5899
BAP, U/L	55.71[b]	57.30[b]	66.90[a]	2.85	0.0481
CTX-1, U/L	0.34[a]	0.29[ab]	0.24[b]	0.017	0.0143
BAP:CTX	222.45[b]	219.78[b]	331.94[a]	25.44	0.0279
Urea, mg/dl	13.52[a]	10.88[ab]	9.61[b]	0.82	0.0251
Creatinine, umol/L	204.96[a]	182.81[ab]	160.50[b]	8.49	0.0266
NEFA, umol/L	127.89	130.05	125.67	8.97	0.9460
TG, mg/ml	0.55	0.53	0.45	0.08	0.7405

[abc] Means within a row lacking a common superscript differ ($P < 0.05$)

[1]LS means adjusted for pre-treatment values as covariates

Table 4-19. Concentrations of blood variables measured at the end of Phase 1 (Pre) and the end of Phase 2 (Post) in horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means)

Item	LO		MA		GA		SEM	*P*-value		
	Pre	Post	Pre	Post	Pre	Post		Trt	Time	Trt*Time
Ca, mg/dl	11.21	11.99	12.02	12.09	12.00	12.48	0.38	0.1887	0.1400	0.6012
P, mg/dl	3.30	3.27^{a}	3.20	3.22^{a}	2.95	2.71^{b}	0.17	0.0409	0.5903	0.7496
Ca:P	3.42	3.67^{b}	3.79	3.81^{b}	4.14	4.67^{a}	0.25	0.0133	0.2060	0.6037
BAP, U/L	64.41	58.06^{b}	59.10	55.29^{c}	61.13*	66.56^{*a}	4.19	0.4306	0.5163	0.1424
CTX-1, U/L	0.42	0.39	0.34*	0.26*	0.34*	0.21*	0.1018	0.6400	<0.0001	0.0118
BAP:CTX	226.19	247.23	192.07	212.94	180.08*	313.99*	38.36	0.6182	0.0070	0.0380
Urea, mg/dl	12.60	13.73	10.49	10.48	12.48	9.79	0.7607	0.0271	0.2908	0.0174
Creatinine, umol/L	180.03	198.74	180.79	177.27	200.11*	172.27*	18.90	0.8860	0.5385	0.0446
NEFA, umol/L	202.69	131.79	202.49	133.89	164.54	117.93	29.47	0.6084	0.0078	0.8552
TG, mg/ml	0.43	0.46	0.43	0.43	0.66	0.62	0.10	0.2445	0.9747	0.7907

*Means within a treatment differ between pre and post ($P < 0.05$)

^{abc}Means within a timepoint differ among treatments ($P < 0.05$)

Table 4-20. Phosphorus concentrations in samples of muscle, fat, hide, and salivary glands from one pony

Tissue	Phosphorus, % DM
Muscle	
Triceps brachii m.	0.58
Lastissimus dorsi m.	0.59
Adipose	
Subcutaneous fat over triceps	0.019
Inter-muscular fat between triceps and lastissimus	0.016
Other	
Hide (skin and hair)	0.14
Sublingual salivary gland	0.47
Mandibular salivary gland	0.65
Parotid salivary gland	0.65

Table 4-21. Relationships between fecal P excretion (g), serum Ca and P, and markers of bone (serum BAP and CTX-1), protein (serum creatinine and plasma urea-N [PUN]), and fat (plasma NEFA and TG) metabolism

Dependent variable	Intercept	Slope	R^2-value	*P*-value for Model
Ca, mg/dl	10.46	0.56	0.0192	0.6224
P, mg/dl	10.39	2.24	0.0954	0.2626
BAP, U/L	18.94	-0.028	0.0078	0.7538
CTX-1, U/L	14.35	10.15	0.5477	0.0016
Creatinine, umol/L	9.27	0.04	0.2656	0.0493
PUN, mg/dl	10.91	0.56	0.1847	0.1098
NEFA, umol/L	20.36	-0.024	0.0512	0.4172
TG, mg/ml	17.73	-0.90	0.0052	0.7980

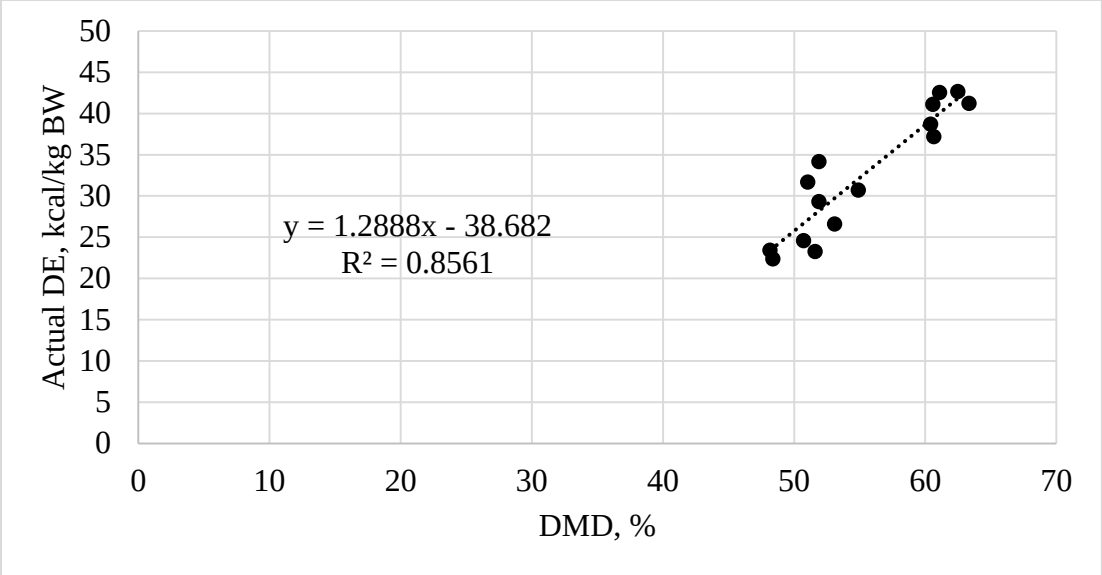


Figure 4-1. Regression of actual DE intake (kcal/kg BW) as a function of DM digestibility. $P < 0.001$

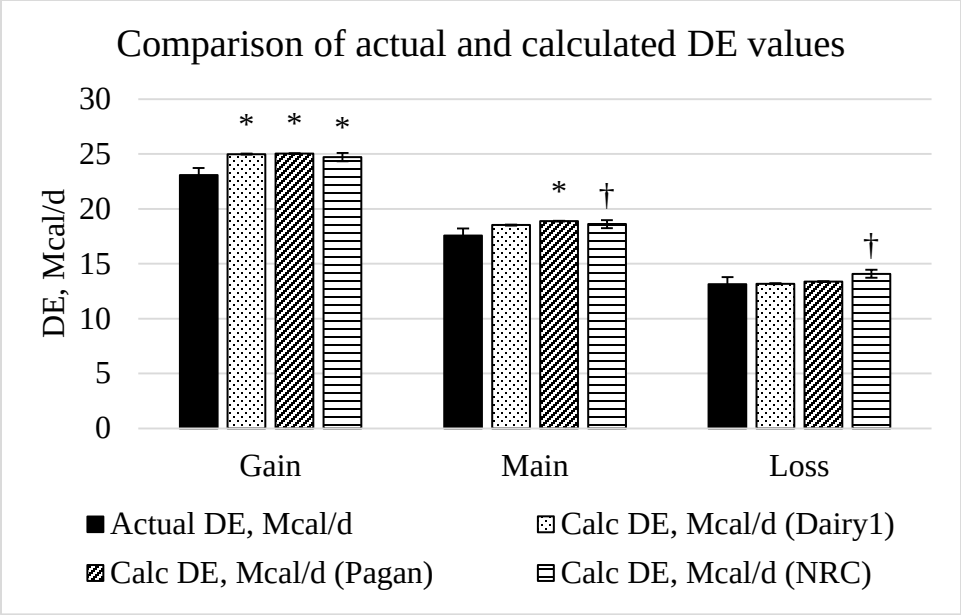


Figure 4-2. Comparison of actual (measured) versus calculated DE values (Mcal/d) for horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means)

*Indicates difference from actual DE ($P < 0.05$).
†Indicates a trend for difference from actual DE ($0.05 < P < 0.10$).

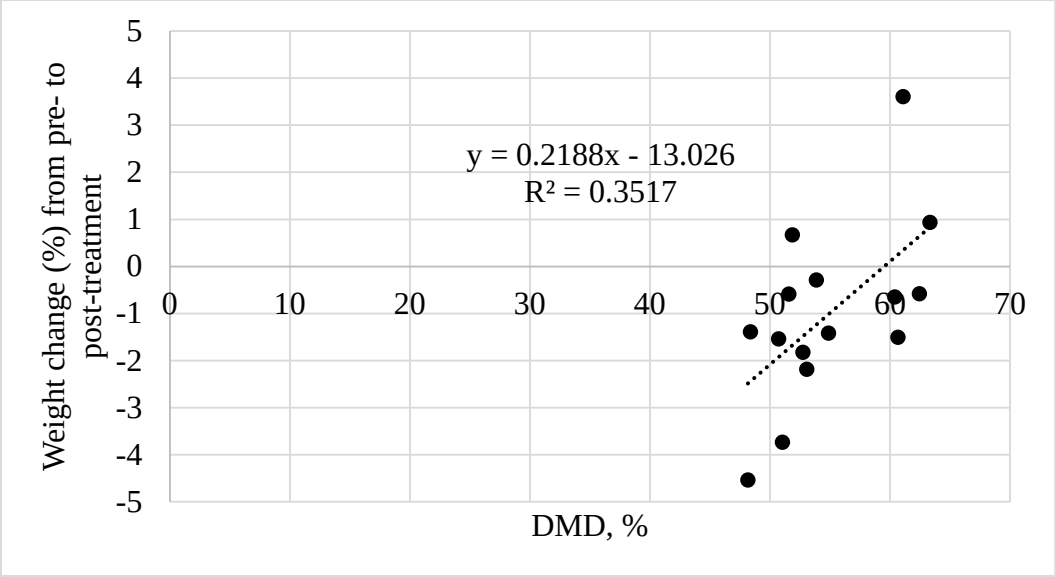


Figure 4-3. Regression of percent weight change from pre- to post-treatment and DM digestibility. $P < 0.05$

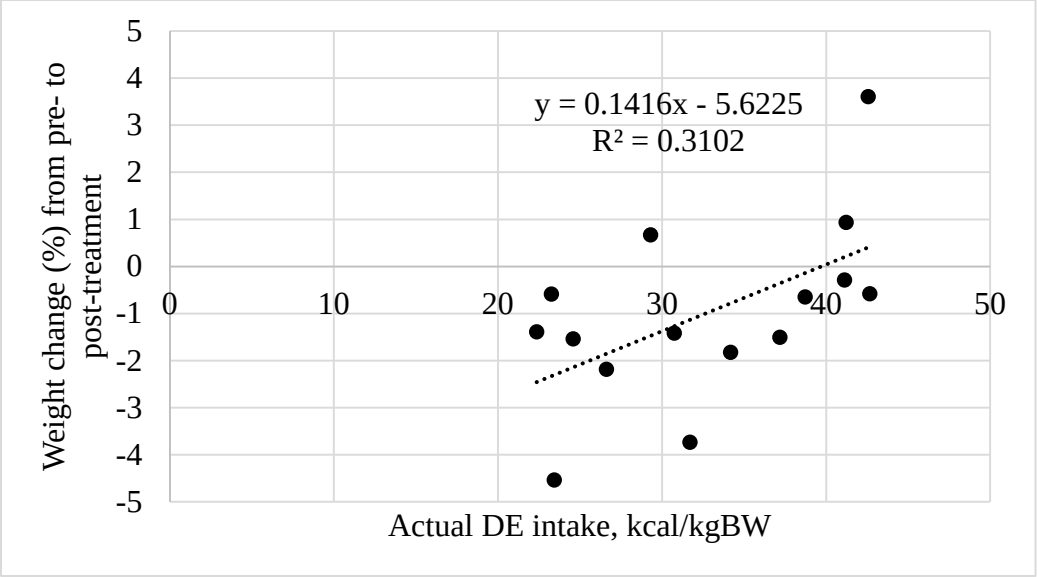


Figure 4-4. Regression of percent weight change from pre- to post-treatment and actual DE intake (kcal/kg BW). $P < 0.05$

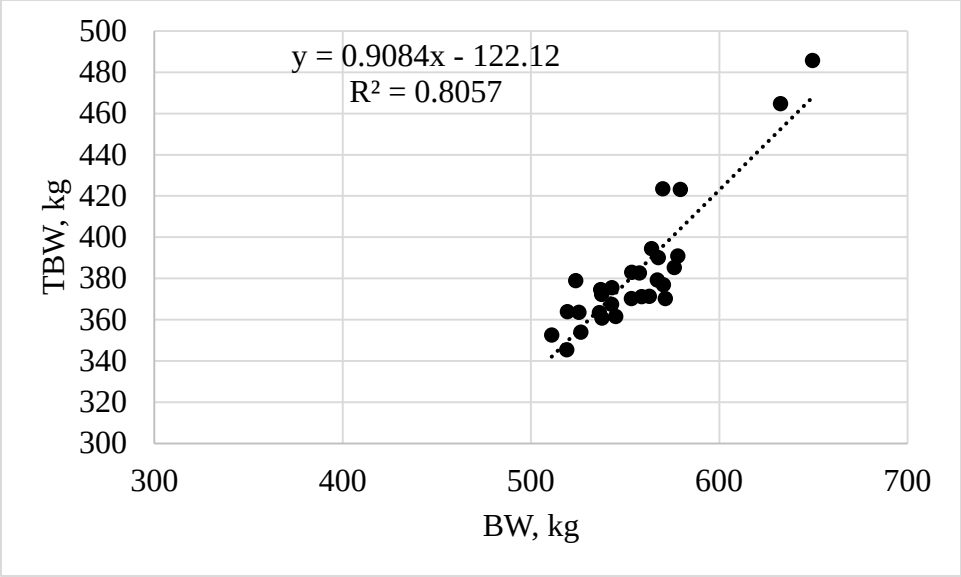


Figure 4-5. Relationship between total body water (TBW) and body weight (BW). P < 0.05.

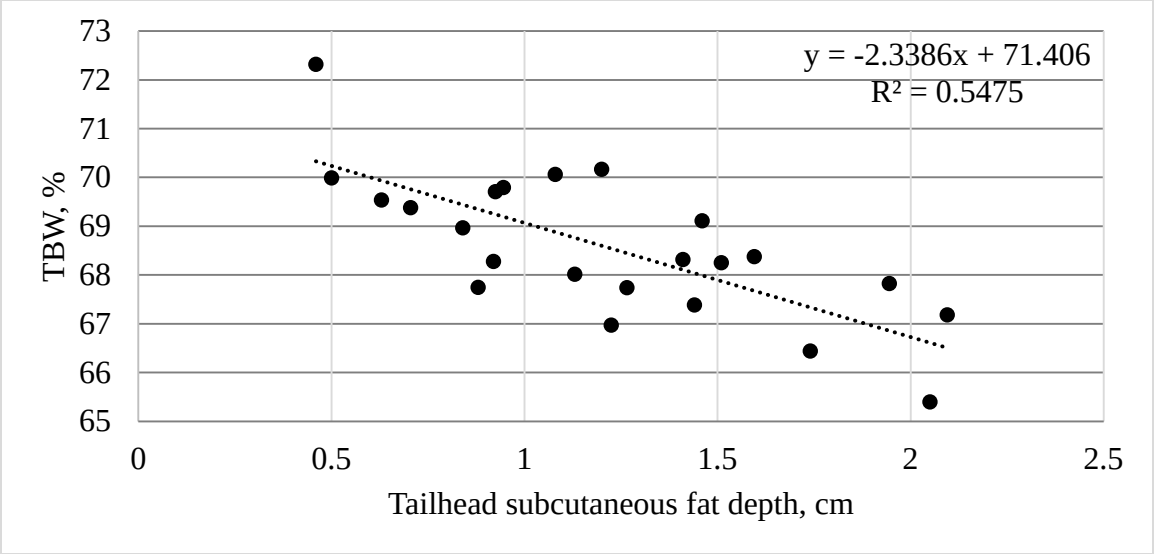


Figure 4-6. Relationship between subcutaneous fat depth at the tailhead and total body water (TBW) for horses with BCS $\geq$ 5. $P < 0.05$

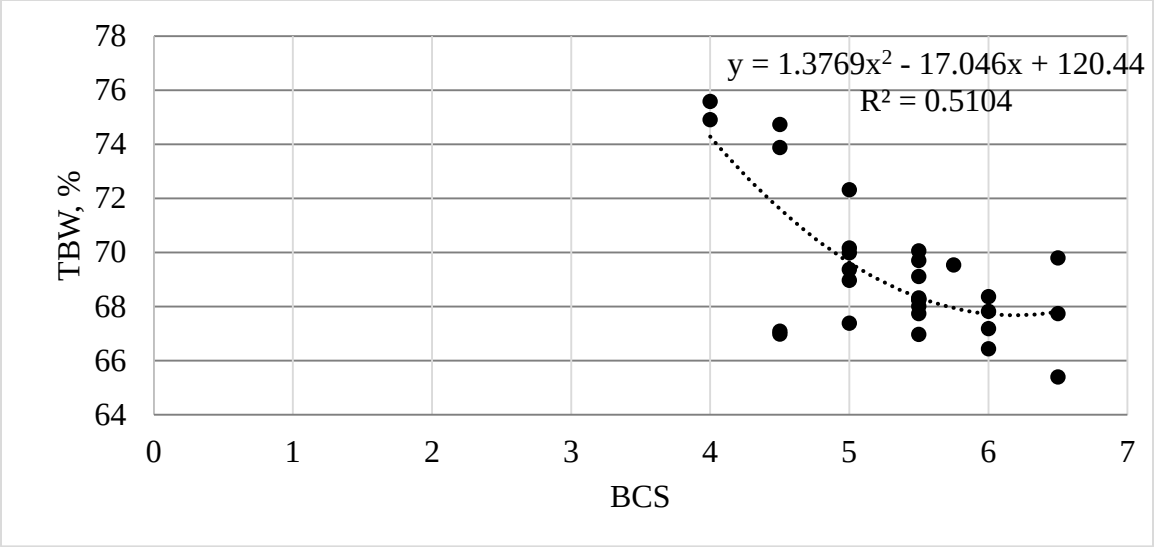


Figure 4-7. Relationship between BCS and total body water (TBW) for all horses. P < 0.05

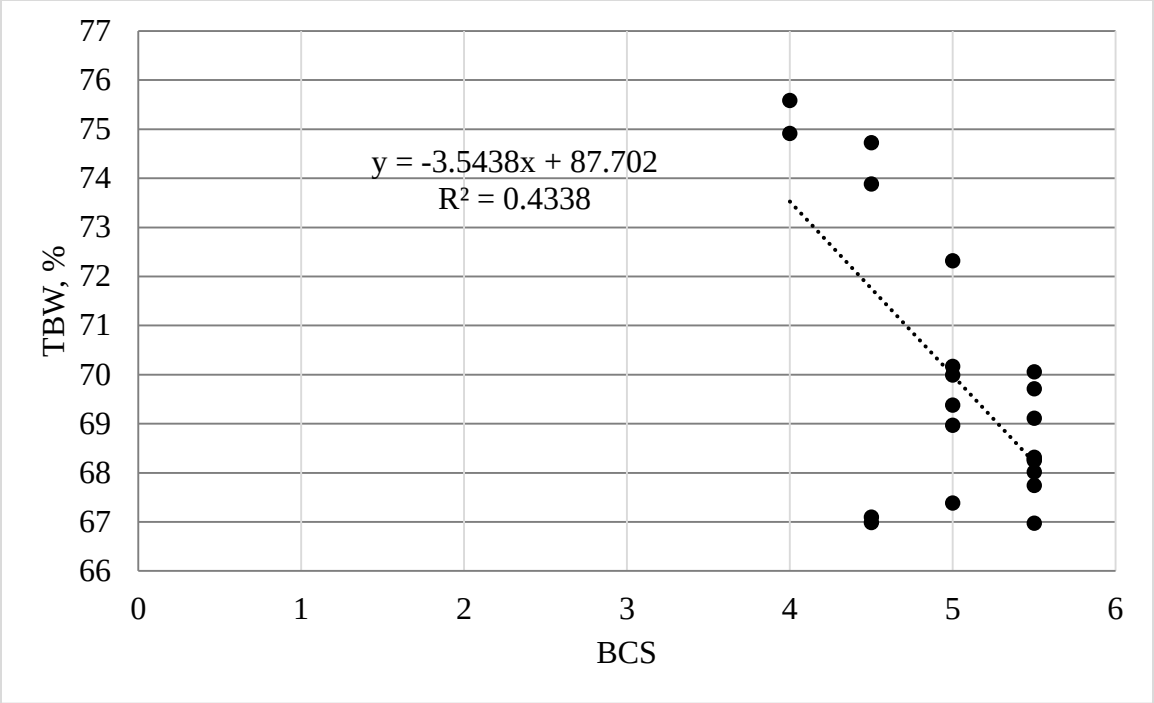


Figure 4-8. Relationship between total body water (TBW) and BCS for horses with a BCS from 4 to 5.5. $P < 0.01$.

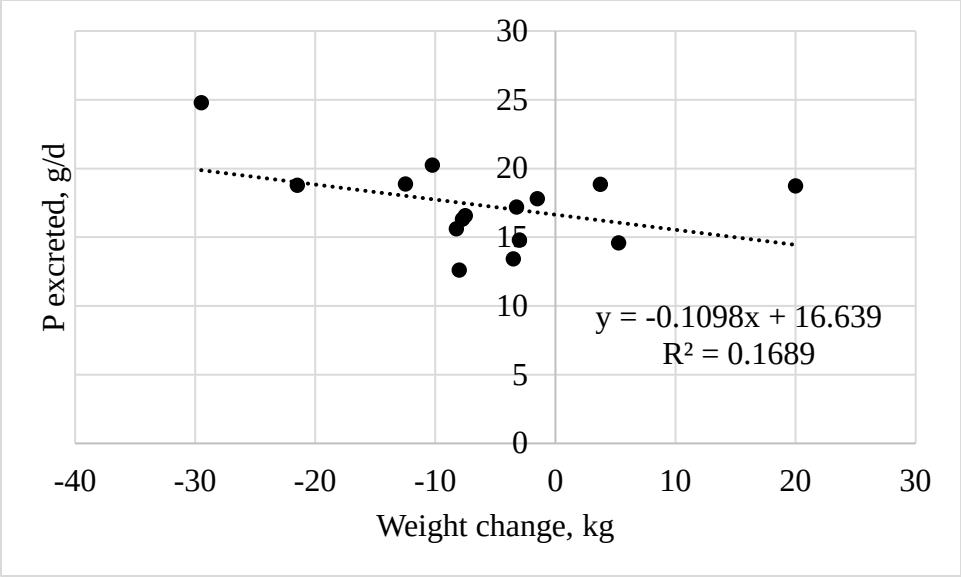


Figure 4-9. Relationship between weight change (kg) over Phase 2 and fecal P excretion (g/d). P = 0.09.

CHAPTER 5: THE USE OF EXTERNAL MARKERS IN HORSES FOR DETERMINING FECAL OUTPUT

Introduction

Indigestible markers have been used in many species as a way to indirectly determine diet digestibility. Exogenous, or externally applied markers, are added to the diet and fecal output and digestibility are calculated by measuring the concentration of the marker in the feces. If every sample of feces has a similar concentration of marker, then the need to collect all of the excreted feces is eliminated.

The main benefit of using external markers is that total fecal collections do not need to be performed; only fecal grab samples are needed. Total fecal collections are not only time and labor intensive, they can also disrupt the normal management of horses, which can lead to feed refusals, reduced exercise, or other stress-related responses. The use of markers also allows digestibility to be determined in horses that would be difficult to maintain in a fecal collection harness, such as lactating mares or exercising horses. Additionally, the sampling period can be extended beyond the normal 5 to 7 d as there is less labor involved in collecting fecal grab samples.

Two external markers are common in animal digestibility studies: chromic oxide (Cr_2O_3) and titanium dioxide (TiO_2) (Sales, 2012). While Cr_2O_3 is probably the most regularly used external marker in horses (Sales, 2012), TiO_2 is gaining popularity in the equine literature (Schaafstra et al., 2012; Winsco et al., 2013).

Excretion of Cr_2O_3 has been shown to vary diurnally and to be related to time of marker dosing (every 12 h) (Haenlein et al., 1966; Parkins et al., 1982; Cuddeford and Hughes, 1990). However, this diurnal excretion pattern could also be related to diurnal

fecal excretion, as large intestinal motility also varies throughout the day (Williams et al., 2011). Most studies only dose external markers once or twice daily in conjunction with feeding (Haenlein et al., 1966; Holland et al., 1998), and an increased frequency in meal feeding and marker dosing may eliminate some of the diurnal effects observed.

To date, there have been no studies validating the use of TiO_2 to indirectly determine fecal output from horses via fecal grab samples. Winsco et al. (2013) used TiO_2 to determine voluntary dry matter intake in gestating mares, but no validation was described. Schaafstra et al. (2012) fed TiO_2 to ponies and used TiO_2 concentration in the total collected feces to calculate total fecal output. The authors reported that 97% of the TiO_2 was recovered in the feces and calculated total fecal output was not different than actual fecal output, but the use of grab samples to estimate total fecal output was not evaluated.

Our long term objective is to develop a system to accurately assess true P digestibility in horse feeds. However, because of the GI recycling of absorbed P, the best experimental design would be to use animals that are either retaining P (i.e. growing or pregnant) or are secreting P into milk (i.e. lactating). In each of these cases, long term confinement and use of fecal collection harnesses present logistical issues. Thus, the objective of this study was to evaluate the use of Cr_2O_3 and TiO_2 for calculating total fecal output using fecal grab samples in horses so that P digestibility could be studied more effectively.

Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Experiment 1

This experiment was performed in conjunction with the study described in Chapter 3. Briefly, eight Thoroughbred mares were used to evaluate the use of two indigestible markers in determining digestibility indirectly. Four mares were assigned to receive Cr_2O_3 and four mares were assigned to receive TiO_2. Experimental diets consisted of timothy cubes, a pelleted concentrate, and soybean oil. All horses received the same ration at the same DMI on a BW basis. Daily rations were divided into two equal parts and fed twice daily. The assigned marker was top-dressed on the timothy cubes at each feeding (10 g TiO_2/d or 6.84 g Cr_2O_3/d) and complete consumption was ensured by careful observation of the feed buckets.

The experiment was conducted in two periods with four mares in each period (two mares receiving TiO_2 and two mares receiving Cr_2O_3). Each period consisted of a 22-d diet adaptation and a 4-d total fecal collection. For the first 12 d of adaptation, horses were gradually adjusted to their experimental diet and adapted to their housing conditions. On d 13 of adaptation (10 d before total fecal collections began), horses were fully transitioned to the experimental diet and began receiving their assigned marker. Two days before total fecal collections began, the mares were fed half of their daily ration and half of their daily marker dose every 12 h.

During the 4-d fecal collection period, horses were fitted with fecal collection harnesses (Bun-bag, Sagle, ID) and monitored closely to ensure that all feces were collected in the bags. To ensure that urine did not enter the fecal collection bag, a plastic shield was attached under the tail of the mare to block urine from entering the fecal collection bag, but allowed feces to fall into the bag.

A 250-g fecal sample was removed from the fecal collection bag every 4 h for determination of marker concentration. To simulate a fecal "grab sample," feces were not mixed prior to removing the 250-g sample. The remaining daily output of feces was compiled for each horse, thoroughly mixed, and a 10% subsample was saved for analysis at the end of each 24-h period. The weight of each grab sample was added to the weight of the remaining feces collected to determine total fecal output.

Experiment 2

This experiment was performed in conjunction with the study described in Chapter 4. Briefly, 15 horses were assigned to three treatment diets designed to cause weight gain, weight maintenance or weight loss. The diets were formulated to contain low forage (LO), medium forage (MED), or high forage (HI). Dry matter intakes were similar for MED and LO (14 g/kg BW) and lower for HI (12 g/kg BW). Horses were fed these diets for 3 to 4 wk before a 5-d total fecal collection period was performed.

At the beginning of the adaptation period, the horses' daily rations were split into two equal meals fed at 0700 h and 1530 h. All feed ingredients for each meal were combined into one bucket and thoroughly mixed together, resembling a total mixed ration (TMR). At least 12 d prior to the beginning of total fecal collections, the daily ration was

split into 3 equal meals fed at 0700 h, 1500 h, and 2300 h. Concurrently, horses began to be dosed with 2.00 g TiO_2/kg DM each day. The daily dose of TiO_2 was split into three equal portions and thoroughly mixed into each of the three daily TMR meals.

During the total fecal collection period, geldings were fitted with fecal collection harnesses (The Horse Diaper, Equisan Marketing Pty Ltd., Victoria, Australia; Nappy, Stablemaid Horse Hygeine and Waste Management, Australia) that allowed for the separate capture of all feces and urine. Mares were fitted with fecal collection harnesses (BunBag, Sagle, ID) as in the previous experiment to allow for capture of all feces. Horses were adapted to their collection apparatuses for at least 2 wk prior to the collection period. During the collection period, horses remained in stalls and were hand-walked for 15 min twice daily. Horses were monitored throughout the collection period. Spot samples of feces (approximately 250 g each) were planned to be taken every 4 h for indirect determination of digestibility by measuring TiO_2. Bags were checked at least every 2 h and if feces was present, the time was noted and the feces were saved. At the 4-h timepoint, the fecal sample that was collected closest to the planned timepoint was weighed and saved for analysis. All remaining feces were composited for each horse over each 24-h period. At the end of a 24-h period, feces were thoroughly mixed, weighed, and a 10% subsample was saved for analysis. Total daily fecal output was calculated by adding the weight of composite feces and the weights of all the spot samples of feces in the 24-h period.

Sample analysis

Fecal DM was determined for each spot sample by drying the 250 g samples in a 55° C forced air oven for at least 72 h. Dry matter of feed and daily composite feces were determined by drying 1 g of sample in duplicate for 24 h at 55° C.

Titanium dioxide was determined in duplicate spot samples from Experiment 1 using a method described by Short et al. (1996), as modified by Titgemeyer et al. (2001) (Appendix B). Chromic oxide was determined in composite fecal samples and selected spot samples by atomic absorption spectrophotometry using a method described by Williams et al. (1962).

Due to greater fecal TiO_2 concentrations in Experiment 2 compared with Experiment 1, complete dissolution of TiO_2 was not achieved using the method described by Short et al. (1996). Thus, some adjustments were made to the method as suggested by Myers et al. (2004) to ensure complete digestion of the samples. The complete method is described in detail in Appendix C. Duplicate spot samples of feces from Experiment 2 were analyzed using this method.

Calculations and statistics

For both experiments, actual daily fecal output (aFO) was measured during total fecal collections and was also calculated using markers (cFO). Fecal output was calculated by using the following equation: cFO (kg/d) = X (g/d) ÷ $[X]_{feces}$ (g/kg), where X represents amount of marker dosed per day and $[X]_{feces}$ represents the concentration of marker in the feces. This value (cFO) was calculated for fecal composite samples from Experiment 1 for both TiO_2 and Cr_2O_3, as well as in every spot sample of feces for TiO_2

in Experiment 1 and 2. Percent accuracy of the marker in predicting aFO was calculated by dividing cFO by aFO and multiplying by 100. Fecal recovery of the TiO_2 was calculated as: $\frac{\text{avg \% Ti in spot samples} \times \text{aFO,kg} \times 10}{\text{Ti intake,g}} \times 100$. Fecal recovery of Cr_2O_3 was calculated as: $\frac{\text{\% Cr in composited feces} \times \text{aFO,kg} \times 10}{\text{Cr intake,g}} \times 100$. Calculated fecal output was then corrected for fecal recovery of the marker (cFO x % recovery). Calculated fecal output corrected for fecal recovery could not be accurately calculated for Cr_2O_3 because Cr_2O_3 concentrations in composite samples were used to determine percent recovery (based on aFO) as well as cFO, so corrected cFO would be equal to aFO in this case.

Data were examined in a progressive fashion. First, all available data were averaged across all horses to obtain an overall average. Then data were averaged by horse to examine variation among individuals. Third, data were averaged by day and by horse to investigate whether there were differences among days of collection for individual horses. Next, data were examined by day. Finally, percent accuracy was evaluated by hour of collection for individual horses.

For the methods of examining data as described above, the following statistics were employed (SAS 9.4; SAS Institute, Inc, Cary, NC). For all experiments, aFO was compared to cFO using a paired t-test. Percent accuracy was tested to determine if the value was different from 100% by using a t-test with the null hypothesis equal to 100. Additionally for Experiment 2, data were also separated by treatment, and the effect of dietary treatment on aFO, cFO, percent accuracy, fecal recovery was analyzed using repeated measures ANOVA with block as the random effect and day of collection as the repeated measure. If there were significant differences, means were separated using an

LSD test. Additionally, simple linear regression was used to examine the relationship between aFO and cFO. Data are presented as LS means ± SE. Significance was considered when $P < 0.05$.

Results and Discussion

Experiment 1 – Chromic oxide

Out of the four horses fed Cr_2O_3, one horse's composited feces on d 1 was discarded before a sample could be taken, but the remaining 3 d for this horse were included. Chromic oxide was analyzed in composite samples first, to evaluate the efficacy of Cr_2O_3 in estimating aFO.

Table 5-1 shows aFO, cFO, percent accuracy of cFO in predicting aFO, and fecal Cr_2O_3 recovery, for each horse on each day, as well as overall average values for horses fed Cr_2O_3. Overall, aFO for all horses averaged 2.72 kg DM and was greater than cFO, which was 2.25 kg DM ($P < 0.05$). Overall accuracy of cFO in predicting aFO averaged 84.6%, but ranged from 70.0 to 107.4% for individual horses on individual days. Fecal recovery of Cr_2O_3 averaged 82.25% and ranged from 71.87 to 97.76% for individual horses on individual days.

There was a significant relationship between aFO and cFO when using the values for each horse on each day (Figure 5-1), with a moderate R^2 ($R^2 = 0.4360$). This suggests that cFO was able to account for less than 50% of the variation in aFO. In order to investigate where the variation might be originating from, the horse or the day of collection, data were averaged by day of collection.

Table 5-2 shows aFO, cFO, percent accuracy, and fecal Cr_2O_3 recovery averaged by day of collection. Percent accuracy was poor on d 1 and d 3 as these values were different than 100% ($P < 0.05$), but percent accuracy was not different from 100% on d 2 and d 4 ($P > 0.10$). The lowest accuracy was observed on d 1 and the greatest was observed on d 4. This overall increase in accuracy may suggest that longer fecal collection periods could result in even greater increases in percent accuracy. In this study, Cr_2O_3 was fed for 10 d before total fecal collections and the 12 h feeding schedule was started just 48 h before fecal collections. Although mean retention time in horses is usually less than 30 h (Hansen, 2014), the 48-h period prior to marker collections may not have been long enough to stabilize excretion. It is possible that Cr_2O_3 needs to be fed longer for a stable excretion in the feces to be obtained, so Cr_2O_3 excretion over time (concentration in fecal spot samples) was examined next.

Before all fecal spot samples were analyzed for Cr_2O_3, spot samples were selected for each horse to examine any trends in fecal excretion. One sample per d per horse was selected and the time of collection each d was advanced by 4 h in an attempt to observe any diurnal variation. Figure 5-2 shows the Cr_2O_3 concentrations from fecal spot samples for each horse. Fecal Cr_2O_3 concentration in the first samples collected was close to 0.2%. For three horses, the fecal Cr_2O_3 concentration appeared to stay relatively constant after d 2, but increased to 0.35% for one horse. These data suggest that stable excretion was not established until at least d 2 of fecal collection. An alternate explanation is that despite being adapted to fecal collections harnesses for a few h each d before total collections began, fecal excretion may have been impacted by the change in routine during the collection period. As shown in Table 5-2, aFO on d 1 was 3.07 kg and

decreased to 2.52 kg on d 2. It is possible that this decrease in fecal output was also caused by horses adapting to their new management routine.

Experiment 1 – Titanium dioxide

Similar to Cr_2O_3, concentration of TiO_2 was first evaluated in the fecal composite samples to evaluate the potential of cFO to predict aFO. For the composite samples, cFO was 2.67 ± 0.17 kg DM and aFO was 2.57 ± 0.19 kg DM (data not shown). These values were not different from each other ($P > 0.10$). Percent accuracy of cFO in predicting aFO was 104.5 ± 6.9% and fecal recovery of the marker in the composite samples was 100.1 ± 6.8%. Similarly, Schaafstra et al. (2012) reported fecal recovery of TiO_2 in composite samples to be 97% and that organic matter digestibilities were similar when calculated with aFO and cFO. These results suggest that TiO_2 is more promising than Cr_2O_3 as a candidate for use as an external marker. Thus, TiO_2 was analyzed in every collected spot sample to look for patterns in excretion and to determine the ability of fecal TiO_2 concentration of spot samples to determine fecal output.

Using data generated from spot samples, Table 5-3 shows aFO, cFO, percent accuracy, fecal TiO_2 recovery, and cFO corrected for fecal TiO_2 recovery for each horse on each day. Across all horses and all days, average aFO was 2.57 kg DM and cFO was 2.79 kg DM ($P < 0.05$). Percent accuracy of cFO in calculating aFO was 110.2% and ranged from 85.7 to 144.0% among horses and days. The over-prediction of aFO by cFO could be explained by fecal recovery of the marker. Fecal recovery in spot samples averaged 94.70 ± 7.22% and ranged from 70.3 to 117.6%. When cFO was corrected for fecal recovery, cFO averaged 2.62 ± 0.20 kg and was not different from aFO ($P > 0.10$).

Percent accuracy of cFO corrected for fecal recovery averaged 101.99 ± 1.05 and ranged from 100.16 to 105.12%. The inaccuracy of uncorrected cFO is likely due to inaccuracies in recovering marker in the feces. However, in order to calculate fecal recovery values, the aFO needs to be known. Because the goal of using external markers is to eliminate the need for total fecal collections, fecal recoveries would not be known for studies solely relying on spot samples for determining FO. Thus, in those studies, uncorrected cFO would be the only obtainable value. One could assume a constant fecal recovery in an attempt to correct cFO, however the large range in fecal recoveries observed in the current study (70.3 to 117.6%) indicates that this may not be a practical solution.

Concentrations of TiO_2 in spot samples were averaged by horse on each day and the relationship between aFO and cFO was examined. The relationship was not significant and very weak ($R^2 = 0.1484$; Figure 5-3), indicating other sources of variation, such as day of collection or individual animal, may be influencing the relationship. Therefore, data were then averaged by day.

Using TiO_2 concentrations in spot samples, Table 5-4 shows the cFO and percent accuracy of cFO at predicting aFO by day. There was no effect of day on aFO or cFO ($P > 0.10$). On d 1 of collection, the percent accuracy is 123% and exhibits the largest variation compared to the rest of the days. As with Cr_2O_3 on d 1, the large amount of variation may be due to horses having to adapt to fecal collection harnesses or to the change in feeding schedule 48 h before d 1 of fecal collections. The percent accuracy decreases to the lowest observed value (102%) by d 3. However on d 4, accuracy is decreased, straying further from 100%.

Figure 5-4 shows fecal TiO_2 concentrations in fecal spot samples collected for all horses over the course of the experiment. Fecal TiO_2 concentrations show a large amount of variation for most horses during the first 40 h of the experiment, ranging from 0.2 to 0.45%. However, fecal concentrations appear to become more stable after h 40 for three of the horses, staying mainly in the range of 0.3 to 0.45%. One horse (Lizzy) continued to have large amounts of variation in fecal excretion patterns.

There appear to be peaks and valleys in the fecal concentrations of TiO_2. Haenlein et al. (1966) noted diurnal variation in fecal excretion of Cr_2O_3 that was related to meal time. Because horses in this experiment and in the experiment by Haenlein et al. (1966) were only fed twice per d, it is possible that fecal TiO_2 excretion exhibited fluctuations due to meal time. The marker was top-dressed on the timothy cubes and concentrate was offered in a separate bucket in this experiment. The horses may have consumed their concentrate first, then began to eat the timothy cubes. The top-dressing of the marker on the timothy cubes may have caused the horses to ingest a large amount of the marker at the start of eating the timothy cubes, and little marker towards the end of the meal. Additionally, eating rate could vary among horses. This feeding behavior may have resulted in incomplete mixing in the digestive tract, which could cause the spikes in fecal excretion observed in this experiment. Additionally, horses in this experiment consumed their meal in under 2 h, so they were without food for approximately 10 h before the next meal was fed. As meal feeding can influence gut motility and rate of passage (Van Weyenberg et al., 2006), it is possible that fecal excretion was influenced by meal feeding. Feeding more frequent meals and thoroughly mixing the marker into the

complete ration may help eliminate some of the daily variation seen in fecal TiO_2 concentration.

Overall, TiO_2 appeared to have more potential for use as an external marker due to better fecal recovery rates. Therefore, the next experiment utilized more horses, more frequent meal feedings, and homogenous mixing of the marker into the meal in an attempt to improve the accuracy of TiO_2 as a potential external marker.

Experiment 2

Titanium dioxide was only analyzed in the spot samples in Experiment 2. For all horses averaged across d, aFO (3.31 kg DM) was not different from cFO (3.21 kg DM; $P > 0.10$; Table 5-5). When separated by treatment diet, the average values of cFO and aFO for each horse were compared and horses fed MED and LO diets did not show differences between aFO and cFO ($P > 0.05$; Table 5-5). However, for horses fed the HI diet, aFO was greater than cFO ($P < 0.05$; Table 5-5). The accuracy of cFO in predicting aFO averaged 98.1% overall and was not different among treatments (Table 5-5; $P > 0.10$). Fecal recovery of TiO_2 was 106% and was not different among treatments (Table 5-5; $P > 0.10$). When cFO was corrected for fecal recovery, accuracy of cFO in predicting aFO averaged 101% and was not different among treatments (Table 5-5; $P > 0.10$), but was different from 100% ($P < 0.05$). However, accuracy of cFO in predicting aFO was not different from 100% for individual horses or for each treatment ($P > 0.10$). Low variation within treatment but larger variation between treatments could have caused the average percent accuracy to differ, while individual treatments were not different.

Table 5-6 shows accuracy of cFO and fecal recovery of TiO_2 by d for each treatment as well as the overall average. On average, percent accuracy of cFO in predicting aFO was not different from 100% on d 1, 3, and 4 ($P > 0.10$). Percent accuracy was less than 100% on d 2 and 5 ($P < 0.05$). Horses fed HI appeared to have less variation in accuracy from day to day, only ranging from 91 to 100%. Horses fed MED and LO both have wider ranges for accuracy, with MED accuracy ranging from 90 to 108% and LO ranging from 91 to 120%. Less variation in percent accuracy in horses fed a high-forage diet may indicate that fecal excretion of the marker was more stable across days, whereas horses fed medium forage and low forage diets had a more pulsatile excretion of marker across days. Because the medium forage and low forage diets had more ingredients in the mixed ration, it is possible that these horses were able to sort ingredients as they ate. Feed sorting could result in a less homogenous mixture of feed in the GI tract.

Fecal TiO_2 recovery also appeared to vary by day, with recovery for all horses increasing on d 2, then decreasing on d 3, then increasing again on d 4 and 5 (Table 5-6). Again, fecal recovery for horses fed HI had a range from 101 to 113%, while the ranges for MED and LO were wider. These data also suggest that fecal excretion of TiO_2 was not steady, but may be more pulsatile across days, particularly for horses fed MED and LO.

To further examine the variation among horses fed different diets, data were averaged for each horse on each day and aFO was plotted against cFO. When all horses were included, the relationship between aFO and cFO was significant but very poor (Figure 5-5a; $P < 0.05$; $R^2 = 0.0855$). Overall, cFO overestimated aFO, which was

potentially caused by fecal recoveries greater than 100%. However, there were three horses that had feed refusals during the fecal collection period (two from LO diet and one from HI), which may have affected fecal TiO_2 excretion. While TiO_2 in the orts was measured and subtracted from TiO_2 offered to obtain actual TiO_2 intakes, differences in TiO_2 intakes among days could have had an effect on fecal excretion of TiO_2. Therefore, the relationship between cFO and aFO was also examined with only horses that did not have feed refusals. When only horses that did not have feed refusals were included, the R^2 increased to 0.1373 (Figure 5-5b; $P < 0.05$), which suggests that refusals may have slightly influenced excretion of TiO_2, but did not explain much of the variation in the relationship.

Data for each horse were then averaged across the entire fecal collection period in order to remove variation that may be due to day. When all horses were included, the relationship between aFO and cFO tended to be significant, but was relatively weak (Figure 5-6a; $P = 0.0854$; $R^2 = 0.2104$). When only horses that did not have feed refusals were included, the relationship became significant, the R^2 increased to 0.3611 and the slope increased to 0.64 (Figure 5-6b; $P < 0.05$). It is possible that interday variation resulted in overestimation of aFO, so when values were averaged over 5 d, the variation decreased among horses.

Relationships between aFO and cFO were then examined by treatment to determine if the diet could explain some of the variation. First the average values for each horse on each day were examined (Table 5-7). When all horses were included, there was no relationship between aFO and cFO for horses fed MED and LO ($P > 0.10$) and only a weak relationship ($R^2 = 0.1671$) for horses fed HI ($P < 0.05$). When horses with refusals

were removed, more of the variation in the relationship for HI was explained (R^2 = 0.2243), but still only accounted for less than 25%.

Therefore, data for each treatment was averaged over all days for each horse and relationships were re-examined (Table 5-8). There were no relationships between aFO and cFO when data included all horses for each treatment ($P > 0.10$). When horses with refusals were removed, the only relationship existed for horses fed LO ($P < 0.05$) and the variation explained by cFO was very high (R^2 = 0.9992). However, there were only three data points used in this analysis, so results should be interpreted with caution. While the relationships were not significant, when data were averaged across days, the slopes appeared to be closer to 1 than those observed when data were averaged for each horse on each day.

Lastly, concentrations of TiO_2 in individual fecal spot samples were examined over time for each horse to determine if there were any patterns of excretion that may have caused the variation among days. For the horses fed LO, TiO_2 concentrations appeared to be more stable during the first 30 to 40 h of the experiment (Figure 5-7). Then two horses began to have erratic patterns of excretion. One horse (Vision) did have refusals during the last days of the collection period, so that may have influenced the fecal excretion of TiO_2. The other horse with large variation in fecal excretion (Matty) did not have refusals, differences in fecal output among days, or differences in fecal DM among days (data not shown), so it is unclear what caused the erratic pattern observed.

For horses fed MED, the excretion of TiO_2 appears more stable than LO across time, ranging from approximately 3 to 6 g TiO_2/kg DM (Figure 5-8). However, there are

some peaks and valleys for individual horses. The most stable marker excretion overall was observed for horses fed LO, with fecal TiO_2 concentrations ranging from approximately 3 to 5 g TiO_2/kg DM (Figure 5-9). There were two horses (Emi and Rancho) that started with concentrations around 3 to 3.5 g TiO_2/kg DM at the beginning of the collection period, and then increased to around 4 to 5 g TiO_2/kg DM by h 40 of the experiment. One of these horses (Rancho) did have orts on d 2, which may explain some of the lower excretion during the beginning of the experiment. The remaining horses all had relatively stable marker excretion across the experiment, with concentrations staying between 4 and 5 g TiO_2/kg DM throughout.

The relatively stable marker excretion seen in HI, but not in LO or MED may have been influenced by rate of meal consumption or differences in passage rates among the diets. While time to finish each meal was not recorded in this study, other researchers have observed that increasing the amount of chopped hay to concentrate decreased the rate of intake, leading to longer feeding times (Campbell et al., 2005). Additionally, rate of passage of high-forage diets is typically faster than for high concentrate diets (Jouany et al., 2008), which may impact the rate of fecal excretion.

Conclusion

Results from Experiment 1 showed promise for TiO_2 as an acceptable marker for estimating FO. However, due to large variation among individual fecal samples, one random fecal sample was not able to accurately predict total fecal output, so Experiment 2 had more frequent marker dosing in an attempt to reduce intraday variation. While more frequent marker dosing did not result in stable fecal excretion, when averaged across 5 d, accuracy of TiO_2 to predict aFO is increased. Additionally, diet composition

may also influence accuracy of markers in predicting fecal output, with high forage diets showing more constant fecal excretion patterns compared to low forage diets.

Improvements for future research in this area might include feeding TiO_2 for a longer period of time prior to fecal sampling, increase the feeding frequency, and combining all feed ingredients and TiO_2 into a complete pelleted feed to reduce the instances of sorting. Additionally, meal size could be increased or feeding rate reduced so that horses spend more time eating throughout the day. This practice might reduce the variation in fecal excretion patterns observed post-prandially.

Tables and Figures

Table 5-1. Actual fecal output (aFO), calculated fecal output (cFO), percent accuracy of cFO, and fecal recovery for horses fed Cr_2O_3 in Experiment 1. Values were calculated from marker concentrations in the composite fecal sample for each horse on each day.

Horse	Day	aFO, kg DM	cFO, kg DM	Accuracy of cFO, %	Fecal recovery, %
Dara	1	2.78	2.37	85.24	80.25
Dara	2	2.45	2.19	89.28	76.61
Dara	3	2.78	2.10	75.62	90.45
Dara	4	3.00	2.28	76.02	89.97
Dara AVG		**2.75[a]**	**2.24[b]**	**81.54***	**84.32**
Silk	1[1]	--	--	--	--
Silk	2	2.02	2.09	103.43	66.13
Silk	3	2.53	2.03	80.14	85.35
Silk	4	2.42	2.13	87.86	77.85
Silk AVG		**2.33**	**2.08**	**90.48**	**76.45**
Sirocco	1	3.72	2.70	72.51	94.33
Sirocco	2	3.24	2.64	81.66	83.76
Sirocco	3	2.72	2.16	79.36	86.19
Sirocco	4	1.98	2.13	107.44	63.66
Sirocco AVG		**2.91**	**2.41**	**85.24**	**81.99**
Calling	1	2.72	2.55	93.76	72.95
Calling	2	2.37	2.25	95.17	71.87
Calling	3	3.08	2.18	70.80	96.61
Calling	4	3.06	2.14	69.97	97.76
Calling AVG		**2.81**	**2.28**	**82.42**	**84.80**
Overall AVG		**2.72 ± 0.23[a]**	**2.25 ± 0.11[b]**	**84.55 ± 5.79***	**82.25 ± 6.84**

[1]Data for Silk on day 1 is not available due to accidental discarding of composited feces
[ab]Average aFO differs from average cFO ($P < 0.05$)
*Accuracy of average cFO in predicting average aFO is different from 100% ($P < 0.05$)

Table 5-2. Actual fecal ouput (aFO), calculated fecal output (cFO), and percent accuracy of cFO in predicting aFO averaged by day for horses fed Cr_2O_3 in Experiment 1. Means represent the average value for all horses calculated from marker concentrations in the composite fecal samples. Data presented as LS means ± SE.

Day	**aFO, kg DM**	**cFO, kg DM**	**Accuracy, %**	**Fecal recovery, %**
1	3.07 ± 0.28	2.54 ± 0.08	76.90 ± 3.61*	74.24 ± 11.83
2	2.52 ± 0.26	2.29 ± 0.12	86.56 ± 6.66	74.86 ± 2.68
3	2.78 ± 0.11[a]	2.12 ± 0.03[b]	82.93 ± 4.66*	89.65 ± 2.58
4	2.61 ± 0.25	2.17 ± 0.04	92.27 ± 6.73	82.31 ± 7.44
AVG	**2.72 ± 0.23[a]**	**2.26 ± 0.105[b]**	**84.55 ± 5.79***	**82.25 ± 6.84**

[ab]Actual fecal output (aFO) is different than calculated fecal output (cFO, $P < 0.05$)
*Accuracy of average cFO in predicting average aFO is different from 100% ($P < 0.05$)

Table 5-3. Actual fecal output (aFO), calculated fecal output (cFO), percent accuracy of cFO, fecal recovery, cFO corrected for fecal recovery (corr. cFO) for horses fed TiO_2 in Experiment 1. Values represent the average of spot samples over a day for each horse.

Horse	Day	aFO, kg DM	cFO, kg DM	Accuracy of cFO, %	Fecal recovery, %	Corr. cFO, kg DM	Accuracy of corrected cFO, %
Lizzy	1	2.02	2.91	144.04	70.33	2.05	101.31
Lizzy	2	2.29	2.62	114.51	87.47	2.29	100.16
Lizzy	3	2.01	2.60	129.55	77.62	2.02	100.55
Lizzy	4	2.58	2.91	112.97	90.81	2.65	102.59
AVG		**2.23[b]**	**2.76[a]**	**125.27***	**81.56**	**2.25**	**101.15**
Phil	1	2.92	2.50	85.66	117.64	2.94	100.77
Phil	2	2.18	2.41	110.76	90.87	2.19	100.64
Phil	3	2.54	2.43	95.81	105.77	2.57	101.35
Phil	4	2.63	2.54	96.49	104.41	2.65	100.75
AVG		**2.57**	**2.47**	**97.18**	**104.67**	**2.59**	**100.86**
Quick	1	2.55	3.47	136.18	74.56	2.59	101.54
Quick	2	3.04	3.05	100.37	107.34	3.27	107.73
Quick	3	2.9	2.68	92.36	109.45	2.93	101.09
Quick	4	2.29	2.84	123.82	84.55	2.40	104.69
AVG		**2.70**	**3.01**	**113.18**	**93.98**	**2.80**	**103.76**
Skindy	1	2.92	3.69	126.43	81.03	2.99	102.45
Skindy	2	3.19	3.22	100.86	104.23	3.35	105.12
Skindy	3	2.57	2.35	91.39	110.04	2.58	100.56
Skindy	4	2.42	2.46	101.58	99.033	2.43	100.60
AVG		**2.78**	**2.93**	**105.07**	**98.58**	**2.84**	**102.18**
OverallAVG		**2.57 ± 0.18[b]**	**2.79 ± 0.20[a]**	**110.17 ± 8.76***	**94.70 ± 7.22**	**2.62 ± 0.20[b]**	**101.99 ± 1.05***

[ab] Means for aFO, cFO and corrected cFO (corr. cFO) lacking a common letter differ ($P < 0.05$)

*Accuracy of average cFO in predicting average aFO is different from 100% ($P < 0.05$)

Table 5-4. Actual fecal ouput (aFO), calculated fecal output (cFO), and percent accuracy of cFO in predicting aFO averaged by day for horses fed TiO_2 in Experiment 1. Means represent the average value for all horses on each day (calculated from spot samples). Data presented as LS means ± SE.

Day	aFO, kg DM	cFO, kg DM	Accuracy, %	Fecal recovery, %
1	2.60 ± 0.21	3.14 ± 0.27	123.08 ± 12.98	85.89 ± 10.81
2	2.68 ± 0.26	2.93 ± 0.19	106.62 ± 3.56	97.48 ± 4.89
3	2.51 ± 0.18	2.52 ± 0.08	102.28 ± 9.14	100.72 ± 7.76
4	2.48 ± 0.08	2.69 ±0.11	108.72 ± 6.10	94.70 ± 4.39
AVG	**2.57 ± 0.18[b]**	**2.79 ± 0.20[a]**	**110.17 ± 8.76***	**94.70 ± 7.22**

[ab]Average cFO differs from average cFO ($P < 0.05$)
*Accuracy of average cFO in predicting average aFO is different from 100% ($P < 0.05$)

Table 5-5. Actual (aFO), calculated (cFO), corrected fecal output (corr. cFO) and percent fecal recoveries for individual horses fed TiO_2 in Experiment 2. Means represent averaged values from spot samples for each horse across all days.

Horse	Trt[1]	aFO, kg DM	cFO, kg DM	Accuracy for cFO , %	Fecal recovery, %	Corr. cFO, kg DM	Accuracy for corr. cFO , %
Emi	HI	2.91[a]	3.07[a]	106.12	96.59	2.95	101.48
Moses	HI	3.85[a]	3.16[b]	82.41*	122.52	3.87	100.47
Paddy	HI	3.22[a]	3.03[b]	94.58*	106.72	3.23	100.24
Rancho	HI	3.37[a]	3.08[b]	91.84*	110.57	3.39	100.40
Tad	HI	3.10[a]	3.08[a]	100.12	101.36	3.11	100.55
AVG	**HI**	**3.29 ± 0.037[a]**	**3.08 ± 0.022[b]**	**95.01 ± 1.00***	**107.63 ± 1.09**	**3.40 ± 0.038**	**100.63 ± 0.048**
Dino	MED	3.55[a]	3.27[b]	92.14*	110.83	3.61	101.67
Leroy	MED	3.98[a]	3.47[b]	88.07*	115.57	4.01	100.86
Maestro	MED	3.77[a]	3.51[a]	94.95	111.66	3.82	101.36
Sirocco	MED	3.64[a]	3.75[a]	103.43	99.96	3.72	102.23
Susanna	MED	3.02[b]	3.17[a]	105.59*	95.97	3.04	100.73
AVG	**MED**	**3.59 ± 0.036[a]**	**3.43 ± 0.029[a]**	**96.84 ± 1.17***	**105.42 ± 1.24**	**3.60 ± 0.036**	**101.37 ± 0.092**
Eggs	LO	3.00[a]	2.66[b]	88.71*	114.74	3.04	101.22
George	LO	3.02[a]	2.74[b]	91.72*	112.58	3.06	101.47
Matty	LO	3.30[a]	3.78[a]	116.28	92.95	3.45	104.84
Oliver	LO	3.00[a]	2.66[b]	88.79*	114.59	3.04	101.17
Vision	LO	2.94[b]	3.71[a]	127.05*	85.85	3.01	102.64
AVG	**LO**	**3.05 ± 0.026[a]**	**3.11 ± 0.062[a]**	**102.47 ± 2.16**	**104.37 ± 1.52**	**3.15 ± 0.028**	**102.27 ± 0.39**
All horses		**3.31 ± 0.022[a]**	**3.21 ± 0.033[a]**	**98.10 ± 1.10**	**106.16 ± 0.74**	**3.36 ± 0.022**	**101.42 ± 0.14***

[1]HI = high forage diet; LO = low forage diet; MED = intermediate level of forage diet

[ab]Means for aFO and cFO lacking a common letter differ ($P < 0.05$). Treatment and overall effects were analyzed using average values by horse. Each horse effects were analyzed using all collected spot samples.

*Percent accuracy is different from 100% ($P < 0.05$)

Table 5-6. Accuracy of calculated fecal output (cFO) in predicting actual fecal output (aFO) and percent fecal recovery of TiO_2 averaged by day of collection for each treatment in Experiment 2. Means represent values averaged for each day across all horses in each treatment (calculated from spot samples). (LS means ± SE)

Day of collection	**HI[1]**	**MED[1]**	**LO[1]**	**All horses**
Accuracy for cFO, %				
1	97.09 ± 1.71	101.43 ± 0.71	105.19 ± 1.10	101.24 ± 0.53
2	92.18 ± 2.10	93.92 ± 1.20	90.80 ± 1.43	92.32 ± 0.67*
3	99.86 ± 1.02	108.27 ± 2.23	94.64 ± 1.03	100.92 ± 0.69
4	90.85 ± 0.40	89.85 ± 1.05	119.58 ± 5.26	100.09 ± 1.45
5	95.09 ± 0.93	90.70 ± 1.48	101.75 ± 2.86	95.85 ± 0.83†
Fecal recovery, %				
1	106.40 ± 2.10	99.89 ± 0.82	98.08 ± 0.92	101.46 ± 0.61
2	112.69 ± 2.04	109.55 ± 1.49	113.90 ± 1.62	112.04 ± 0.72
3	101.27 ± 1.08	96.66 ± 2.01	106.81 ± 1.24	101.58 ± 0.65
4	111.05 ± 0.42	114.21 ± 1.41	99.07 ± 3.31	108.11 ± 0.92
5	106.34 ± 0.98	113.68 ± 1.71	102.86 ± 2.22	107.63 ± 0.74

[1]HI = high forage diet; LO = low forage diet; MED = intermediate level of forage diet
*Average accuracy for all horses differs from 100% ($P < 0.05$)
†Average accuracy for all horses tends to differ from 100% ($P = 0.0527$)

Table 5-7. Relationships between actual fecal output (aFO) and calculated fecal output (cFO) from TiO_2 for each treatment in Experiment 2. Data were averaged by horse on each day.

	HI[1]	**MED[1]**	**LO[1]**
All horses			
Intercept	1.14	2.94	2.78
Slope	0.70	0.19	0.088
R^2	0.1671	0.0224	0.0431
P-value	0.0425	0.4751	0.3192
Horses with refusals removed			
Intercept	0.60	2.94	2.61
Slope	0.87	0.19	0.16
R^2	0.2243	0.0224	0.0952
P-value	0.0349	0.4751	0.2632

[1]HI = high forage diet; LO = low forage diet; MED = intermediate level of forage diet

Table 5-8. Relationships between actual fecal output (aFO) and calculated fecal output (cFO) from TiO_2 for each treatment in Experiment 2. Data were averaged for each horse across the fecal collection period.

	HI[1]	**MED[1]**	**LO[1]**
All horses			
Intercept	-14.87	0.23	2.69
Slope	5.89	0.98	0.12
R^2	0.5580	0.3879	0.2209
P-value	0.1468	0.2617	0.4244
Horses with refusals removed			
Intercept	-15.01	0.23	2.28
Slope	5.92	0.98	0.27
R^2	0.5741	0.3879	0.9992
P-value	0.2423	0.2617	0.0181

[1]HI = high forage diet; LO = low forage diet; MED = intermediate level of forage diet

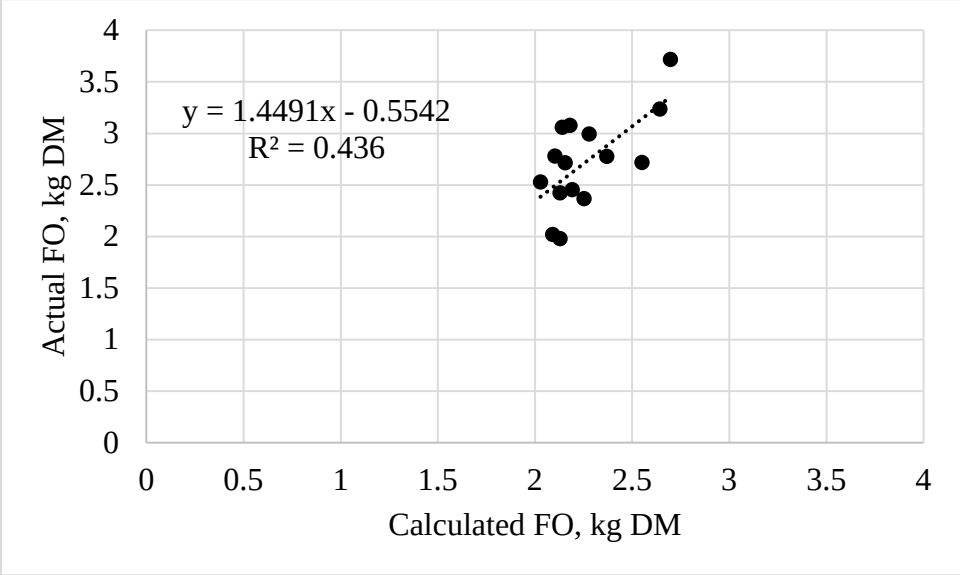


Figure 5-1. Relationship between actual FO and calculated FO from composite samples for horses fed Cr_2O_3 in Experiment 1. Each point represents the average of one horse on one day. $P < 0.05$.

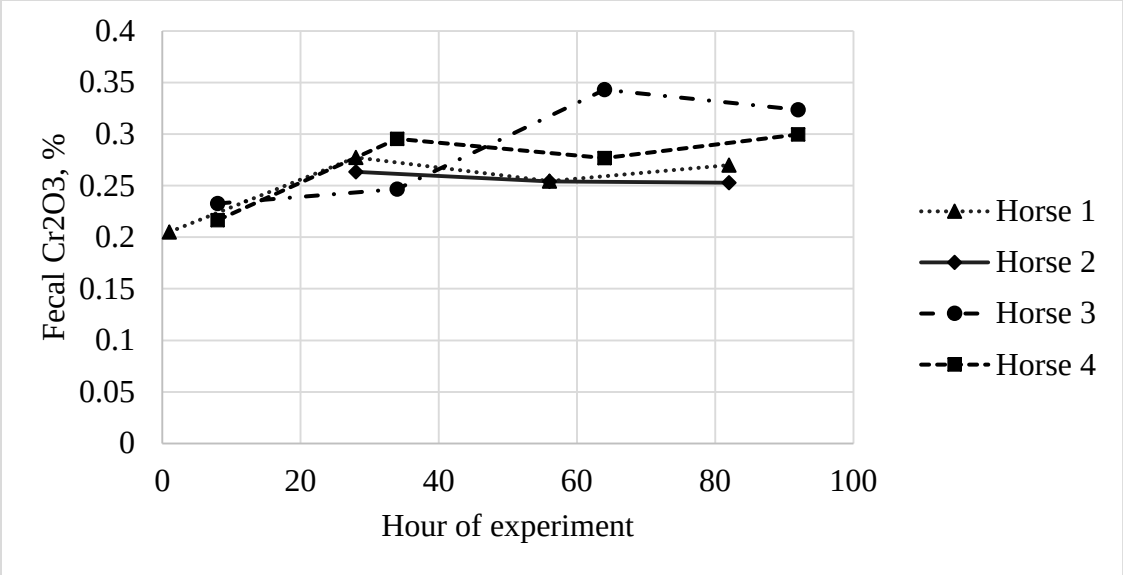


Figure 5-2. Fecal excretion of Cr_2O_3 in selected spot samples over the fecal collection period. Each data point represents the fecal concentration in selected spot samples for each horse. A meal was fed at h 4 and then every 12 h thereafter.

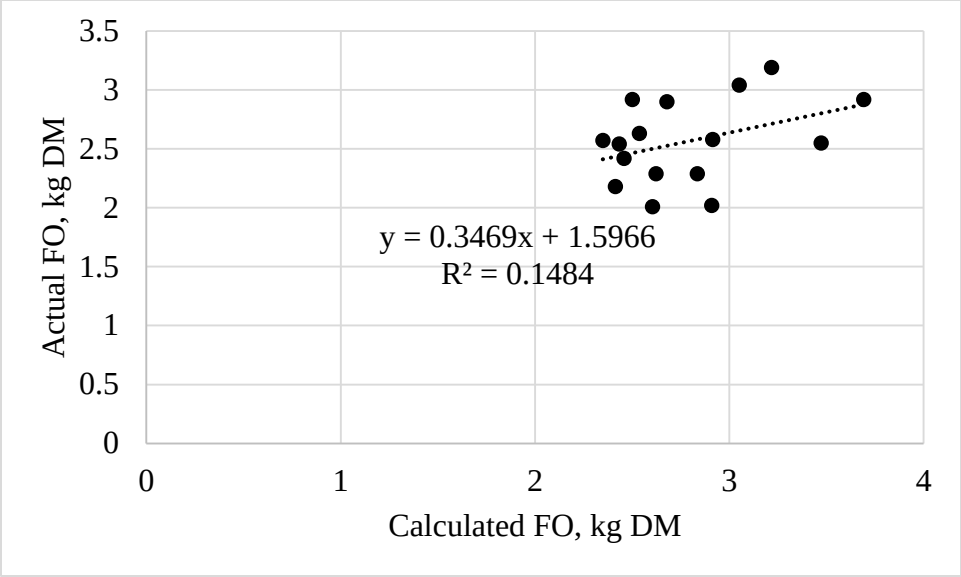


Figure 5-3. Relationship between actual FO and calculated FO for horses fed TiO_2 in Experiment 1. Each point represents the average of spot samples collected on one day for one horse. P = 0.1406.

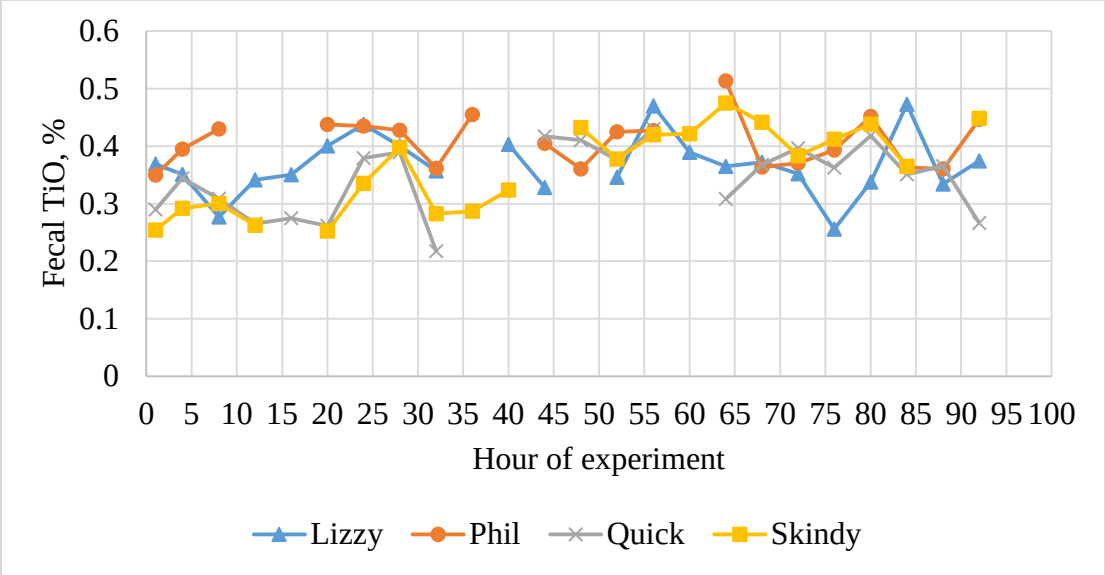
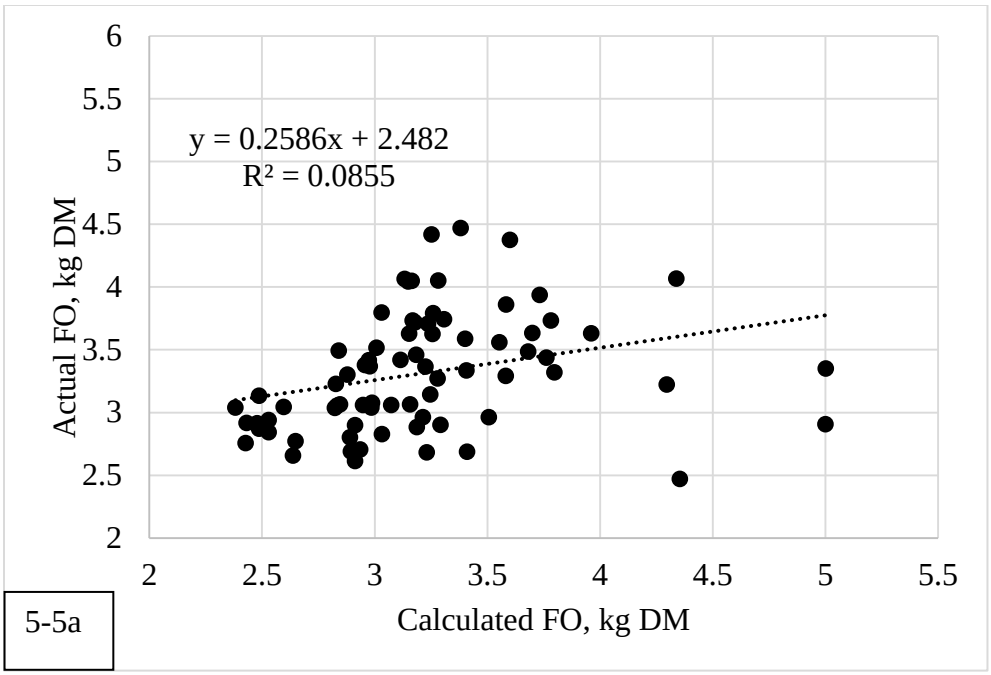


Figure 5-4. Fecal TiO_2 excretion patterns over the course of the fecal collection period in Experiment 1. Each data point represents fecal concentration in individual fecal spot samples collected. Horses were fed at h 4 and then every 12 h.



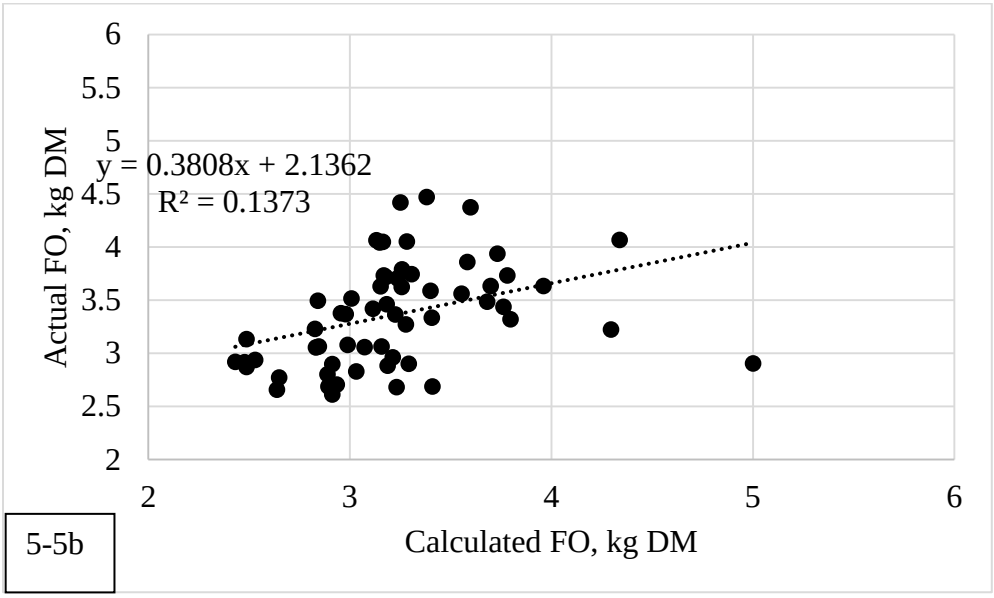
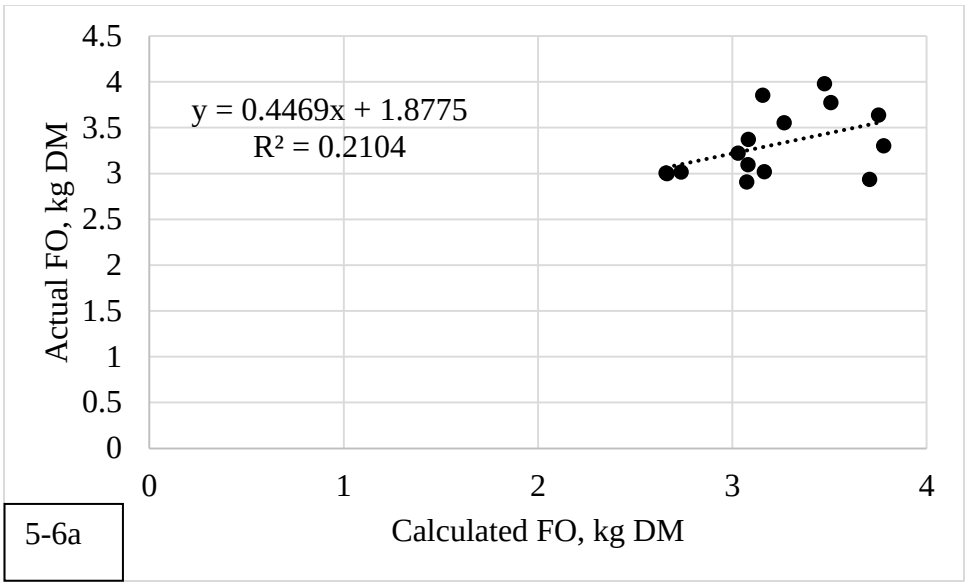


Figure 5-5, ab. Relationship between calculated FO and actual FO for horses fed TiO_2 in Experiment 2 for (a) all horses and (b) with horses that had feed refusals removed. Each point represents one horse's values averaged per d of collection.



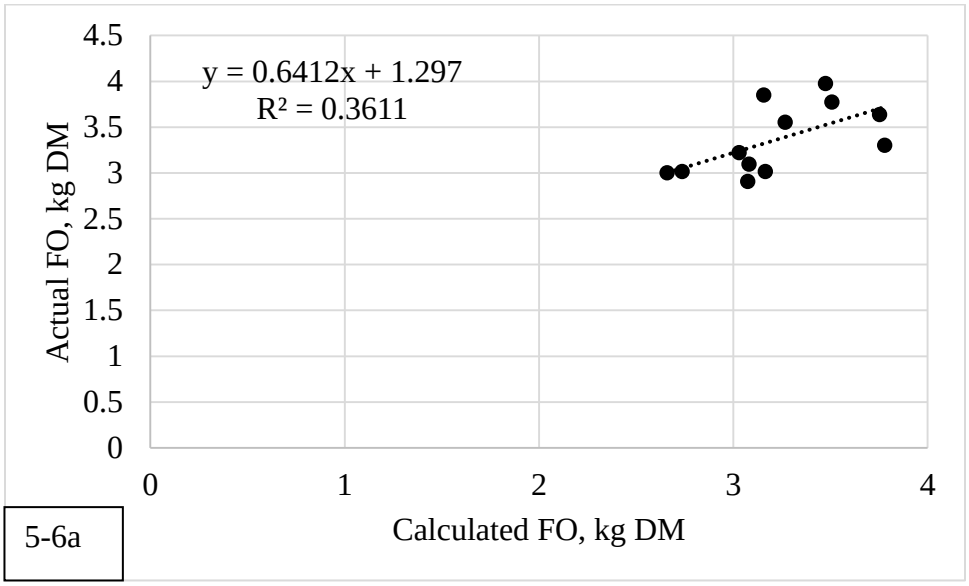


Figure 5-6, ab. Relationship between calculated FO and actual FO for horses fed TiO_2 in Experiment 2 for (a) all horses and (b) with horses that had feed refusals removed. Each point represents one horse's values averaged over the entire collection period.

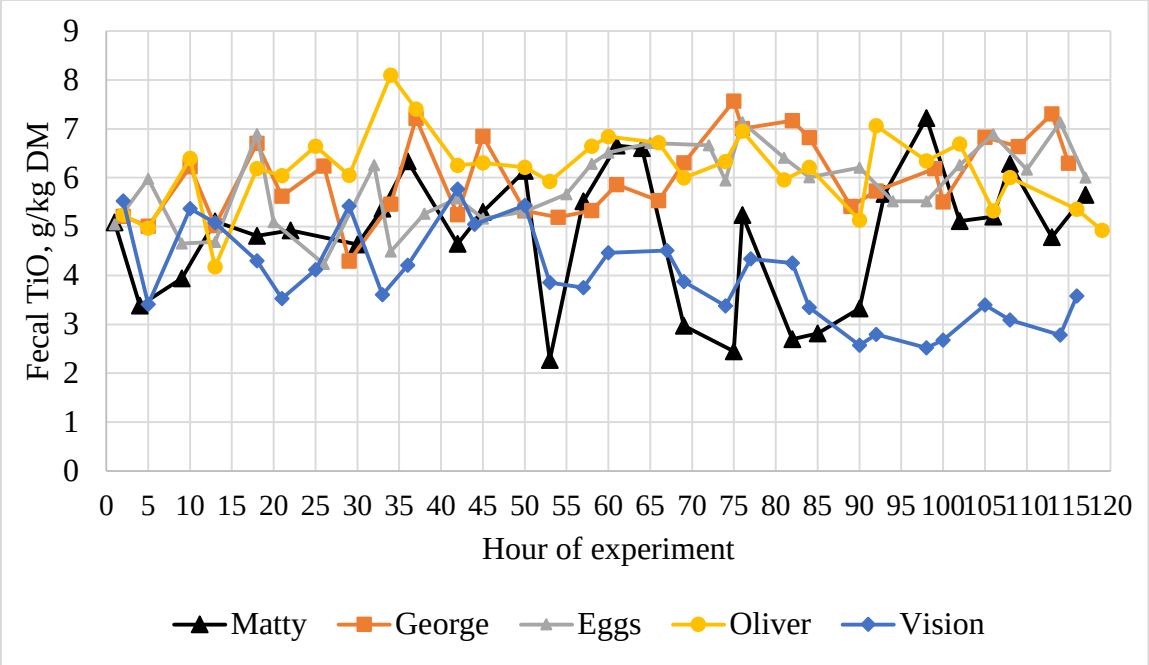


Figure 5-7. Fecal TiO_2 concentration (g/kg DM) for horses fed LO diet in Experiment 2. Meals were fed every 8 h, starting at h1.

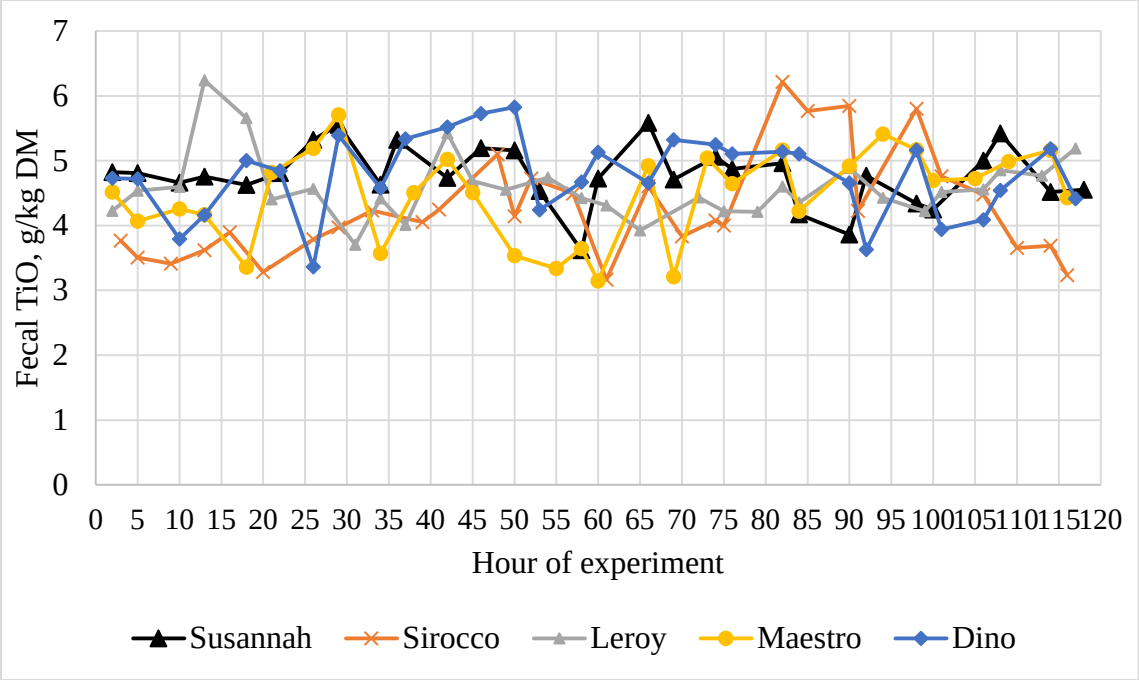


Figure 5-8. Fecal TiO_2 concentration (g/kg DM) for horses fed MED diet in Experiment 2. Meals were fed every 8 h, starting at h1.

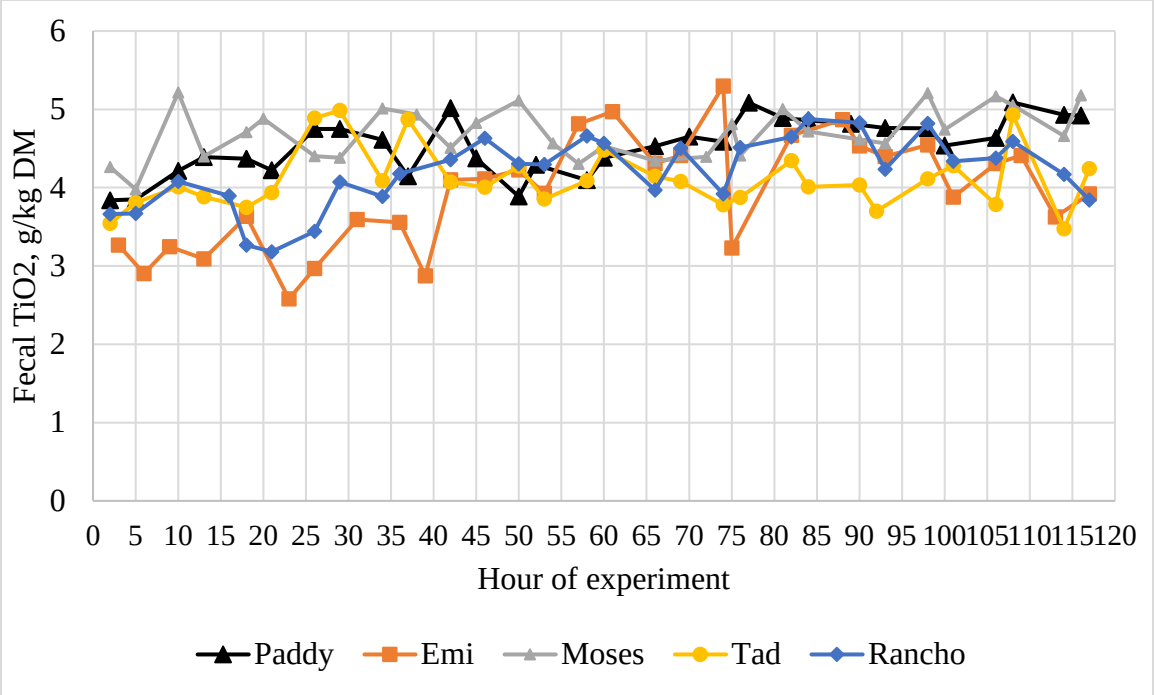


Figure 5-9. Fecal TiO_2 concentration (g/kg DM) for horses fed HI diet in Experiment 2. Meals were fed every 8 h, starting at h1.

CHAPTER 6: SEASONAL VARIATION OF MINERAL CONTENT IN COOL-SEASON GRASSES

Introduction

In Kentucky, many economically valuable horses are raised and maintained in pastures consisting primarily of cool-season grasses. The macromineral (Ca, P, Mg) content of the pasture is important, especially for growing and lactating horses, to ensure proper skeletal growth and milk production, respectively. To ensure no deficiencies occur, commercial concentrates containing additional macrominerals are often fed to these horses. However depending on the concentrations of these minerals in the pasture, minerals provided by concentrate may result in excess intakes. Excess mineral not needed by the animal is excreted and, for P in particular, could be harmful to the environment. Therefore, an understanding of pasture mineral content may allow horse operations to strategically supplement horses to complement their pastures.

Pasture mineral concentrations may vary during the growing season, making it difficult to precisely formulate supplementation programs for grazing animals. Ideally, pasture samples would be taken throughout the year to evaluate the changing mineral profiles. However, frequent pasture sampling and analysis can be tedious, expensive, and time-consuming. The use of near infrared spectroscopy (NIRS) for determination of plant nutrient concentrations is quicker and less expensive than wet chemistry analysis.

Near infrared spectroscopy has been successfully used to predict moisture, fiber, and protein of feeds, but has not been widely used for mineral analysis. Because NIRS responds to rotational and vibrational bonding energies of hydrogen, use of NIRS to detect inorganic minerals is limited. However, NIRS can detect some mineral forms if

they are associated with organic or hydrated inorganic molecules (Clark et al., 1987). Accurate use of NIRS for predicting forage mineral concentration has been suggested to be limited to macro minerals (Ca, P, Mg, and K) (Clark et al., 1987), potentially due to the larger concentrations of these minerals as well as their associations with organic molecules. Chemical forms of minerals may also differ among forages, being dependent on species as well as maturity (Spears, 1994). Therefore, developing equations using NIRS to predict forage macro minerals in a highly specific population have provided the most accurate results (Clark et al., 1987; Saiga et al., 1989).

The goal of this study was to develop NIRS equations to predict Ca, P, and Mg concentrations in cool-season grasses (bluegrass, orchardgrass, perennial ryegrass and tall fescue) and then use NIRS analysis to examine seasonal changes, species differences, and effect of N fertility on cool-season grass pasture mineral content. Species common in Kentucky horse pastures (tall fescue, Kentucky bluegrass, and orchardgrass) were included in the study in order to be relevant for horse farms in the Kentucky region (Smith et al., 2009).

Materials and Methods

The NIRS equations for predicting mineral content were calibrated using samples collected over two consecutive years as part of another study (Prince, 2017). In the first year, samples were collected from variety test plots maintained by the Department of Plant and Soil Science at the University of Kentucky North Farm. The cool-season grasses that were sampled were: Kentucky bluegrass (varieties ‘Barderby’ and ‘Ginger’), orchardgrass (varieties ‘Persist’ and Profit’), tall fescue (varieties ‘Cajun’ and ‘Bronson’),

and perennial ryegrass (varieties ‘Calibra’ and ‘Linn’). Samples were collected on 8 different days (Table 6-1) by clipping forage at 5 cm height from randomly selected locations throughout three plots per variety (approximately 100 g of wet sample). Samples were collected in the afternoon (1500 to 1600 h). Samples were diced, placed in Ziploc plastic bags, and frozen at -20 °C until they could be processed. When samples were ready to be analyzed, they were thawed, dried in a forced air oven at 55 °C, and then ground to pass through a 1 mm screen in an UDY Cyclone Sample Mill (UDY Corporation, Fort Collins, CO). These samples were used only in the development of the NIRS equations and were not included in analyses to evaluate species or seasonal effects on mineral concentrations.

The second year of sampling occurred in 2015. Research plots were seeded in September 2014 and consisted of Kentucky bluegrass (varieties ‘Barderby’ and ‘Ginger’), orchardgrass (varieties ‘Persist’, ‘Proft’, and ‘Quickdraw’), tall fescue (varieties ‘Cajun II’ and ‘Bronson’), and perennial ryegrass (varieties ‘Calibra’, ‘Linn’ and ‘Aberzest’). Each of the 10 cultivars were arranged in a randomized complete block design replicated over four blocks. Blocks were split and nitrogen (N) was applied to half of each plot as the split block, while the other half of the plot received no N. The N treatments were applied on March 16, 2015 (56.07 kg N/ha), May 13, 2015 (39.23 kg/N ha), and August 19, 2015 (56.07 kg N/ha) for a total of 157 kg N/ha over the year. Plots were mowed to a height of 10 to 12.5 cm every 2 wk to keep grasses in a vegetative state. Depending on grass growth, samples were collected every 2 to 4 wk from April 15, 2015 to November 3, 2015 (Table 6-1) in a similar manner as described for 2014. Samples were collected in the morning (0800 to 0900h) as well as the afternoon (1500 to 1600 h)

as part of another study (Prince, 2017); however, only samples collected in the afternoon were analyzed for this study. Once collected, samples were immediately frozen in liquid N and stored at -20 °C until they could be freeze-dried and then ground as described above. Due to freeze-dryer malfunction, all of harvest 2 and some of harvests 3 and 4 developed mold while in the freeze-dryer and therefore were not analyzed. Additionally, Kentucky bluegrass varieties were not sampled at harvest 1 (April 15, 2015) due to limited growth. As a result, only samples from harvest 3 (May 13, 2015) through harvest 12 (November 3, 2015) were included in the study.

NIRS and wet chemistry

All samples from 2014 and 2015 were scanned using FOSS 6500 NIRS (Foss, Inc., Hillderod, Denmark; n = 1050). Wet chemistry was performed on a sub-set of the 2014 samples (n = 27) selected at random and a sub-set 2015 samples (n = 100) selected from FOSS 6500 NIRS ISI software (Infrasoft International, L.L.C., State College, PA). Phosphorus was analyzed in ground samples using a gravimetric method (Shaver, 2008). Calcium and Mg were analyzed using atomic absorption (Bowers Jr and Rains, 1988).

Prediction equations were then developed for Ca, P, and Mg using modified partial least square regression (WinISI® software v.4.4). Outliers were removed from the equation if they fell beyond the standard deviation limit as determined by the software. Table 6-2 shows statistics for the equations calibrated to predict Ca, P, and Mg. The R^2 for all equations was ≥ 0.90, indicating good accuracy of predicting unknown samples. The standard errors of calibration are low for all equations (≤ 0.02). The 1-VR values, which are similar to an R^2-value for a prediction regression, were lower than R^2 values:

0.508 for Mg, 0.594 for Ca, and 0.601 for P. The 1-VR values indicate predictability of unknown samples and the values observed in this study suggest that predictability may be lower than desired.

Statistical analysis of sample data

The sample set for the 2015 year included 787 observations for each mineral. The main effects of species, sample day and N fertility were analyzed using repeated measures ANOVA (SAS 9.4, SAS Institute Inc., Cary, NC) with a random effect of block. If differences between means were found to be significant ($P < 0.05$), means were separated using an LSD test. Unless otherwise noted, mean values in tables are presented as LS means.

Results and Discussion

NIRS equations

To further examine the efficacy of the equations, the calibration population from 2014 and 2015 was predicted by each equation and plotted against wet chemistry values (Figure 6-1, 6-2, 6-3). A slope of 1 would indicate a perfect relationship between actual and predicted values.

Because NIRS detects chemical bonds, accurate prediction of minerals can be difficult due to differences in mineral chemical form within the plant and also across plant species. Calcium can exist in plants as calcium phosphate, calcium oxalate, or bound to pectin and lignin (Butler and Jones, 1973; Hazell, 1985). The chemical forms of Ca depends on forage species and maturity. For example, the proportion of total Ca in the

cell wall fraction of tall fescue has been reported to be 14.1% compared to 45.2% in white clover (Whitehead et al., 1985). Additionally, the availability of Ca from forages in the rumen is dependent on chemical Ca form and has been shown to be highly variable (range 30 to 78%) depending on forage species and maturity (Spears, 1994). However despite the multiple forms of Ca that may exist within plant tissue, the equation could accurately predict actual Ca concentrations when outliers were removed (R^2 = 0.9715; Figure 6-1).

The relationship between actual and predicted values for P is not as robust as that for Ca (R^2 = 0.8836; Figure 6-2). Phosphorus also exists in plants in many chemical forms. It can be present as inorganic phosphate, RNA, phospholipids, phosphate ester, and phytic acid (Butler and Jones, 1973; Hazell, 1985). Ruminal release of P from different forages ranges from 61 to 88%, suggesting that P is readily available and is less likely to be associated with cell wall fractions than Ca (Spears, 1994). In fact, Whitehead et al. (1985) demonstrated that only 5% of total P is found in the cell wall fraction of tall fescue and white clover, which is lower than that observed for Ca. As described for Ca, variation among chemical forms of P could lead to inaccurate predictions of P concentrations among species by NIRS.

The relationship between actual and predicted Mg concentrations is relatively robust (R^2 = 0.9230; Figure 6-3). Magnesium has the least number of potential chemical forms in the plant compared to Ca and P, being mainly associated with chlorophyll but can also bind to lignin (Butler and Jones, 1973; Hazell, 1985). In fact, the NIRS wavelengths used for predicting Mg are very similar to peaks in the chlorophyll spectrum (Tremblay et al., 2009). Additionally, ruminal release of Mg is relatively constant across

forage species, further suggesting that mineral form of Mg is similar in different forages (Spears, 1994). Because the chemical forms of Mg are so few, it is possible that the chemical forms were very similar across species and the NIRS was better able to recognize Mg bonds and therefore was relatively accurate in predicting Mg concentrations.

Concentrations of Ca, P, and Mg in cool-season grasses

Table 6-3 shows the average mineral concentrations for each species, as well as the average concentration across species, that was analyzed via wet chemistry and as predicted by NIRS. For Ca, the overall average analyzed concentration was 0.552 ± 0.151% and the equation predicted the concentration to be 0.538 ± 0.099%. For P, the average analyzed concentrations was 0.486 ± 0.098% and the predicted concentration was 0.480 ± 0.066%. For Mg, the average analyzed concentration was 0.218 ± 0.06% and the predicted concentration was 0.222 ± 0.048%. There were no differences between average analyzed and predicted concentrations for any of the minerals ($P > 0.10$).

The greatest concentration of Ca was in perennial ryegrass and lowest in tall fescue (Table 6-3). A 500-kg mare in early lactation consuming 2% of her BW in pasture (DM) would have a Ca intake of 79.8 g if she was consuming perennial ryegrass pasture and 51.4 g if she was consuming mostly tall fescue (using wet chemistry values). The Ca requirement for a mare in early lactation is 59 g (NR, 2007), so she would be meeting her requirement if her pasture was mostly perennial ryegrass, but may require supplementation on a tall fescue pasture.

For P, the greatest concentration was in orchardgrass and the lowest was in Kentucky bluegrass. The same mare as described above would be consuming 54.9 g of P on an orchardgrass pasture and 42.2 g on a Kentucky bluegrass pasture (using wet chemistry values). Her P requirement would be 38 g, which would be met by both grasses, indicating no need for P supplementation.

Tall fescue had the greatest concentration of Mg and Kentucky bluegrass had the lowest. The lactating mare would be consuming 25.2 g of Mg on a tall fescue pasture and 14.3 g on a Kentucky bluegrass pasture (using wet chemistry values). This mare would be meeting her Mg requirement (11.2 g) if fed either of these grasses.

On average, these species of cool-season grasses grown in central Kentucky provide adequate P and Mg for most horses. Calcium may need to be supplemented in some cases, but the Ca in cool-season grasses will likely meet requirements for horses with lower nutrient needs. However, other variables, such as time of harvest and fertility, may also impact mineral concentrations. Thus, the following sections will discuss the effects of species in more depth, as well as harvest date and N fertilization on mineral concentrations of these cool-season grasses using values predicted from equations developed using NIRS. While these equations may not be as robust as other equations for organic plant compounds, they at least provide an indication of the relative responses of each mineral, even if the actual values are not perfectly predicted.

Effect of species

Across all harvests and fertility levels, Ca, P, and Mg concentrations were all affected by species (Figure 6-4). Perennial ryegrass had the greatest Ca concentrations,

followed by orchardgrass, and tall fescue had the lowest Ca concentration ($P < 0.05$). Orchardgrass had the greatest concentrations of P and Mg and perennial ryegrass had the second greatest concentrations of P and Mg ($P < 0.05$). Kentucky bluegrass had the lowest concentrations of P and Mg ($P < 0.05$).

As a result of varying Ca and P concentrations, the Ca:P ratio was also affected by species (Figure 6-5). Perennial ryegrass had the greatest ratio of 1.13:1 and orchardgrass had the lowest ratio of 0.91:1. In equine nutrition, a dietary Ca:P ratio of at least 1:1 is desired and ideally the ratio would be closer to 2:1 (NRC, 2007). All species were relatively close to 1:1 however, both orchardgrass and tall fescue had inverted Ca:P ratios (less than 1:1). It is reasonably common for horse pastures to contain legumes, particularly clover (Smith et al., 2009), which would increase the Ca intake of grazing animals. However, if legumes are not present, horses grazing these species may need to be supplemented with Ca to achieve a more desirable ratio. In the event that horses were supplemented with an unfortified grain, such as oats, the potential for an inverted Ca:P ratio would be exacerbated. For example, the Ca:P ratio of oats is reported to be 0.275:1 (NRC, 2007), so the addition of unfortified oats to meet calorie needs for growth or lactation could have a marked impact on P intake and on the Ca:P ratio.

Effect of harvest date

Calcium, P, and Mg were all affected by harvest date, as their concentrations varied across the season. Across harvest, Ca concentrations ranged from 0.45 to 0.60% with concentrations being lowest in the first part of May, staying relatively stable throughout late spring and summer, and then increasing into the fall (Figure 6-6).

Phosphorus concentrations had a more narrow range compared to Ca (0.48 to 0.55%). Similarly to Ca, the lowest P concentrations occurred in early May and the greatest concentrations are observed in fall (Figure 6-7). However, P concentrations increased throughout June up to a peak in early July, then declined in late summer, which is different than the pattern observed for Ca. Seasonal variation in P concentrations have been observed to be lowest in the summer when growth of cool-season grasses is reduced (Greene et al., 1987). However in that study which was performed in Texas, P concentrations were much lower (0.07 to 0.17% P) (Greene et al., 1987), than those seen in the current study, which is likely an effect of the high soil P found in central Kentucky compared to other locations in the United States. Additionally, differences in climate as well as different varieties that may be more heat tolerant, could also produce different results among studies.

As a result of varying Ca and P concentrations, the Ca:P ratio also varies among harvests (Figure 6-8). The ratio stays relatively close to 1:1 throughout the sampling period, but inverted Ca:P ratios are seen in early May and also in July, so the inclusion of Ca in the diet, either by the addition of legumes or through the concentrate, may be needed during these times, especially for animals grazing tall fescue or orchardgrass, as those species generally tend to have inverted ratios overall. Again, there is no need for P supplementation, as additional P would further decrease the Ca:P ratio.

Seasonal variation in Mg concentrations follow a similar pattern to P (Figure 6-9). The lowest concentration of 0.20% was observed in early May, a peak of 0.26% in early July, then a decrease through the late summer and another increase in October and November.

Effect of nitrogen fertilizer

Overall, N fertilization increased P and Mg concentrations ($P < 0.05$), but had no effect on Ca ($P > 0.10$; Figure 6-10). Even when broken out by harvest or species, N fertilizer had no effect on Ca concentrations (data not shown). Hemingway (1961) reported that Mg concentrations in grass species were influenced by application of ammonium sulfate, but Ca concentrations were not significantly impacted. Another study also reported that P concentrations in cool-season grasses were significantly increased by N fertilization in the vegetative stages (Williams, 1953). The differences between overall P and Mg concentrations from fertilized and non-fertilized plots appear to be relatively small (around 0.03%), however, there was a significant effect of harvest which shows greater differences depending on sampling date (Figure 6-11).

Phosphorus concentrations between fertilized and non-fertilized plots were significantly different on May 27, June 10, June 24, July 22, October 13, and November 3 (Figure 6-11). Fertilizer was applied on March 16, May 13, and again on August 19. The May application was followed by an immediate increase in P concentration at the next sampling date and the effect remained throughout the following two sampling dates. The fall application of fertilizer did not affect P concentrations until approximately 8 wk later in October.

Magnesium concentrations between fertilized and non-fertilized plots were significantly different at all harvest dates except for July (Figure 6-11). Interestingly, there was no effect of fertilizer for P on this date as well. The May application of fertilizer increased the difference between fertilized and non-fertilized plots at the next

sampling date (May 27), and then the difference was reduced between the two treatments at the next sampling date, but still remained significant.

When broken out by species, N fertilizer significantly increased P and Mg concentrations for all species (Figure 6-12, 6-13). There was no effect of N fertilization on Ca concentrations for any species.

Strategic supplementation of horses maintained on pasture in central Kentucky

In this study, pasture mineral concentrations were observed to vary with species, date, and in some cases, N fertility. These observations suggest that there is potential to develop strategic mineral supplementation programs that meet animal needs, but minimize the excretion of minerals, particularly P, into the environment.

Generally, P concentrations were lowest in early May and in Kentucky bluegrass. If a growing Thoroughbred yearling is consuming an unfertilized pasture mainly containing Kentucky bluegrass in May (P = .43%; DM intake of 1.5% BW), it would be consuming 20.7 g P, which more or less meets its P requirement (P requirement = 21 g/d, NRC, 2007). This yearling would need to be supplemented with 0.3 g of P per day in order to exactly meet the daily P requirement. Typically, yearlings are fed approximately 1% of the BW in concentrate in order to meet energy requirements, which would equate to 3 kg in this scenario. In order to meet but not exceed the P requirement of this yearling, the concentrate should only contain 0.010% P. However, current commercial concentrates formulated for growing horses often contain around 0.60 to 0.80% P (Fowler, 2013). A yearling being fed 3 kg of these higher P concentrates will be consuming 18 to 24 g of P from the concentrate alone, which well exceeds the 0.3 g P

needed from the concentrate to meet requirements and in some cases, P intake from the concentrate alone exceeds the P requirement. The excess P that the yearling is consuming will be excreted and by feeding over the requirement, more P than necessary is excreted in the feces.

If a yearling was consuming a mostly orchardgrass pasture in October (P = 0.63%), the pasture would be meeting its requirement without any supplementation. However, if the same concentrates as described above are fed to meet energy requirements, the P intake could be 230 to 260% of the P requirement.

These examples illustrate the potential to develop lower P concentrates that still meet energy needs for horses grazing pasture. One such concentrate could be fed in the fall when pasture P is high to reduce the excessive P intakes that are likely to exist at that time of year. Concentrates developed for spring feeding could contain more P than concentrates developed for fall feeding in order to meet P requirements that are not met by pasture alone.

Influence of estimates of true P digestibility on P excretion in horses maintained on central Kentucky pastures

The example above calculated daily P requirements for a growing horse (P requirement = 21 g/d) based on estimates of true P digestibility as described by the NRC (2007) of 45% for growing horses. However, if true P digestibility is greater than previously believed due to P recycling and differences in endogenous losses, then P requirements may be overestimated and supplementation programs on for horses on pasture may need to be altered even further. For example, if true P digestibility is actually

80% for growing horses, as suggested by the NRC (1966), then the daily P requirement for a yearling would be 11.8 g P/d, almost half of the requirement calculated by NRC (2007). If a requirement of 11.8 g P/d is assumed, then even yearlings grazing unfertilized Kentucky bluegrass in May (P = 0.43%) would be consuming 20.7 g P/d, which is still 8.9 g P/d more than the newly calculated P requirement. On the other extreme, yearlings grazing a pasture with large concentrations of P (i.e. fertilized orchardgrass in October; P = 0.63%) would be consuming 30.3 g P/d, which is 18.5 g P over the newly calculated requirement. Phosphorus supplementation would not be needed for any yearling in this scenario, even if grazing pasture with the lowest P concentrations.

This exercise highlights the importance of not only knowing how much P the pasture is providing to the horse, but also the importance of knowing the actual true P digestibility by horses. Because the calculation of P requirements rely so heavily on the value chosen for true P digestibility, it is imperative that the correct value is chosen so P requirements can be accurately calculated and overfeeding of P can be avoided.

Conclusion

Rapid NIRS determination of pasture minerals could be useful for equine operations to understand how pasture mineral profiles change over time. A handheld NIRS would be ideal for rapid evaluation in the field. With knowledge of pasture mineral concentrations, producers can tailor mineral supplementation programs to complement pasture mineral composition, thereby reducing the impact of mineral excretion on the environment, as well as ensuring their horses are obtaining optimal mineral nutrition.

Tables and Figures

Table 6-1. Dates that samples were collected for 2014 and 2015 studies

Harvest #	**Harvest date**
Spring 2014	4/18, 4/21, 4/23, 5/6, 5/7, 5/21, 5/30
Fall 2014	10/17
Harvest 3 2015	5/13
Harvest 4 2015	5/27
Harvest 5 2015	6/10
Harvest 6 2015	6/24
Harvest 7 2015	7/8
Harvest 8 2015	7/22
Harvest 9 2015	8/19
Harvest 10 2015	9/15
Harvest 11 2015	10/13
Harvest 12 2015	11/3

Table 6-2. Equation statistics for quantification of calcium (Ca, %), phosphorus (P, %), and magnesium (Mg, %) by near infrared reflectance spectroscopy (NIRS)

Equation	**n**	**Mean ± SD**	**Range**	**R^2**	**1-VR**	**SEC**	**SECV**
Ca	64	0.533 ± 0.097	0.241 – 0.825	0.9884	0.594	0.0105	0.0616
P	73	0.468 ±0.064	0.277 – 0.667	0.8968	0.601	0.0205	0.0401
Mg	52	0.219 ± 0.045	0.085 – 0.353	0.9352	0.508	0.0114	0.0310

Table 6-3. Average mineral concentrations of the same cool-season grass population when analyzed using wet chemistry and NIRS (mean ± SD)

	Calcium, %		**Phosphorus, %**		**Magnesium, %**	
	Lab[1]	**NIRS[2]**	**Lab[1]**	**NIRS[2]**	**Lab[1]**	**NIRS[2]**
Kentucky bluegrass	0.532 ± 0.142	0.545 ± 0.099	0.422 ± 0.061	0.421 ± 0.040	0.143 ± 0.040	0.173 ± 0.036
Orchardgrass	0.548 ± 0.092	0.551 ± 0.080	0.549 ± 0.109	0.545 ± 0.054	0.242 ± 0.038	0.258 ± 0.035
Perennial ryegrass	0.798 ± 0.139	0.631 ± 0.104	0.484 ± 0.081	0.475 ± 0.053	0.222 ± 0.052	0.215 ± 0.038
Tall fescue	0.514 ± 0.111	0.543 ± 0.094	0.475 ± 0.091	0.463 ± 0.051	0.252 ± 0.049	0.236 ± 0.041
Average of all species	**0.552 ± 0.151**	**0.538 ± 0.099**	**0.486 ± 0.098**	**0.480 ± 0.066**	**0.218 ± 0.060**	**0.222 ± 0.048**

[1]Concentrations analyzed in the laboratory using wet chemistry

[2]Concentrations predicted using NIRS equation (using same samples analyzed for wet chemistry)

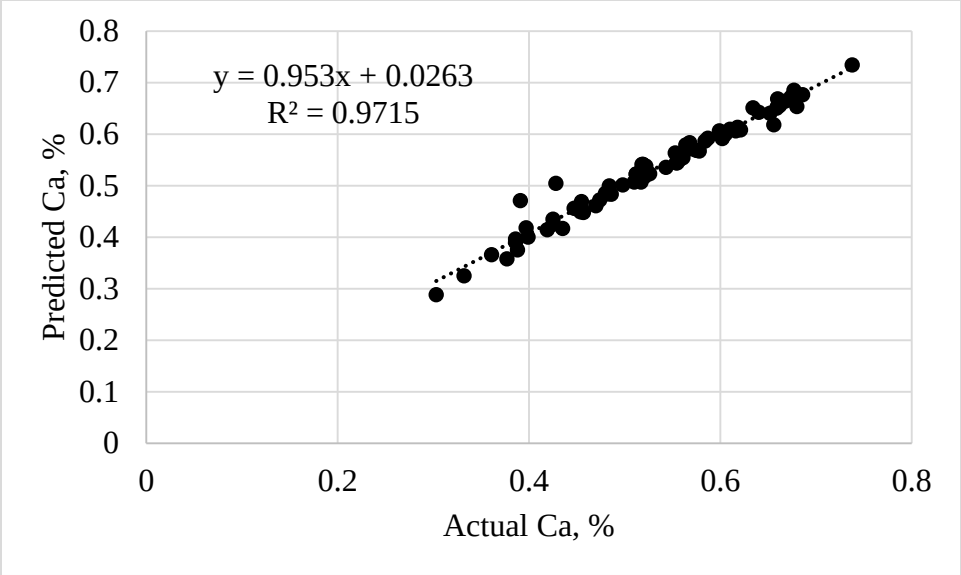


Figure 6-1. Relationship between actual laboratory analyzed calcium concentrations and near infrared reflectance spectroscopy (NIRS) equation-predicted Ca concentrations of cool-season grasses harvested 2014 – 2015. Outliers were excluded from the equation if the absolute residual was greater than 0.09 (as determined by modified partial least squares regression using WinISI® v4.4 software).

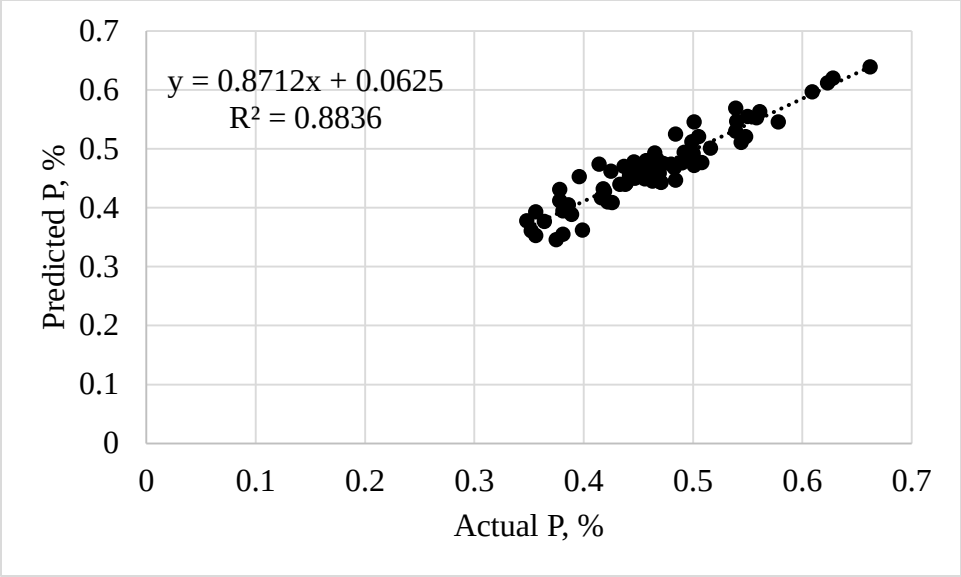


Figure 6-2. Relationship between actual laboratory analyzed phosphorus concentrations and near infrared reflectance spectroscopy (NIRS) equation-predicted P concentrations of cool-season grasses harvested 2014 – 2015. Outliers were excluded from the equation if the absolute residual was greater than 0.061 (as determined by modified partial least squares regression using WinISI® v4.4 software).

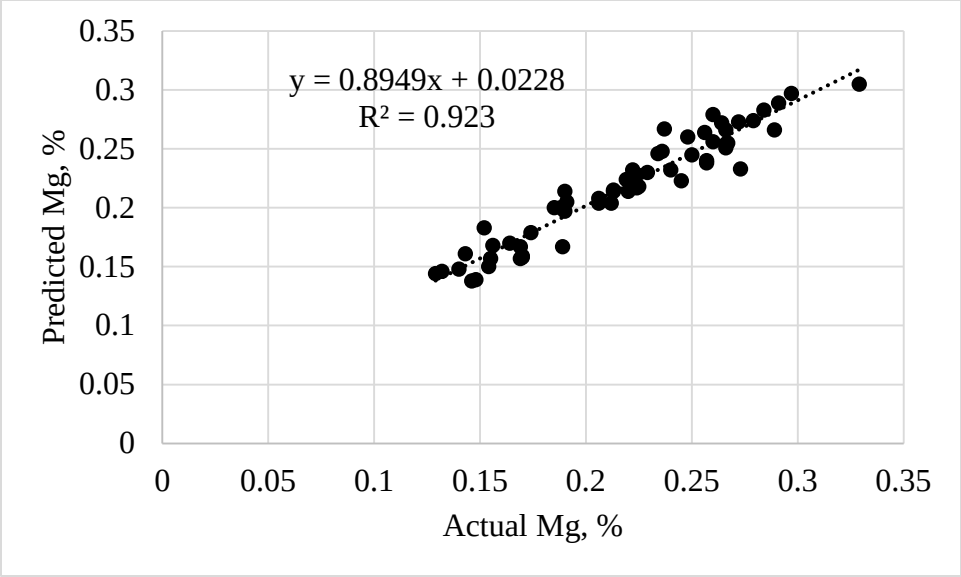


Figure 6-3. Relationship between actual laboratory analyzed magnesium concentrations and near infrared reflectance spectroscopy (NIRS) equation-predicted Mg concentrations of cool-season grasses harvested 2014 – 2015. Outliers were excluded from the equation if the absolute residual was greater than 0.04 (as determined by modified partial least squares regression using WinISI® v4.4 software).

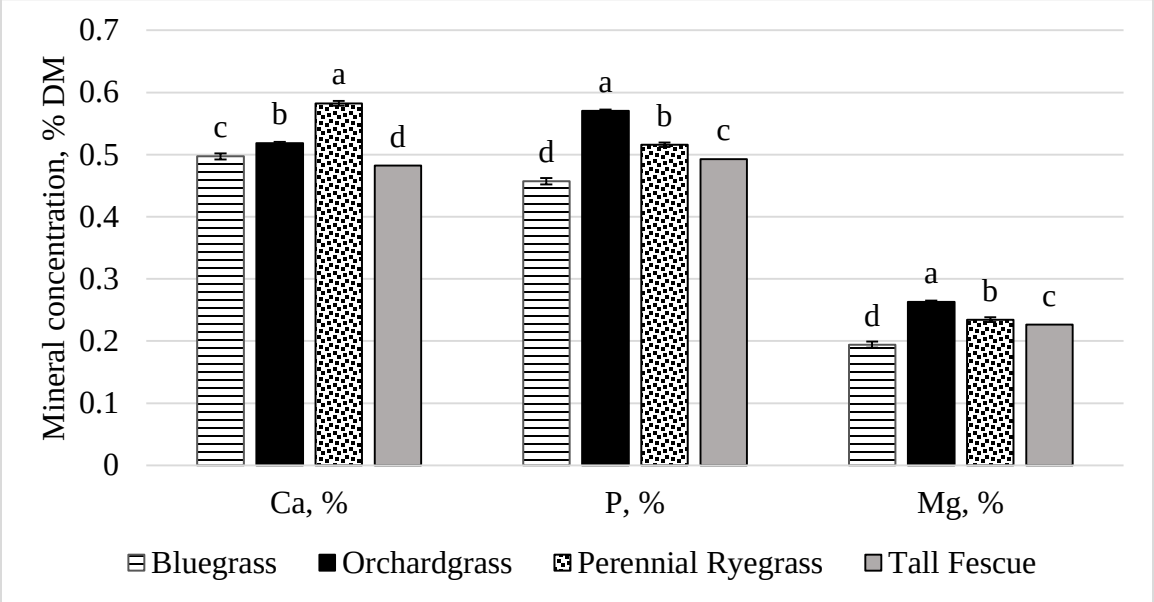


Figure 6-4. Effect of species on concentrations of calcium (Ca), phosphorus (P), and magnesium (Mg) in four species of cool-season grasses sampled in 2015. Means within a mineral lacking a common letter differ ($P < 0.05$).

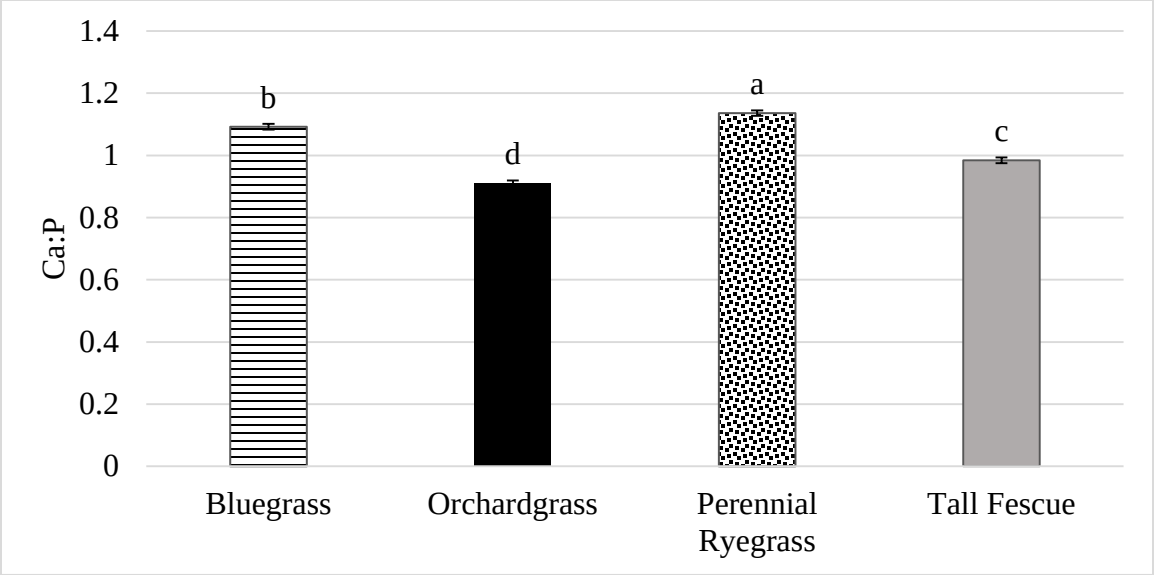


Figure 6-5. Effect of species on Ca:P ratio in four species of cool-season grasses sampled in 2015. Means lacking a common letter differ ($P < 0.05$).

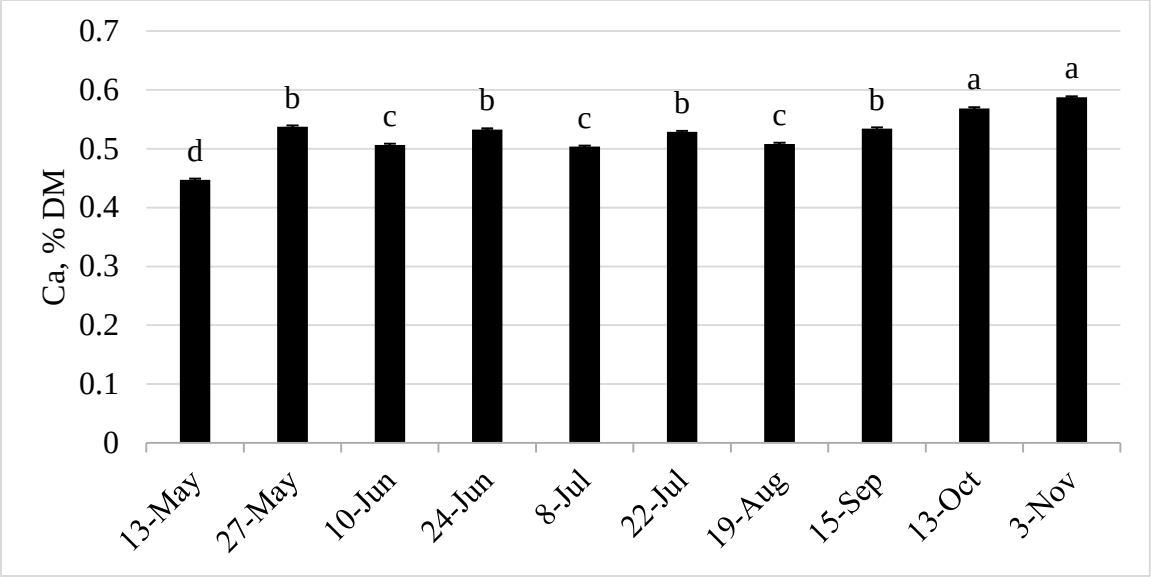


Figure 6-6. Effect of harvest on calcium (Ca) concentration in all four species of cool-season grasses sampled in 2015. Means lacking a common letter differ ($P < 0.05$).

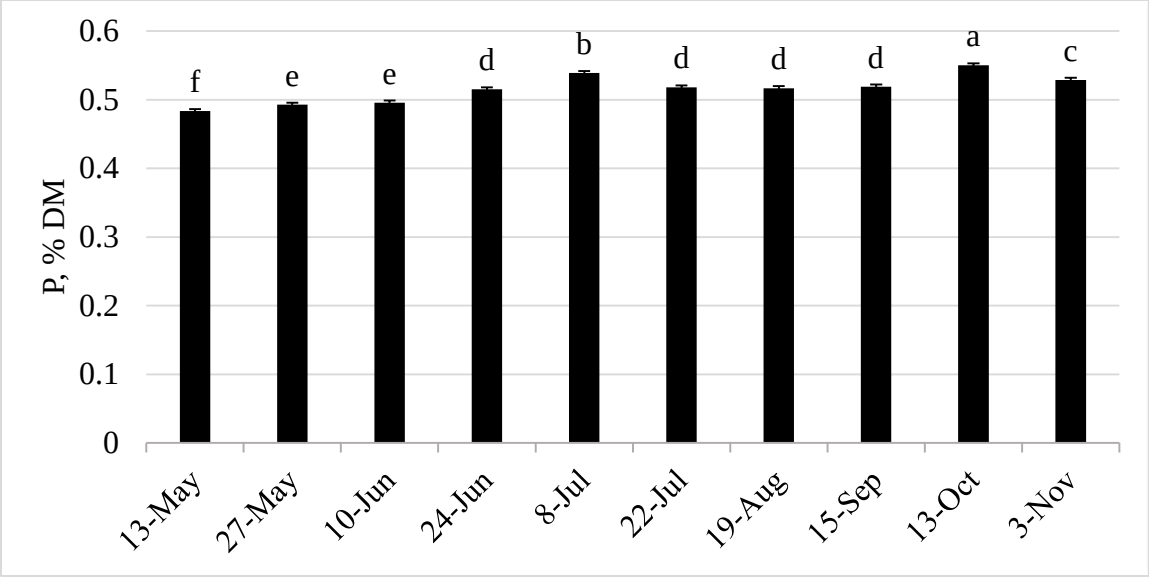


Figure 6-7. Effect of harvest on phosphorus (P) concentration in all four species of cool-season grasses sampled in 2015. Means lacking a common letter differ ($P < 0.05$).

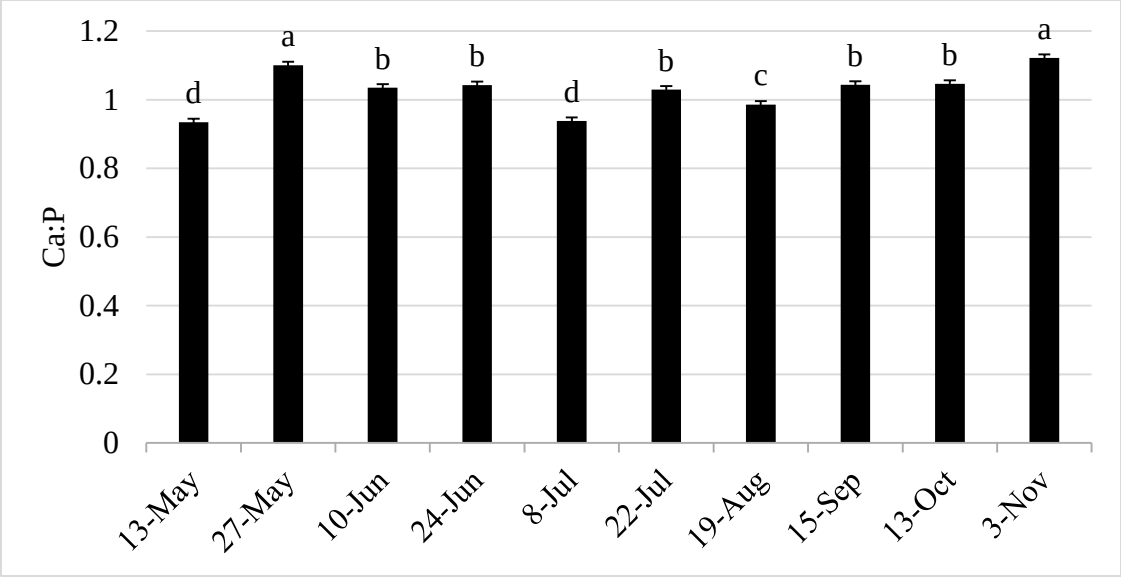


Figure 6-8. Effect of harvest on Ca:P concentration in all four species of cool-season grasses sampled in 2015. Means lacking a common letter differ ($P < 0.05$).

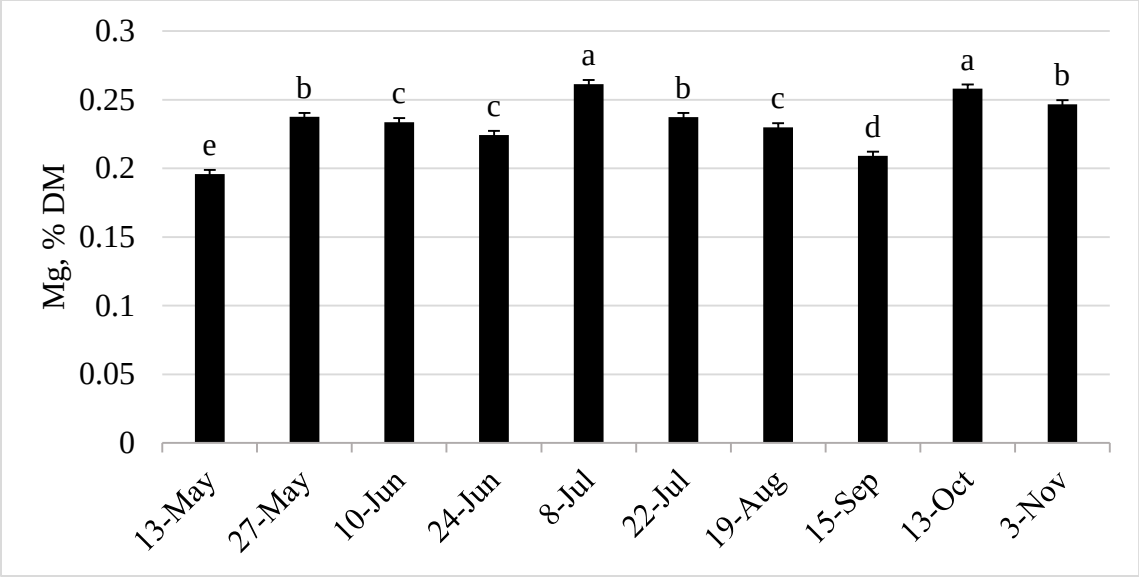


Figure 6-9. Effect of harvest on magnesium (Mg) concentration in all four species of cool-season grasses sampled in 2015. Means lacking a common letter differ ($P < 0.05$).

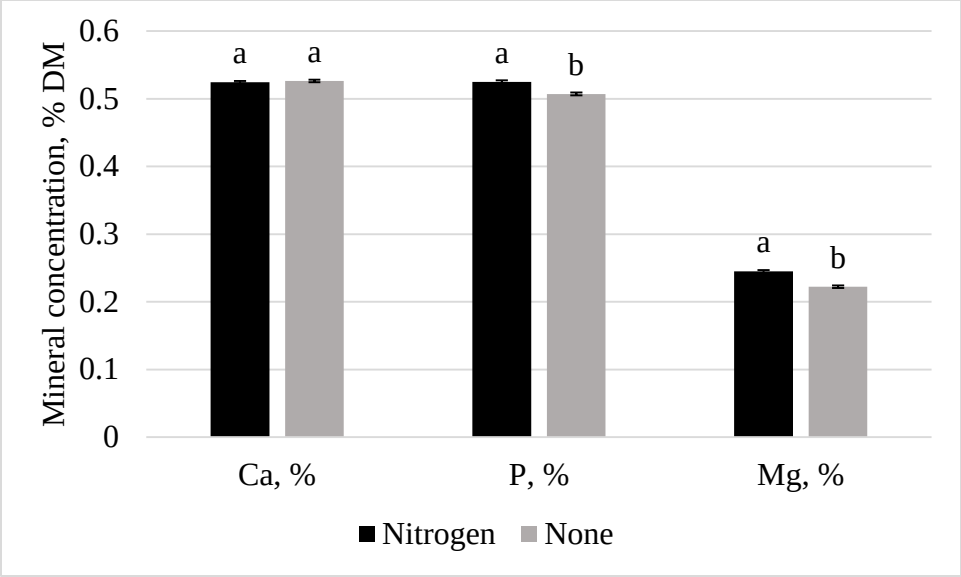


Figure 6-10. Effect of nitrogen fertilizer or no fertilizer on concentrations of calcium (Ca), phosphorus (P), and magnesium (Mg) in all four species of cool-season grasses sampled in 2015. Means within a mineral lacking a common letter differ ($P < 0.05$).

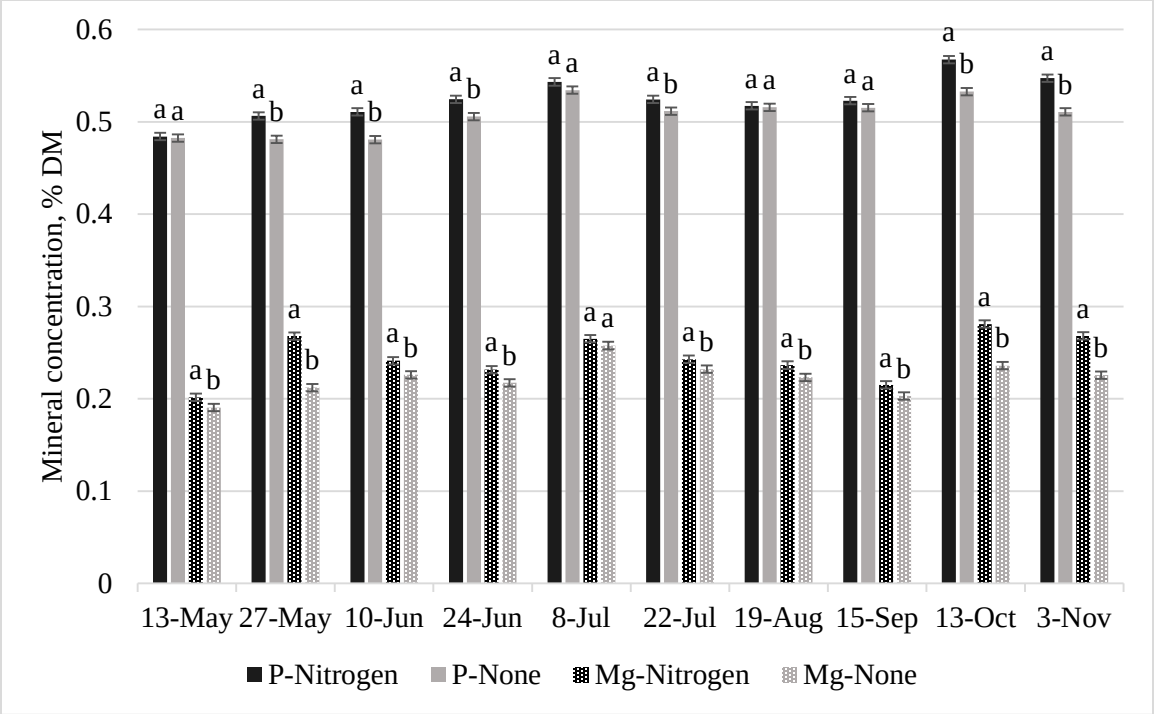


Figure 6-11. Effect of nitrogen fertilizer or no fertilizer on phosphorus (P) and magnesium (Mg) concentrations across harvests for all cool-season grasses sampled in 2015. Means within a harvest and mineral lacking a common letter differ ($P < 0.05$).

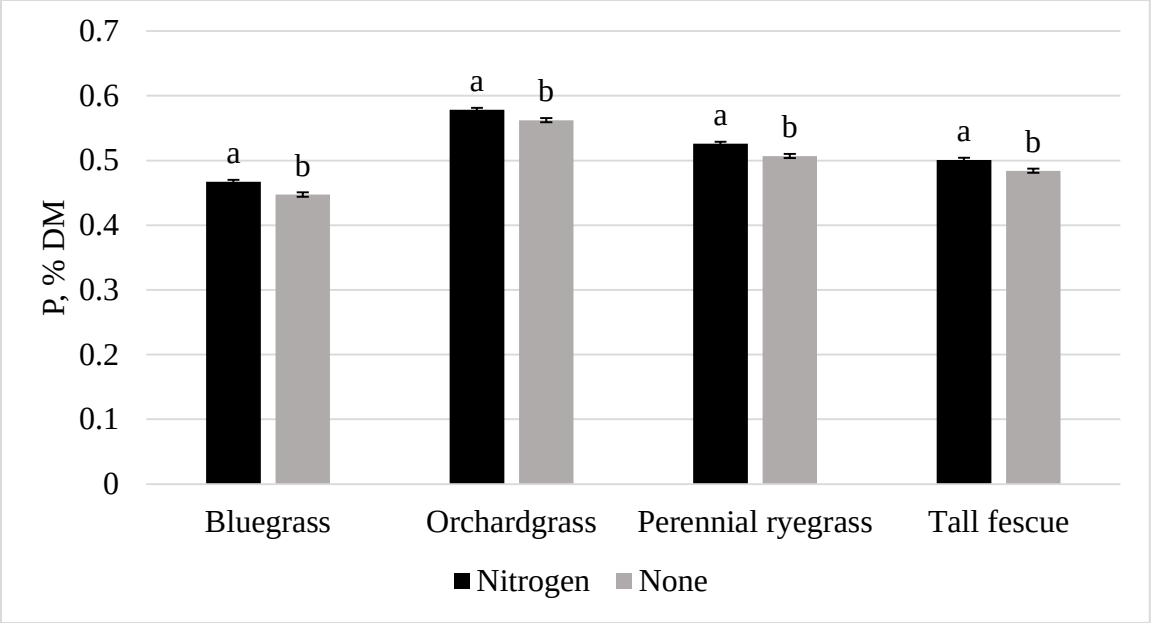


Figure 6-12. Effect of N fertilizer of no fertilizer on phosphorus concentrations averaged by species. Means within a species lacking a common letter differ ($P < 0.05$).

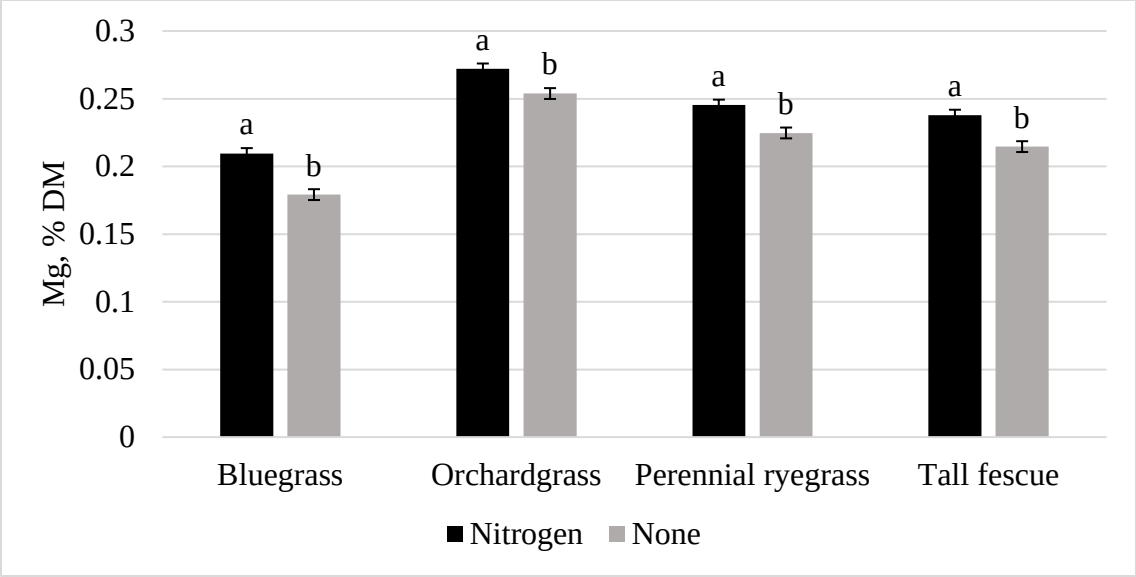


Figure 6-13. Effect of N fertilizer of no fertilizer on magnesium concentrations averaged by species. Means within a species lacking a common letter differ ($P < 0.05$).

CHAPTER 7: CONCLUSION

True P digestibility in horses is likely higher than currently suggested by NRC (2007), due to P recycling into the GI tract. If true P digestibility is actually greater than currently believed, then current recommendations for P intakes by horses are probably high. In order to truly elucidate true P digestibility, P intake should be below the actual requirement of the horse, in order to maximize P retention and decrease recycling. Out of all the published papers reporting P digestibility in mature horses, only two experiments have fed P below the current P requirement of 28 mg P/kg BW (Kichura et al., 1983; Buchholz-Bryant et al., 2001), which may be high based on the low true P digestibilities used to calculate that requirement (NRC, 2007). Buchholz-Bryant et al. (2001) found that mature horses fed at their P requirement had no difference in apparent P digestibility than horses consuming 200% of their P requirement, further suggesting that the current requirement is too high to reduce P recycling within the gut.

Formulating a low P diet for mature horses at maintenance in an attempt to maximize retention is difficult due to the low P requirement of horses at maintenance. The goal of this dissertation was to attempt to create a model that would increase P retention and reduce P recycling to better understand actual P digestibility of the feed. Post-lactational mares and horses undergoing weight gain were the two physiological states that we hypothesized would increase P retention and reduce P recycling into the gut. While post-lactational mares increased fecal P excretion, they were also losing weight which may have reduced their need to retain P. Horses gaining weight did tend to excrete less P than horses losing weight, suggesting the P retained in the body was greater in these horses. Horses that were assigned to gain weight only gained a modest amount of

weight (6.8 kg), which may not have been enough to eliminate active P secretion into the GI tract.

Ideally, future studies would utilize horses with even greater requirements, such as lactating or exercising horses. However, performing total fecal collections with these animals is difficult. Therefore, external markers were evaluated in tandem with the animal studies in order to validate the accuracy of their ability to predict fecal output, so that P digestibility could eventually be calculated in lactating mares and exercising horses. Titanium dioxide may be an appropriate marker to use to calculate fecal output, but multiple spot samples over many days are needed to obtain reliable results.

Many horses likely consume much more than their P requirement based on current NRC (2007) recommendations due to the dietary addition of fortified concentrates containing P. To demonstrate how much P from pasture is already available to horses throughout the growing season in Kentucky, cool-season grasses were evaluated for mineral content. Pasture mineral content does vary throughout the year. Despite seasonal fluctuations, pasture provides sufficient P for most horses without the need for supplementation. Horses with greater P requirements (e.g. pregnant, lactating, growing) may only need to be supplemented in certain periods of the year, when pasture P is at its lowest. Lowering P intake by reducing or eliminating P supplementation would reduce excess P excretion and be more environmentally sustainable.

In conclusion, the current P requirement for horses likely exceeds the actual P required by the horse due to P recycling occurring in the studies that were used to calculate the current P requirement. True P digestibility is likely greater than currently

suggested, but more studies that feed P below the horses' requirements are needed in order to fully determine actual P digestibility by horses.

APPENDICES

Appendix A. Water balance and digestibility of organic matter, NDF and ADF from Chapter 4 experiment

Organic matter intakes were similar among treatments, but OM digestibility was greatest in GA and lowest in LO and MA (Table A-1). Organic matter digestibilities were numerically greater than DM digestibilities for all treatments, but the difference between DM digestibility and OM digestibility was greatest in LO (50.48 versus 54.61%, respectively) and lowest in GA (61.57 versus 61.79%, respectively).

Intake of the fiber components, NDF and ADF, were greatest in LO group and lowest in the GA group (Table A-2; $P < 0.05$). Despite differences in intake, digestibility of these fiber components were similar among treatments ($P > 0.10$).

One gelding in the LO treatment group consumed twice as much water than all other horses. The gelding consumed an average of 84 ml water/kg BW per day and the remaining 14 horses consumed an average of 41.5 ± 5.1 ml water/kg BW (mean ± SD). The one gelding consumed more than 8 times the SD from the overall mean and was treated as an outlier. This horse was removed from analysis of water intake. Water intake did not differ among treatments (Table A-3; $P > 0.10$). However there was a trend for water intake in relation to feed DM to be greatest in LO horses and lowest in GA horses ($P = 0.0875$). There was also a trend for water intake in relation to ADF intake to be greatest in GA and lowest in LO horses ($P = 0.0941$).

Table A-1. Organic matter digestibility over the fecal collection period for horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means)

Item	LO	MA	GA	SEM	*P*-value
Intake, kg	6.56	7.27	6.99	0.32	0.3340
Intake, g/kg BW	11.81	13.30	12.84	0.49	0.1294
Fecal excretion, kg	2.95[b]	3.19[a]	2.63[c]	0.09	0.0001
Fecal excretion, g/kg BW	5.31[b]	5.83[a]	4.85[c]	0.11	<0.0001
OMD, %	54.61[b]	56.08[b]	61.79[a]	1.71	0.0232

[abc] Means within a row lacking a common superscript differ (P < 0.05)

Table A-2. NDF and ADF digestibility over the fecal collection period for horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means)

Item	LO	MA	GA	SEM	*P*-value
NDF intake, kg	4.19[a]	4.40[a]	3.59[b]	0.105	0.0016
NDF intake, g/kg BW	7.67[a]	8.06[a]	6.50[b]	0.155	0.0003
NDF excreted, kg	2.21[ab]	2.52[a]	1.96[b]	0.095	0.0102
NDF excreted, g/kg BW	4.04[b]	4.60[a]	3.55[b]	0.153	0.0043
NDF digestibility, %	47.22	42.85	45.39	1.572	0.2038
ADF intake, kg	2.38[a]	2.48[a]	1.98[b]	0.067	0.0018
ADF intake, g/kg BW	4.36[a]	4.53[a]	3.58[b]	0.099	0.0003
ADF excreted, kg	1.41[a]	1.56[a]	1.19[b]	0.060	0.0064
ADF excreted, g/kg BW	2.57[a]	2.85[a]	2.15[b]	0.094	0.0023
ADF digestibility, %	40.72	36.69	40.03	1.701	0.2600

[abc] Means within a row lacking a common superscript differ (P < 0.05)

Table A-3. Water intake over the fecal collection period for horses fed loss (LO), maintenance (MA) and gain (GA) diets (LS means)

Item	LO[1]	MA[2]	GA[2]	SEM	*P*-value
Water intake, L/d	20.95	24.15	21.53	1.17	0.2094
Water intake, ml/kg BW	39.34	44.84	38.54	2.48	0.2260
Water intake, L/kg feed DM	3.27	3.09	2.71	0.14	0.0875
Water intake, L/kg NDF	5.10	5.49	6.00	0.27	0.1577
Water intake, L/kg ADF	9.04	9.78	10.88	0.47	0.0941

[1] n=4; outlier from LO treatment removed due to value being 8 SD from overall mean
[2] n=5

Appendix B. Determination of titanium dioxide in equine fecal samples: Method 1

Reagents:

Standard titanium solution (0.5 mg/ml)

Dissolve 250 mg titanium dioxide in 100 ml concentrated sulphuric acid by heating in a beaker to just below boiling. Adding a stir bar to assist in dissolution is useful. It may take ~1 hr to 2 h to dissolve completely. Rinse the contents of the beaker into a 500 ml volumetric flask containing approximately 200 ml nanopure water. Add another 100 ml concentrated sulphuric acid to the mixture and dilute to 500 ml with nanopure water.

Sulphuric acid solution (7.4 M)

Add 400 ml of concentrated sulphuric acid to 400 ml of nanopure water in a 1 L volumetric flask and dilute to 1 L.

Procedure:

1. Weigh 0.2 g of fecal material into a quartz crucible and ash overnight at 600°C.
    a. A sample size of 0.1 g is also acceptable, but sample sizes greater than ~0.25 g make it more difficult to digest and dissolve TiO_2 in sulfuric acid. A sample size of 0.2 g is used here because the concentration of TiO_2 in horse feces is relatively low.
2. Add 20 ml of the 7.4 M H_2SO_4 solution to each crucible upon cooling. Gently swirl to ensure there are no clumps of ash in the crucible.
3. Heat the samples to just below boiling (~390°C or until samples steam, but don't bubble) for approximately 90 min or until white particles (TiO_2) are dissolved.
4. Allow crucibles to cool completely. Very slowly and gently, squirt nanopure water into the crucible by letting it flow down the inside wall of the crucible. If spattering occurs, stop and gently swirl crucible contents, let cool and try again even more slowly. Add about 15 mL of nanopure water in total, but no need to be exact.
5. Pour the contents of the crucibles into 100 ml volumetric flasks.
    a. You may pour through filter paper (Whatman 541) at this step, or you may skip the filtering and just allow the flasks to sit overnight before analyzing. Better recoveries have been achieved with horse feces by skipping the filtering and letting the flasks sit overnight.
6. Add 10 ml of 30% hydrogen peroxide (H_2O_2) to the flasks and the dilute to 100 ml with nanopure water.
    a. 10 mL was added as described by Titgemeyer et al. (2001) instead of the amount of 20 mL proposed by Short et al. (1996)
7. Let flasks sit overnight (unless samples were filtered, then ignore this step).
8. Measure aliquots on a spectrophotometer at 410 nm.
    a. If bubbles appear in cuvettes, gently tap on counter until they dissipate.
    b. Wipe outside of cuvette with KimWipe before placing in spectrophotometer. Any dust, debris, bubbles, etc will give inaccurate readings.

Calibration Curve:

1. Pipette 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 ml of the standard titanium solution (0.5 mg/ml) into individual 100 ml volumetric flasks.
2. Add 7.4 M sulphuric acid to each flask so that the combined volume is 10 ml.

Standard sol'n (ml)	H_2SO_4 added (ml)	TiO_2 Concentration (mg/ml)
0	10	0
1	9	0.005
2	8	0.010
3	7	0.015
4	6	0.020
5	5	0.025
6	4	0.030
7	3	0.035
8	2	0.040
9	1	0.045
10	0	0.050

3. Add 10 ml of 30% H_2O_2 to each flask and dilute to volume with nanopure water.
4. Measure aliquots on a spectrophotometer at 410 nm to obtain a calibration curve.

As described by Short, F.J., P. Gorton, J. Wiseman, K.N. Boorman. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Tech 59: 215 – 221.

As modified by Titgemeyer, E.C., C.K. Armendariz, D.J. Bindel, R.H. Greenwood, and C.A. Löest. 2001. Evaluation of titanium dioxide as a digestibility marker for cattle. J. Anim. Sci. 79: 1059 – 1063

Appendix C. Determination of titanium dioxide in equine fecal samples: Method 2

Standard titanium solution (0.5 mg/ml)

Dissolve 250 mg titanium dioxide in 100 ml concentrated sulphuric acid by heating in a beaker to just below boiling. Adding a stir bar to assist in dissolution is useful. It may take ~1 hr to 2 h to dissolve completely. Rinse the contents of the beaker into a 500 ml volumetric flask containing approximately 200 ml nanopure water. Add another 100 ml concentrated sulphuric acid to the mixture and dilute to 500 ml with nanopure water.

Calibration Curve:

5. Pipette 0, 0.5, 1, 1.5, and 2 ml of the standard titanium solution (0.5 mg/ml) into individual 50 ml volumetric flasks.
6. Add concentrated sulphuric acid to each flask so that the combined volume is 10 ml.

Standard sol'n (ml)	H_2SO_4 added (ml)	TiO_2 Concentration (mg/ml)
0	10	0
0.5	9.5	0.005
1.0	9	0.010
1.5	8.5	0.015
2	8	0.020

7. Add 10 ml of 30% H_2O_2 to each flask and dilute to volume with nanopure water.
8. Measure aliquots on a spectrophotometer at 410 nm to obtain a calibration curve.

Procedure:

All glassware needs to be washed in mineral-free detergent (Contrex Acidic Liquid Detergent) and rinsed with distilled water before each use.

Instruction	Notes, details, hints, suggestions, etc
1. Dry sample overnight in 55°C forced-air oven	Results will be on DM basis
2. Weigh 0.15 g of dried sample into quartz crucible in duplicate	Transfer sample from oven to dessicator until ready to weigh. Static electricity can cause problems with samples during weighing and after they are put in the crucibles. Take measures (e.g. dryer sheets, handling with tongs instead of hands, etc) to reduce static if it seems to be a problem.

3. Ash sample overnight at 600°C in ash oven	Overnight or at least 8 hours. Use tongs instead of hands to remove crucibles from oven to avoid causing static. If static is still a problem, washing down the sides of the crucible with a small amount of distilled water is an option.
4. Add 1 g of ammonium sulfate ($(NH_4)_2\ SO_4$) to FOSS 250 ml digestion tubes	Tubes should be individually and uniquely labeled. Easiest to add ammonium sulfate before step 5, but can add after, just make sure to wash the granules down the sides of the tube. One gram of ammonium sulfate is about ¼ teaspoon – so careful addition with ¼ teaspoon can save time, rather than having to weigh enough for each tube.
5. Transfer the contents of the crucible to the 250 ml FOSS digestion tubes.	Wash down sides of crucible with distilled water to ensure all ash is at the bottom of the crucible and to eliminate static. Rinse crucible multiple times with distilled water to ensure all ash has been transferred. Rinse sides of digestion tubes to ensure all ash and ammonium sulfate is at bottom of tube. Up to this step can be completed in advance, tubes can be covered and will be stable for days
6. Add 13 ml of concentrated H_2SO_4 to each digestion tube.	Should be completed only when ready to begin digestion. Addition of acid should be done in a fume hood using acid-resistant repipetter.
7. Place tubes in the FOSS Digestor 2520 and place the exhaust manifold on top of tubes.	If running less than 20 samples, place empty digestion tubes in the empty slots so the exhaust is still collected. See Figure C-1 for complete Digestor set-up and Figure C-2 for condenser set-up. Randomize tubes as they're placed in Digestor so duplicates are not sitting next to each other.
8. Set the machine at 420°C for 3 hours and push Start.	The machine will take approximately 1 hour to come up to temperature before the timer will start.
9. Label 50 ml volumetric flasks with corresponding labels to digestion tubes. Add 10 ml of 30% hydrogen peroxide to each flask.	If flasks are prepared ahead of time (no earlier than 1 hr before the end of digestion), place in refrigerator to keep peroxide cool. Fresh peroxide is required for complete reaction to occur. 10 mL of H_2O_2 was added as described by Titgemeyer et al.

	(2001) instead of the amount of 20 mL proposed by Short et al. (1996)
10. After 3 hours of boiling, remove tubes from Digestor and allow to sit in fume hood until they stop fuming.	Use chemical glove to pull tubes up out of the Digestor and then immediately use a hot pad/glove to grab the tube and transfer to a metal tube rack. If tube is dry or acid is low, add ~6 ml of concentrated acid to help resolubilize. May also be necessary to place tube back on heat block for ~10 min to assist in resolubilization.
11. Pour the contents of the tubes into 50 ml volumetric flasks containing 10 ml of 30% hydrogen peroxide (H_2O_2).	As each tube is processed, first use a squirt bottle to slowly add some distilled water to each tube and swirl to dilute the acid. Place a labeled 50 ml volumetric flask into a small (~tall 150 ml should do) beaker as a holder for the flask. Put a small funnel in the flask and pour the contents of the digestion tube into the corresponding flask. Rinse the tube until all residue is transferred to flask. Rinse funnel into the flask.
12. Let the flasks cool down, dilute to volume and mix.	Parafilm the flasks and pop a hole in the parafilm with a needle. Place thumb over needle hole and mix by inverting and shaking at least 3 times. Allow pressure built up in flasks to be released through needle hole after every inversion to avoid explosions.
13. Let flasks sit overnight.	To let particles settle to the bottom
14. Transfer an aliquot of each sample, standards, and blank into cuvettes. Measure aliquots on a spectrophotometer at 410 nm with the blank standard (0 mg/ml Ti) as the blank used to zero the spectrophotometer	If bubbles appear in cuvettes, use a pipet tip to gently wipe them from the surface of the cuvette. Wipe outside of cuvette with KimWipe before placing in spectrophotometer. Any dust, debris, bubbles, etc will give wacky readings. Let spectrophotometer read at least 3 times in a row before recording absorbance. Any drift in absorbance may indicate the presence of bubbles.

The method was validated by spiking known samples with TiO_2 and measuring percent recovery. Titanium dioxide recovered in the sample increased linearly as the amount of TiO_2 added to the sample increased (Figure C-3). Percent recovery of spiked samples averaged 93.2%. During sample analysis, if the coefficient of variation was above 10% between duplicates, the sample was rerun. Approximately 10% of samples were re-analyzed due to coefficients of variation above 10%.

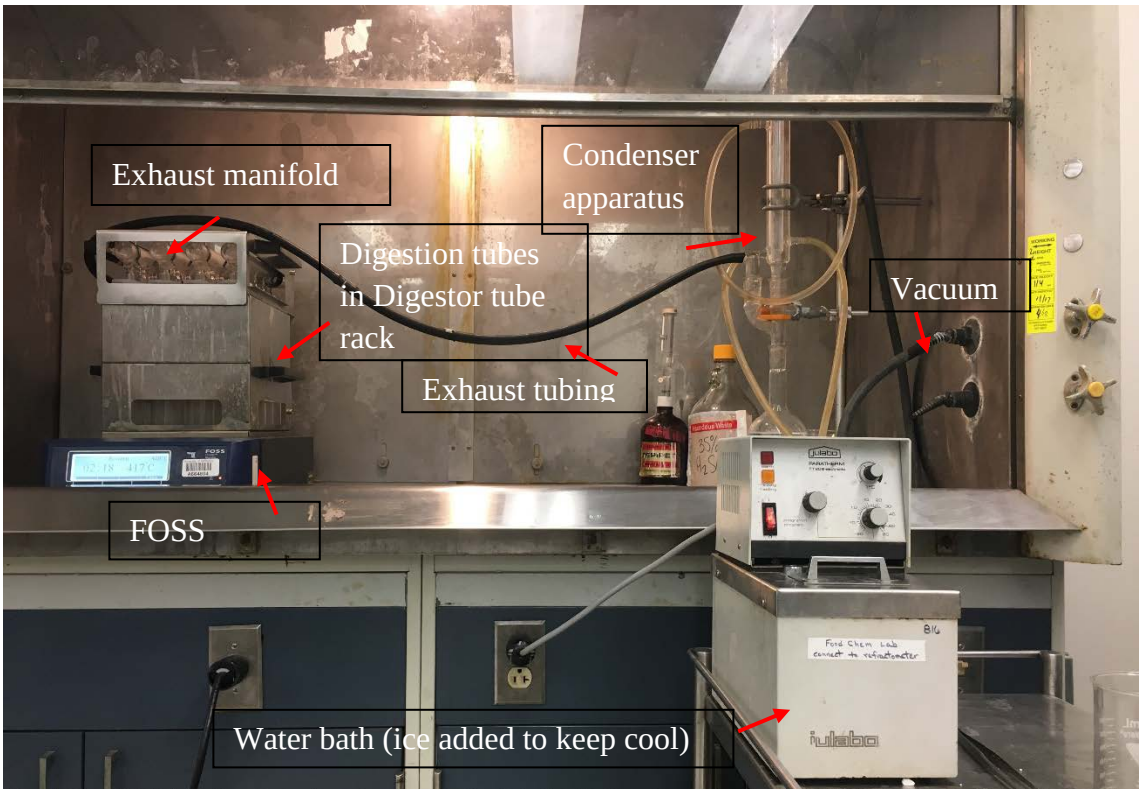


Figure C-1. Digestion set-up in fume hood

* Digestion tube rack is purposely placed upside down on top of the heat block of the FOSS Digestor. If the tube rack is placed right side up, the tubes heat too quickly and evaporate to dryness.

Also note: picture was taken with fume hood manifold up so the entire apparatus could be seen. Manifold was pulled down to working height or lower when digestions were being run.

Figure C-2. Close-up of condenser apparatus

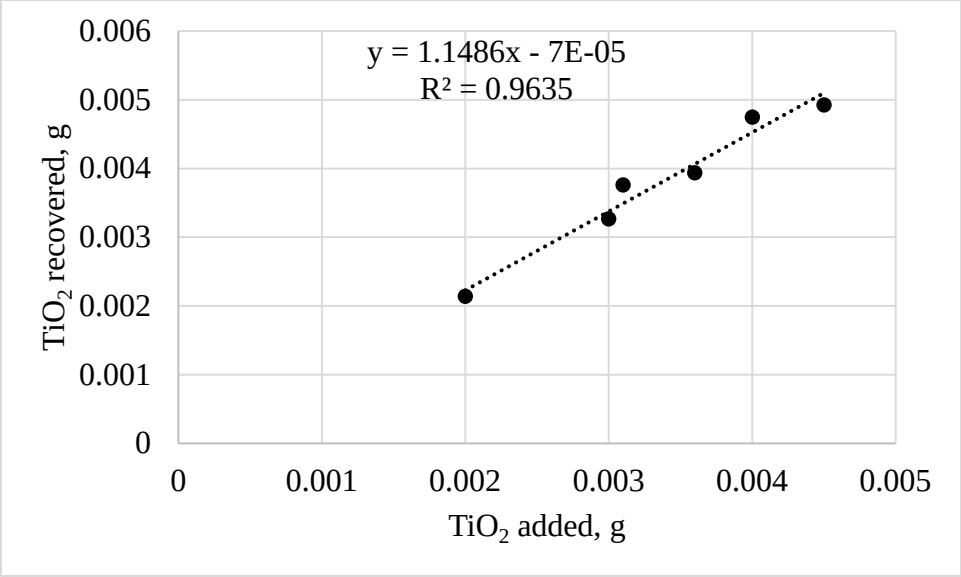


Figure C-3. Relationship of samples spiked with increasing amounts of TiO_2 and amount of TiO_2 recovered using the method described for Experiment 2.

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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. 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 received her M.S. in equine nutrition under Dr. Laurie Lawrence in December 2013. Her main research focus was understanding phosphorus digestibility in horses of different ages as well as developing an *in vitro* system to examine phosphorus digestibility. Ashley remained at the University of Kentucky under the supervision of Dr. Lauire Lawrence to obtain her PhD degree. Her main research focuses remained connected with phosphorus digestibility, but expanded to examine improved models for determining true phosphorus digestibility by horses. She has also completed work examining mineral concentrations in cool-season grasses, examining the efficacy of external markers in predicting fecal output by horses, looking at behavior of muzzled horses, and studying methods to estimate body fat in horses.

In addition to research, Ashley has also completed the Teaching and Learning Certificate for the university, taught an equine behavior class at an independent university, coached the University of Kentucky's Forage Bowl team, and was active in the department's Graduate Student Association. While at the University of Kentucky, Ashley has received the University of Kentucky's Graduate School Academic Year Fellowship for one year, the Kerri Casner Environmental Fellowship for one year, and

the Kentucky Opportunity Fellowship for three consecutive years. She has also received the Outstanding PhD Student award in the Department of Animal and Food Sciences in 2018.

Publications

Book Chapters

1. **Fowler, A.L.,** M. Holder, and L.M. Lawrence. Minerals. Equine Clinical Nutrition. (In preparation)
2. **Fowler, A.L.,** M.B. Pyles, and V. Bill. 2017. Mighty Minerals and Vital Vitamins. Horse Smarts. American Youth Horse Council.

Refereed Journal Publications

1. Kagan, I.A., L.M. Lawrence, D.H. Seman, K.J. Prince, **A.L. Fowler**, S.R. Smith. 2017. Effects of sampling time, cultivar, and methodology on water- and ethanol-soluble carbohydrate profiles of three cool-season grasses in central Kentucky. J. Eq. Vet. Sci. 61: 99-107
2. Calvo-Lorenzo, M.S., L.E. Hulbert, **A.L. Fowler**, A. Louie, L.J. Gershwin, K.E. Pinkerton, M.A. Ballou, K.C. Klasing and F.M. Mitloehner. 2017. Space allowance influences individually housed Holstein bull calf innate immune measures after castration at 3 weeks of age. J. Dairy Sci. 100: 2157-2169.
3. Hansen, T.L., **A.L. Fowler**, L.A. Strasinger, B.E. Harlow, and L.M. Lawrence. 2016. Effect of soaking on nitrate concentration in teff hay. J. Eq. Vet. Sci. 45: 53 – 57.
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5. **Fowler, A.L.**, T.L. Hansen, L.A. Strasinger, B.E. Harlow, and L.M. Lawrence. 2015. Phosphorus digestibility and phytate degradation by yearlings and mature horses. J. Anim. Sci. 93(12): 5735 – 5742.

Extension Publications

1. **Ashley Fowler.** "Phosphorus in my Horse's Diet: What is it Good For?" *Bluegrass Equine Digest.* Jan 30, 2016
2. Brittany Harlow, Tayler Hansen, and **Ashley Fowler**. "How sweet is your sweet feed?" *Bluegrass Equine Digest*. Oct 24, 2015
3. **Ashley Fowler,** Brittany Harlow, Tayler Hansen, and Laura Strasinger. "Hay: To soak or not to soak?" *Bluegrass Equine Digest.* Feb. 21, 2014