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# EVALUATING DIETARY AMINO ACID ADEQUACY IN HORSES USING ISOTOPIC TECHNIQUES

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DISSERTATION

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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
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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

# EVALUATING DIETARY AMINO ACID ADEQUACY IN HORSES USING ISOTOPIC TECHNIQUES

Little is known about amino acid (AA) requirements in horses despite muscle mass accretion being of importance to an athletic species. Isotope methods for determining AA requirements and whole-body protein synthesis (WBPS) had not been previously used in growing horses. The first study herein was the first to apply isotope methods to determine WBPS in growing horses. In the study, weanling colts received two different levels of crude protein. Whole-body protein kinetics indicated that WBPS was greater when the weanlings were fed the diet with a greater crude protein content ($P<0.05$). The second study sought to determine a lysine requirement for yearling horses using the indicator AA oxidation (IAAO) method. Despite using six dietary levels of lysine; three above and three below the current recommendation, no breakpoint could be determined. Phenylalanine kinetics were not affected by lysine level ($P>0.05$), but plasma lysine increased linearly with lysine intake ($P<0.0001$). After comparing dietary AA intakes with current AA requirement recommendations, threonine was a candidate for the limiting AA in the diets used in the first two studies. The objective of the next two studies was to determine if threonine supplementation would increase WBPS. Weanling colts fed a grass forage and commercial concentrate were supplemented with threonine in one study, while adult mares fed a high fiber diet and low threonine concentrate were supplemented with threonine in the other study. In neither case were whole-body protein kinetics affected by threonine supplementation ($P>0.05$). However, multiple plasma AA concentrations were affected by supplementation ($P<0.05$) in both studies, suggesting that supplementation of a single AA can affect the metabolism of other AAs. The final study conducted was aimed at improving the IAAO method for use in horses. Intravenous isotope infusion was compared to a less invasive oral infusion. Both infusion methods produced stable plateaus and by calculation, the splanchnic extraction of phenylalanine was found to be 27%. Additional research is needed to determine AA requirements for horses. These studies add insight into equine AA requirements and metabolism and the confirmation of the oral isotope infusion method will allow future experiments to be less invasive.

KEYWORDS: Equine, Nutrition, Amino Acids, Indicator Amino Acid Oxidation, Isotope Kinetics

Sara L. Tanner
July 11, 2014

# EVALUATING DIETARY AMINO ACID ADEQUACY IN HORSES USING ISOTOPIC TECHNIQUES

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*Research horses at Maine Chance Farm:*

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## Chapter 1. Introduction

The horse is unlike other agricultural species because the value of a horse is often determined by its athletic prowess, whereas species such as chickens, pigs, or cattle are valued for the nutrition they provide to the human consumer. As athletic prowess is a particularly difficult endpoint to quantify and lacks impact on human nutrition, research on equine nutrient requirements has lagged behind the research in other species.

As an athletic species, protein accretion and turnover are particularly important to the horse industry. Currently, protein requirements are expressed as an amount of crude protein, which is based on nitrogen content rather than amino acid content. Horses cannot use all forms of nitrogen equally, especially because they are hindgut fermenters and cannot use microbial proteins as ruminants can. Furthermore, mammals do not have protein requirements per se, but instead have amino acid requirements. As the majority of protein accretion occurs during growth, the amino acid requirements of growing horses are of particular importance.

This dissertation includes a review of pertinent literature and describes the first study to apply isotope methodology to determine whole-body protein synthesis in growing horses. Subsequent studies attempted to determine a lysine requirement using the indicator amino acid oxidation method and investigate the effects of threonine supplementation. The final study involves the determination of the splanchnic extraction of phenylalanine and compared measurements of oral versus intravenous infusion to improve the methodology for future studies in this area.

## Chapter 2. Literature review

This literature review covers the current use of protein in the diets of horses, bioavailability of amino acids, the ideal protein concept, commonly limiting amino acids, common protein sources for equine feeds, and methods of determining amino acid requirements.

### Dietary protein use in the equine industry

#### *Consumer perception of protein in equine diets*

The amount of protein in horse feeds is often used as a marketing tool with the protein percentage sometimes being part of the name of the feed. Examples of this practice include: Purina's Nature's Essentials® Enrich 12® (Purina, 2010), Triple Crown's 14% Performance Horse Feed Formula (Triple Crown Nutrition, 2010), and Blue Seal's SBP 16-10TM (Blue Seal Feeds, 2012). Consumers often assume that more protein for their horse is better as a result of this marketing.

#### *Overuse of protein in the equine industry*

The equine industry has a history of overfeeding protein. Surveys of racing Standardbreds found that protein was fed an average of 127% of requirement (Gallagher et al., 1992). A regional survey in North Carolina found that 70% of horses were provided crude protein in excess of requirements (Honore and Uhlinger, 1994). Both of these studies used the requirements put forth by the NRC (1989). A more recent study comparing industry practices and the most recent NRC (2007) requirements showed that equine rations in the Chesapeake Bay area provided 157± 21.6% of the 2007 NRC requirement for crude protein (Harper et al., 2009). Overfeeding protein in the equine

industry has been a persistent trend. Whether the overfeeding is because of marketing or to meet amino acid requirements remains unclear.

### *Potential problems resulting from overfeeding protein to horses*

There are several potential consequences of overfeeding protein to horses: 1) an increase in the cost of feed, as protein is one of the most expensive nutrients; 2) an increase in nitrogenous waste excretion; 3) the nitrogen that is currently overfed to horses could be utilized in other animal industries; and 4) overfeeding protein can cause health problems.

#### *Cost of feeding horses*

Feed cost has historically been a substantial expense of keeping horses (Cooper, 1917; Harris, 1999; Langdon, 1982; Łojek et al., 2009). One group of researchers found that by more efficiently managing feed costs, the total cost of rearing a young horse could be decreased by 11.3% (Dajlidenok et al., 2011). Higher protein content is associated with higher feed prices. For example, Relative Feed Value (RFV) is positively correlated with CP content within alfalfa hay (Canbolat et al., 2006) and price. Feeding protein amounts closer to the horses' requirements could be an effective method in cost saving for the industry.

#### *Role of the equine industry in nitrogenous waste production*

Both urine and feces contain nitrogen, primarily in the forms of urea, ammonia, uric acid and undigested protein. Over a billion tons of manure per year is produced by the domestic livestock production industries (EPA, 2011). There are 9.2 million horses in the United States (American Horse Council, 2006), making their contribution

consequential. Concerns as to the use of animal manure as fertilizer, including the over application of nutrients to a small space (Fertilizer Industry Federation of Australia, 2001) and water runoff (Council for Agricultural Science and Technology, 1996), are abundant. Animal manure varies as to its components by species. Horse manure contains 0.4-0.65% N on a total basis, which is comparable to other species, such as cows, sheep, pigs, chickens, and rabbits, which contains 0.6, 0.6-1.00, 0.50-0.6, 1.1-3, and 2.4%, respectively (Bradley and Ellis, 2008; EPA, 2001; Gowariker et al., 2009).

The negative effects of nitrogenous waste on the environment have been reviewed elsewhere (Fenn et al., 2003) and include increased greenhouse gas emissions (Chowdhury, 2013), accumulation of nitrogen content in plant tissues causing nitrate poisoning (Weightman and Hudson, 2013), eutrophication (Nixon, 1995), and odorous gases that may affect animal and employee health (Chowdhury, 2013). Regulation in the future regarding nitrogenous waste may focus on areas of high equine concentration as well as areas with the potential to affect sensitive ecosystems. One such sensitive ecosystem is the Chesapeake Bay area, where nutrient intake in horses has already been studied in order to gain more insight into ways of decreasing nitrogenous waste of horse farms (Harper et al., 2009).

### *Use of nitrogen in other industries*

Many agricultural industries are dependent on nitrogen. Many of these industries produce human foodstuffs directly or indirectly, which ties the input cost of dietary nitrogen to human food prices (Swanson et al., 2013). For this reason, in addition to the negative impact of nitrogenous waste, many other industries have focused on ways to more efficiently utilize nitrogen. The United States Department of Agriculture released a

report in 2011 reviewing policy strategies to encourage better management of nitrogen, underscoring the importance of this nutrient (Ribaudo et al., 2011). Efficiency in nitrogen management in growing cereal grains (Raun and Johnson, 1999) and agricultural animal species (Rotz, 2004) has been reviewed elsewhere. Despite efforts to become more efficient, nitrogen inputs in agriculture have continued to rise due to demand (Howarth et al., 2002).

## *Adverse effects on equine health and performance*

Overfeeding protein to horses can have negative impacts on health and performance. Increased water consumption, increased ammonia production, and disrupted acid-base balance are some of the ways that a diet containing excess protein can be detrimental to the horse.

### *Increased water consumption and urine output*

Metabolism of excess protein necessitates that the excess nitrogen be excreted. Water is necessary for the elimination of the excess nitrogen. Therefore, researchers have found that feeding additional protein to other species resulted in increased water intake and increased urine output (Funaba et al., 1996; Kim et al., 2011). For example, human body builders, who consume relatively high protein amounts, had a daily urine output of approximately 1775 mL (Kim et al., 2011) compared to an average of 1500 mL for healthy adults (Alexander et al., 2009). Increased urine output associated with excess protein, due to the body's need to remove excess nitrogen, can decrease retention of calcium as reviewed by Delimaris (2013), Heaney (2002) and Roughead (2003), as it is also excreted in the urine (Hashimoto et al., 1996; Heaney, 2002; Roughead, 2003). The result is decreased mineralization of bone as reviewed by Barzel and Massey (1998) and

Delimaris (2013), which is of particular importance for the exercising horse as lower bone density is associated with stress fractures in human athletes (Myburgh et al., 1990) and Quarter Horses in race training (Nielsen et al., 1997).

*Ammonia inhalation*

In addition to impacting the outside environment, excess nitrogenous waste may also affect animal housing, which is the workplace for animal caretakers. Ambient ammonia was found to be 100-200 ppb higher in equine stables than in pastures, and equine breath pH was also significantly higher in stabled than in pastured ponies with equine breath pH of 4.8 and 4.5, respectively (Whittaker et al., 2009). Ammonia inhalation is known to exacerbate symptoms of chronic obstructive pulmonary disease in horses (Vandenput et al., 1998) and cause respiratory symptoms in people at higher concentrations (Arwood et al., 1985). The Center for Disease Control has set a short term exposure limit for ammonia of 15 minutes at 35 ppm (CDC, 2011).

*Decreased athletic performance*

Protein needs are greater for the horse in work (NRC, 2007), but too much protein may actually decrease athletic performance. Acid-base balance is altered when horses are fed higher amounts of protein, as protein contains sulfur and phosphorus that alters the dietary cation-anion balance, resulting in metabolic acidosis (Graham-Thiers and Kronfeld, 2005b; Graham-Thiers et al., 1999). The metabolic acidosis related to the diets with higher protein content may correlate with fatigue (Mainwood and Renaud, 1985). Additionally, heat load may be of concern for athletic horses needing to dissipate heat during exercise, especially in warm climates. Excess protein increases heat production in

pigs (Kerr et al., 2003b) and intravenously administered amino acids are used to help prevent hypothermia during surgery in humans as reviewed by Yamaoka (2008).

Increased water consumption and urination associated with excess protein intake may also be detrimental to athletic performance. The horse must dispose of excess nitrogen through urination, which in turn requires additional water. Increased water intake can increase the amount of weight the horse will carry during exercise. It is common to administer diuretics to athletic horses for the purpose of decreasing water weight, as reviewed by Soma and Uboh (1998). Increased urination may additionally dehydrate the horse.

### ***Success in feeding low protein diets to the horse and other species***

#### *Success in other species*

In other species, such as swine, amino acid requirements for various physiological states have been identified (NRC, 2012, 1998) helping the industry to minimize nitrogenous waste (Osada et al., 2011; Panetta et al., 2006) without compromising pig growth (Tuitoek et al., 1997), particularly through supplementing low protein diets with individual amino acids (Kerr et al., 1995; Kerr et al., 2003a). Low protein diets have also been successfully used with broilers (Aftab et al., 2006), ducks (Baeza and Lecerlcq, 1998), and quail (Karaalp, 2009) and less nitrogenous waste is also produced by birds on a low protein diet when compared with other birds with a similar growth rate (Allen, 2003). Aquatic species, such as rainbow trout (Ruohonen, 1999), carp (Jahan et al., 2003), have also been found to produce less nitrogenous waste when fed lower protein diets (Cho and Bureau, 2001).

*Protein deficiency symptoms in the horse*

Protein deficiency in horses is associated with slowed growth (Ott and Kivipelto, 2002), weight loss (Martin et al., 1991), decreased milk production (van Niekerk and van Niekerk, 1997a), and irregular estrus (van Niekerk and van Niekerk, 1997b). As producers wish to avoid these symptoms, formulating diets to avoid deficiency is important.

*Low protein diets fed to horses*

Low protein diets for equines have been met with mixed success. Diets with a crude protein percentage as low as 5.5% have been successfully fed to exercising horses without the horses losing weight over a period of 60 days (Patterson et al., 1985). However, some low protein diets have resulted in signs of deficiency. Yearling horses fed 799 g CP per day had lower weight gain, heart girth gain, and hip height gain compared to horses fed 993 g CP per day (Ott and Kivipelto, 2002). Foal weights were decreased by ~25% in lactating mares fed 2 g CP/kg BW/d (van Niekerk and van Niekerk, 1997a) and ovulation occurred 2-3 weeks later than mares fed 2.75 g CP/kg BW/d (van Niekerk and van Niekerk, 1997b). Lactating Australian stock horse mares fed 92.9 g N (~580 g CP) per day lost 0.32 kg per day over 71 days, which is less weight loss than the group fed the same diet supplemented with urea (Martin et al., 1991). Of particular interest is that horses fed low protein diets supplemented with amino acids likely to be limiting have performed similarly to horses that were fed greater amounts of protein in growth (Staniar et al., 2001).

*Horses and urea utilization*

The horse's ability to use urea as a source of nitrogen is far inferior to that of ruminants. Urea supplementation has been used in conjunction with low protein diets in horses in order to meet nitrogen requirements with mixed success (Hintz and Schryver, 1972; Schubert et al., 1991). One study found improved nitrogen balance with the addition of urea to the diet (Slade et al., 1970). The next year another study found that urea supplemented ponies remained in negative nitrogen balance (Reitnour and Treece, 1971) and more recently urea supplementation to a low protein diet increased weight loss in lactating Australian stock mares (Martin et al., 1991). High levels of urea have proved toxic to ponies, as 7 out of 8 ponies given 140 g of urea orally died (Hintz et al., 1970).

***Current use of amino acid supplements in equine diets***

Amino acid supplementation is actually fairly common in the equine industry. As amino acids are generally regarded as safe, they are marketed as supplements and added to feeds (FDA, 2013). Amino acids are included in commercial concentrates as well as supplements designed to top dress concentrates.

Amino acids are added to the equine diet for a variety of health and performance reasons. Methionine has been added as it has been shown to improve hoof health in horses (Clark and Rakes, 1982). Tryptophan has been added as a calming agent as it is a precursor to serotonin (Kellon, 2008). In practice, 3 g of supplementation five times daily has had no effect on cribbing (O'Reilly, 2006), an equine stereotypy where a horse bites a solid object and sucks in air, and commercial doses (6.3 g) did not change behavior or heart rate in horses introduced to a novel object or stranger, despite elevating plasma concentrations of tryptophan (Noble et al., 2008). Both age and breed influence plasma

tryptophan concentrations (Alberghina et al., 2014). A review noted that no scientific studies have proved tryptophan's effectiveness in horses as a calming agent (Grimmett and Sillence, 2005). Branched chain amino acids have been added to diets as they may have a glycogen sparing action (Harris and Harris, 2005) or may decrease lactate production (Glade, 1989). In the case of leucine alone, supplementation has increased plasma insulin concentrations in horses (Urschel et al., 2010). Both leucine and insulin have been shown to be activators of the mechanistic target of rapamycin pathway responsible for protein synthesis in other species, as reviewed by Avruch et al. (2006). Amino acids, in general, have been fed to horses to attenuate muscle breakdown after exercise as amino acid supplementation has decreased ubiquitin mRNA 4-hrs post exercise in equine muscle (van den Hoven et al., 2011).

*Addition of potentially limiting amino acids*

Both lysine and threonine have been added to equine diets as they may be potentially limiting for protein synthesis. Adding 18 g crystalline lysine to linseed meal increased nitrogen retention 8.9 g/d and average daily gain by 0.41 kg/d in 5.5 month old horses (Hintz et al., 1971). Lysine plasma concentrations increased with increasing crystalline lysine intake in weanlings (Fisher et al., 1989). Increasing dietary threonine content from 0.43 to 0.53% of the diet resulted in an additional girth gain of 1.2 cm over 112 days in yearlings (Graham et al., 1994). However, threonine and lysine supplementation did not increase average daily gain over levels obtained with only lysine supplementation (Graham et al., 1994). No differences in average daily gain or body condition score were detected between growing horses fed a 14% CP pasture supplement

or a 9% CP pasture supplement fortified with 0.6% lysine and 0.4% threonine (Staniar et al., 2001).

## Bioavailability of amino acids to the horse

Bioavailability of amino acids has been defined as "the proportion of ingested dietary amino acids that is absorbed in a chemical form that renders these amino acids potentially suitable for metabolism of protein synthesis" (Stein et al., 2007b). There are a number of factors that can affect the bioavailability of a given amino acid in a given diet. These factors include the form of the amino acid, digestibility, competition for absorption, type of protein in the diet, and other dietary constituents.

### *Amino acid enantiomers*

With the exception of glycine, amino acids have both levorotatory (L) and dextrorotatory (D) forms, which refers to the amino acid's enantiomers (Wong, 2009). Mammals build their proteins in chains of L-amino acids. However, crystalline D-amino acids are common in feed ingredients due to the manufacturing process, particularly methionine (Leuchtenberger, 2008). In some cases transporters and enzymes exist to absorb and then convert the D-amino acid to an L-amino acid, such as through the action of D-amino oxidase as reviewed by D'Aniello et al. (1993). Many mammals have the ability to utilize dietary D-amino acids as reviewed by Baker and D'Mello (1994). The ability of horses to convert D-amino acids to L-amino acids has not been investigated specifically.

***Possible amino acid antagonisms***

Amino acids that are similar in both the structure and charge of their R group are most likely to be antagonists. An antagonism is caused when two or more substances, similar in structure, compete for an enzyme or membrane transporter. In the case of amino acids, there are two main examples of amino acid antagonism that have been identified: 1) lysine/arginine and 2) leucine/isoleucine/valine. Amino acid antagonisms are identified in animal studies when increasing one amino acid results in growth depression that can be corrected when the antagonized amino acid is added to the diet (Harper et al., 1954). Increasing one amino acid also results in symptoms of deficiency of the antagonized amino acid (Czarnecki et al., 1985; O'Dell and Savage, 1966; Ulman et al., 1981). Addition of one amino acid to the diet may also result in reductions in plasma concentration of the antagonized amino acid (Shinnick and Harper, 1977).

Antagonism between lysine and arginine has been extensively studied in poultry (Austic and Scott, 1975; Jones et al., 1967; Kadirvel and Kratzer, 1974; O'Dell and Savage, 1966; Stutz et al., 1971) and has been found to be present in dogs (Czarnecki et al., 1985), but not cats (Fascetti et al., 2004), pigs (Edmonds and Baker, 1987b), calves (Abe et al., 2001) and channel catfish (Robinson et al., 1981). Adverse effects on growth or the creation of deficiency symptoms of the antagonized amino acid have been caused by excess lysine (Czarnecki et al., 1985; O'Dell and Savage, 1966) and by excess arginine (D'Mello and Lewis, 1970). This issue has not been investigated in horses.

The antagonism between branched chain amino acids is at the enzyme level, as these three amino acids share a degradation pathway through the branched chain alpha-keto acid dehydrogenase complex as has been reviewed (Harper et al., 1984; Shimomura

et al., 2006). Reduced plasma concentrations of the antagonized amino acids may be the result of the upregulation of catabolism (May et al., 1991; Smith and Austic, 1978). If one of the branched chained amino acids is supplemented without the others, an antagonism could result between the supplemented amino acid the other two amino acids. This phenomenon has been documented in chicks (D'Mello and Lewis, 1970; Smith and Austic, 1978), turkey poults (D'Mello, 1975), rats (Harper et al., 1954; Shinnick and Harper, 1977), and even ruminal bacteria (Kajikawa et al., 2007). The antagonism remains largely uninvestigated in horses, although leucine supplementation has resulted in a decrease in plasma isoleucine and valine in the horse (Urschel et al., 2010). Many commercial branched chain amino acid supplements for horses contain all three branched chain amino acids (Kellon, 2008), presumably to avoid issues associated with possible antagonism.

***Differences in digestibility of protein bound and free amino acids***

As protein content is decreased in a diet, one or more indispensable amino acids may become limiting. The addition of crystalline amino acids to the low protein diets is frequently done to correct possible deficiencies, making estimates of both protein bound and crystalline amino acid bioavailability important. Determining bioavailability is difficult and therefore digestibility is often used as an estimate and its suitability as an estimate is reviewed by Stein et al. (2007b). Total tract digestibility is the apparent percentage of a feedstuff or a constituent of a feedstuff that is not excreted in the feces. However, due to the actions of microbes, site of amino acid absorption, and endogenous losses into the digestive tract, other measures of digestibility, such as ileal digestibility, which will be discussed later, are sometimes used.

The digestibilities of crystalline and protein bound amino acids in casein have been found to be similar (Chung and Baker, 1992; Plakas and Katayama, 1981). Carp fed casein or a free amino acid diet equivalent had total amino acid total tract digestibilities of 80 and 86% for the casein and free amino acids, respectively (Plakas and Katayama, 1981). Pigs had an average apparent ileal digestibility of 95% and 94% for diets of casein and casein's constituent amino acids in free form, respectively (Chung and Baker, 1992). Depending on the specific protein, amino acid digestibility may vary. For example, in swine fed diets containing either casein or shrimp meal, nitrogen digestibility was 85% and 68%, respectively (Fanimo et al., 2006). Crystalline amino acids are absorbed at a much faster rate than protein bound amino acids in both aquatic (Cowey and Walton, 1988; Plakas and Katayama, 1981; Zarate et al., 1999) and terrestrial (Koopman et al., 2009; Leibholz et al., 1986; Rolls et al., 1972) species. For example, in humans fed either casein or casein hydrolysate, the patients fed casein hydrolysate had plasma amino acid concentration peaks ~25 – 50% higher than those fed the intact casein, but patients ingesting the intact casein had greater plasma amino acid concentrations 4-6 hrs after ingestion (Koopman et al., 2009). Therefore, dietary crystalline amino acids arrive at the tissue level as free amino acids before dietary protein bound amino acids (Fox et al., 2009). This differential in absorption time may result in an imbalance at the tissue level.

In some cases, diets supplemented with crystalline amino acids do not support the same amount of growth (Zarate and Lovell, 1997) or lean gain (Myer and Gorbet, 2004) as intact protein. Also, increased oxidation for crystalline amino acid supplemented diets compared to intact protein indicates lower rates of utilization for protein synthesis (Batterham and Bayley, 1989; Otto and Snejdarkova, 1989; Otto et al., 1989). The effect

of reduced utilization can be attenuated by increasing feeding frequency in swine (Batterham, 1974) or by coating or "protecting" free amino acids (Alam et al., 2004; Kondo and Adriaans, 1982; Murai et al., 1981). Tissue imbalances from the crystalline amino acid supplementation of equine diets may be of particular importance in management situations where a protein rich concentrate feed is provided only twice daily; a common practice in the horse industry (Bezdekova et al., 2008; Hoffman et al., 2009; Nyhart et al., 2011). Even though low protein diets supplemented with amino acids could support adequate growth, nitrogen utilization may not be maximized if tissue imbalances occur from diets fed in meals. Feeding horses little and often would be one way to avoid this potential problem as increased meal frequency from one meal to four meals per day has been shown to decrease urinary nitrogen output in swine fed L-lysine-HCl supplemented diets (Partridge et al., 1985), presumably increasing nitrogen utilization. Plasma amino acid concentrations in horses fed six meals per day did not respond to feeding, while feeding a single meal per day resulted in large post-feeding increases (Russell et al., 1986), indicating that despite varying absorption rates a constant supply of dietary amino acids would be reaching the tissue, minimizing potential tissue imbalance.

### ***Digestion and absorption of protein in the horse***

#### *Aspects of protein digestion unique to the horse*

In the equine stomach, hydrochloric acid is constantly being secreted by the parietal cells in the glandular region (Campbell-Thompson and Merritt, 1990), unlike most animals, which do not have constant secretion. The hydrochloric acid helps to denature the proteins and provide more surface area for proteases to work. Pepsinogen is also secreted in the stomach from the chief cells, which becomes activated by the

hydrochloric acid (Hirschowitz, 1984). Proteolytic enzymes break down the protein into amino acids, which can be absorbed in the small intestine (Ellis and Hill, 2005). Microbes may also play a role in protein breakdown as proteolytic bacteria have been identified in the duodenum, jejunum, ileum, cecum, and colon of the pony, with the highest proportion of proteolytic bacteria present in the duodenum (Mackie and Wilkins, 1988). Protein not absorbed by the horse continues on to the large intestine, where it can be used to support the nitrogen needs of the microbial population of the hindgut (Maczulak et al., 1985). The majority of the equine microbial population resides in the hindgut, which is a contrast to ruminants, where the majority of the microbial population resides in the foregut (Kern et al., 1974). Ponies euthanized 2 hrs post feeding had 642 bacteria per gram $x10^{-7}$ of digesta in the cecum compared to 208 bacteria per gram $x10^{-7}$ of digesta in the ileum ($P<0.01$) (Kern et al., 1974).

*Current knowledge of amino acid absorption in the equine*

In order to reach the bloodstream, amino acids must be transported twice; once through the luminal membrane and once through the serosal membrane. Research on the presence of amino acid transporters in various portions of the equine intestine is lacking, but two papers have found mRNA expression of amino acid transporters in the equine gut (Holcombe et al., 2009; Woodward et al., 2010). The mRNA data supports the theory that most of the amino acid absorption in the horse takes place in the jejunum, although it also indicates that some amino acid absorption may take place in the large intestine (Woodward et al., 2010). Another group examining arteriovenous differences found extraction of dietary glutamine to be greater in the jejunum than the large intestine (Duckworth et al., 1992). Dietary nitrogen that has not been absorbed by the horse can be

converted to microbial protein to help sustain the microbial population of the equine hindgut (Maczulak et al., 1985; Reitnour et al., 1970).

The majority of nitrogen absorption takes place before the cecum, as has been shown to be the case in cecally cannulated horses (Reitnour et al., 1969; Reitnour and Salsbury, 1972). There have been a number of studies that sought to determine if there was any uptake of nitrogen from the large intestine in horses (Martin et al., 1996; Slade et al., 1971). Results indicate that the contribution to absorbed amino acids by the large intestine is minimal at most. By comparing the effect of dietary or cecally infused protein on plasma amino acid concentrations one study found that nitrogen absorbed in the hindgut was not in the form of amino acids (Reitnour and Salsbury, 1975). Another study on tissue from slaughtered ponies found that ammonia passed through colonic tissue, but less than 2% of the amino acids (Bochroder et al., 1994). Unlike ruminants, the bacterial proteins are not available to the horse and are excreted due to their location in the hindgut, posterior to sites of enzymatic digestion and absorption for the horse.

***Available equine ileal digestibility data***

The main portion of the microbial population of the horse's digestive tract is in the hindgut (Kern et al., 1974). Ileal digestibilities largely separate the effect of the host and the microbial population, as they measure the disappearance of feed components from the digesta before it enters the hindgut. Several studies of ileal cannulated horses measured protein digestibilities, but amino acid concentrations in the feed and the digesta were not measured (Farley et al., 1995; Gibbs et al., 1988, 1996; Haley, 1981; Rosenfeld and Austbø, 2009b). Large databases of the ileal digestibilities of amino acids from a variety of feed ingredients are available for swine (NRC, 2012) and chickens (NRC,

1994). No such library exists for horses, as there is only one paper that investigates prececal or ileal amino acid digestibilities in horses (Almeida et al., 1999). In this study, five different levels of CP were investigated in a 50:50 concentrate to *Cynodon dactylon* hay ratio by increasing the amount of soybean meal at the expense of corn in the concentrate (Almeida et al., 1999). Apparent prececal digestibilities ranged from 52%-69% of intake for lysine and from 32% - 62% of intake for threonine increasing with crude protein content of the diet (Almeida et al., 1999). Unfortunately, this study cannot be compared to ileal digestibility values in other species, such as swine, because more than one feedstuff was used.

***Possible effects of fiber on protein digestibility***

Horses have evolved to successfully eat diets high in fiber as reviewed by Janis (1976). High fiber diets have been shown to have decreased total tract protein digestibility in dogs (Burrows et al., 1982) and rats (Shah et al., 1982; Wong and Cheung, 2003). One reason that total tract digestibility may be decreased is that fiber increases endogenous loss of amino acids pre-ileum, as is the case in swine (Stein et al., 2007a) and rats (Shah et al., 1982). Another would be that the majority of protein in most plant-based feedstuffs is surrounded by cell walls (Van Soest et al., 1991), making the protein more difficult for the animal to extract if the cell walls are not degraded first. Type of fiber also has an effect on protein digestibility. True nitrogen digestibility in rats of a control diet was 93%, but this was decreased when 20% of the diet was composed of cellulose, pectin, guar gum, or wheat bran to 86%, 87%, 70%, and 78%, respectively (Shah et al., 1982). In cats, total tract crude protein digestibility of 94% for a basal diet was reduced to

92% or 84% when alfalfa meal or peanut hulls were added, respectively (Fekete et al., 2004). The effect of fiber on protein digestibility is reviewed by Blackburn (1981).

**Protein requirements for horses**

Around the world, there are several different systems to express protein requirements for horses, including crude protein (NRC, 2007), horse digestible crude protein (Martin-Rosset and Tisserand, 2004), apparent digestible crude protein (Ellis, 2004), and prececally digested protein (Coenen et al., 2011). Crude protein is, by definition, a relatively crude way of measuring protein, as it is based on a measurement of nitrogen as opposed to measuring protein, and even more so when using it to gauge a feedstuff's suitability in meeting the nutrient requirements of the horse. Although the NRC (2007) does not take into account differences in availability/digestibility between feedstuffs, other systems have attempted to take these factors into account. However, there is a dearth of knowledge relating to amino acid availability/digestibility in the horse, making it difficult to implement other more descriptive systems to express amino acid requirements, such as on a true ileal digestible basis, which is used to express amino acid requirements for pigs. In light of this knowledge gap, several groups have posited use of other information as proxies. Protein digestibility data from ruminants, namely sheep (Austbo, 2004), and cattle (Coenen et al., 2011) have been used. However, these adjusted values are ultimately linked, at least in part, to the crude protein content of the feed.

The Germans are moving toward a system that would estimate prececal digestibility from analytical protein fractions and amino acid requirements from tissue composition (Coenen et al., 2011). The French correct for the amount of non-protein

nitrogen and site of digestion (small or large intestine), producing a requirement in terms of horse digestible crude protein (Matières Azotées Digestibles Cheval or MADC in French) (Martin-Rosset and Tisserand, 2004). As the complexity of correction factors increases, the protein requirement for these animals decreases. It is expected that if amino acid requirements, in addition to lysine, for the horse were known, the amount of protein required by the horse could be even less.

**Ideal protein concept**

The correlation between amino acid tissue composition of an organism and requirements gave rise to the idea that amino acids should be fed in an ideal ratio (Fisher and Scott, 1954). This concept came to be known as the ideal protein concept. The ideal protein concept has been applied to diet formulation for swine (NRC, 2012), fish (Furuya et al., 2004), and poultry (Baker et al., 2002; Dari et al., 2005). The idea is that there is an optimal balance of amino acids for the animal at maintenance and clearly defined physiological states, which can be expressed as a ratio to lysine (NRC, 2012). Although, amino acid requirements differ based on gender and stress, the ratio stays relatively constant (Van Lunen and Cole, 1996). Lysine is used as the amino acid to which all others are expressed as a ratio because it is the amino acid most likely to be deficient in most diets, as reviewed by Miller (2004).

Use of the ideal protein concept is more common in non-ruminants, such as horses, swine, and humans, because they absorb their amino acids directly from the diet, while ruminants obtain a great deal of their amino acids from microbial protein (Boisen et al., 2000). However ruminally protected amino acids have allowed for more formulation of diets based on the ideal protein concept (Lee et al., 2012; Sun et al., 2007).

### *Effects of dietary amino acid imbalance*

When studying an amino acid imbalance, the limiting amino acid is the same in all treatments. An amino acid imbalance can be created by adding a protein or amino acid mixture devoid of the limiting amino acid to the diet or by adding an amino acid other than the limiting amino acid to the mixture. If only the amino acid that is limiting is added to the imbalanced diet, the imbalance can be alleviated. Adding the same ratio of amino acids to the diet that maintains the ratio of amino acids in the imbalanced diet will not alleviate it despite increasing the protein supply and supply of the limiting amino acid. Amino acid imbalances have been thoroughly reviewed elsewhere (Benevenga and Steele, 1984; D'Mello, 2003; Harper et al., 1970). When supplements containing only one or two amino acids are added to the diet, the possibility of creating an imbalance is increased, as was suggested by a review on human amino acid supplementation by Garlick (2004).

### *Implications for ration balancing in the equine industry*

As amino acid requirements have not been determined for horses, the ideal protein concept cannot be applied directly. Currently, the NRC (2007) recommends the use of the ratio derived from the equine muscle tissue to extrapolate ideal protein ratios. More in depth discussion on using equine muscle as a proxy for empirically determined amino acid requirements is included in the section “Tissue composition”. Benefits of improved diet formulation would include less nitrogenous waste (Hegedüs, 1993; Panetta et al., 2006), reduced overfeeding and avoidance of amino acid deficiency.

Young growing horses and working horses would be the most likely to suffer ill effects from the creation of disproportionate amounts of amino acids due to their greater

need for protein/amino acids. The growing rat is more severely affected by amino acid imbalance caused by tyrosine supplementation than its adult counterparts (Schweizer, 1947). Low protein diets also tend to increase the severity of imbalances as reviewed by Harper et al. (1970). Overnutrition tends to be more likely in the equine industry, although a low protein diet, to which the owner then adds an amino acid supplement, could create an imbalance.

## Commonly limiting amino acids in other species' diets

### *Lysine as a limiting amino acid*

Lysine is a common, if not the most common, first limiting amino acid for a variety of species and diets. Lysine supplementation has been shown to have a positive effect on growth in many species including fish (Li et al., 2011; Viola et al., 1992), rats (Sika and Layman, 1995), humans (Graham et al., 1969; Pereira et al., 1969; Tome and Bos, 2007), cattle (Xue et al., 2011), dogs (Longenecker and Hause, 1959) and swine (Fuller et al., 1979; Thomas and Kornegay, 1972). However, there are also reports where lysine supplementation did not improve upon growth or nitrogen balance in fish (Saavedra et al., 2009), rats (Mekhael et al., 1989), and humans (Hegsted et al., 1955; King et al., 1963). Also lysine supplementation did not attenuate race-associated weight loss in pigeons (Hullár et al., 2008). In horses specifically, supplemental lysine has been shown to increase weight gain in yearlings (Graham et al., 1994; Ott et al., 1981; Potter and Huchton, 1975) and weanlings (Breuer and Golden, 1971). Presumably, lysine was limiting in the diets for the species in that particular physiological state where supplementation resulted in increased growth, but not when supplementation had no effect on growth.

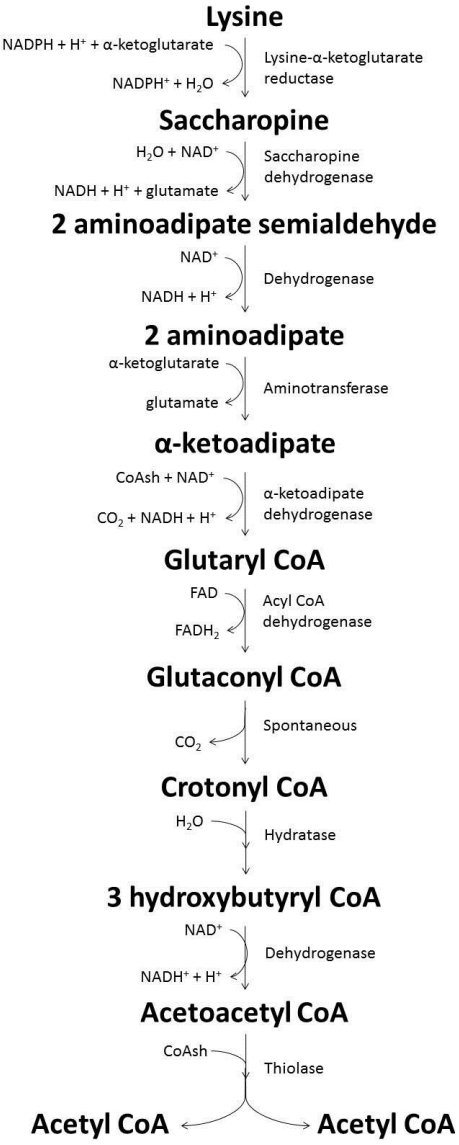
*Overview of lysine pathways in mammals*

Lysine is not made in mammalian tissues as it is an indispensable amino acid. Therefore, lysine only enters metabolism through the diet. If not used for protein synthesis, lysine is either oxidized or converted to other metabolites.

*Lysine oxidation*

Lysine oxidation takes place mainly in the liver, but does occur in other tissues (Manangi et al., 2005). The primary pathway through which lysine is catabolized is via lysine alpha-ketoglutarate reductase to saccharopine and then through a series of intermediates to acetyl-CoA as reviewed by Salway (2004) (Figure 2.1).

**Figure 2.1: Lysine oxidation to acetyl CoA pathway as adapted from Salway (2004)**




*Lysine conversion to other metabolites*

Lysine is a precursor to carnitine (Vaz and Wanders, 2002) and has been supplemented in order to produce additional carnitine in the body. This would be desirable for an athletic species as carnitine aids in moving fatty acids into mitochondria for energy production (Vaz and Wanders, 2002). Carnitine levels have been correlated with lysine in the diets of humans (Krajcovicova-Kudlackova et al., 2000). Carnitine

supplementation of greater than 10 g over 56 days increased plasma concentrations of carnitine, but not muscle concentrations in horses (Foster et al., 1988). However, when supplementation of 10 g carnitine per day was combined with training, carnitine supplemented horses had greater increases in type IIA muscle fibers and capillary to muscle fiber ratio compared to control horses in training (Rivero et al., 2002). Humans also take lysine supplements to aid with athletic performance, but the action is thought to be through the stimulation of growth hormone instead of through the formation of carnitine (Kreider et al., 1993; Lattavo et al., 2007).

***Threonine as a limiting amino acid***

Threonine is often second limiting in species and diets where lysine is the first limiting amino acid. Examples of this have been found in pigs fed a variety of diets (Cuaron et al., 1984; Myer et al., 1996; Soltwedel et al., 2006), rats (Aoyama et al., 1998), and humans (el Lozy et al., 1975). Threonine has also been suggested to be a limiting amino acid in human AIDS patients (Laurichesse et al., 1998). In yearling horses, threonine has been found to be second limiting in one study, based on girth gain that was greater in horses that were supplemented with both lysine and threonine compared to those just supplemented with lysine (Graham et al., 1994). Weight gain was not statistically different between the yearlings that were supplemented with just lysine and those supplemented with both lysine and threonine (Graham et al., 1994). In another study supplementation of 15 g/d threonine with 20 g/d lysine increased subjective muscle scores and decreased plasma urea nitrogen in adult horses (Graham-Thiers and Kronfeld, 2005a). Threonine is considered to be the least toxic amino acid if fed in excess, causing pigs to prefer diets containing excess threonine (4%) over diets containing excess

methionine or tryptophan (Edmonds et al., 1987). Therefore threonine supplementation in commercial diets is likely safe.

*Threonine incorporation into mucins*

Use of threonine by the gut in other species is considerable. Relatively high rates of threonine oxidation in the splanchnic tissues have been demonstrated in piglets (Schaart et al., 2005) and human infants (~70%) (van der Schoor et al., 2007), although this value is less for human adults (~18%) (Chapman et al., 2013) and adult mini-pigs (~37%) (Remond et al., 2009). Ileal losses of threonine also contribute to threonine usage and have been measured at 9-12 mg/kg/d for adult humans (Gaudichon et al., 2002). Greater rates of threonine usage have been reported with enteral feeding versus parenteral feeding resulting in lower threonine requirements for parenterally fed neonatal piglets (Bertolo et al., 1998) and neonatal infants (Chapman et al., 2009) when compared to their enterally fed counterparts. Mucin production is one reason for the use of threonine by the splanchnic tissues and the endogenous loss of threonine in the gut.

Intestinal mucin production is considered a major metabolic fate for threonine (Wu, 2009). For rats (Fahim et al., 1987), swine (Fogg et al., 1996), and humans (Roberton et al., 1996), threonine makes up a considerable portion of the mucin proteins, 18, 15, and 19% respectively. When threonine in the diet is restricted, mucin synthesis is reduced in rats (Faure et al., 2005) and swine (Law et al., 2007). Threonine imbalance has also been associated with decreased protein synthesis in the jejunal mucosa of swine (Wang et al., 2007) and decreased small intestine barrier function (Wang et al., 2010).

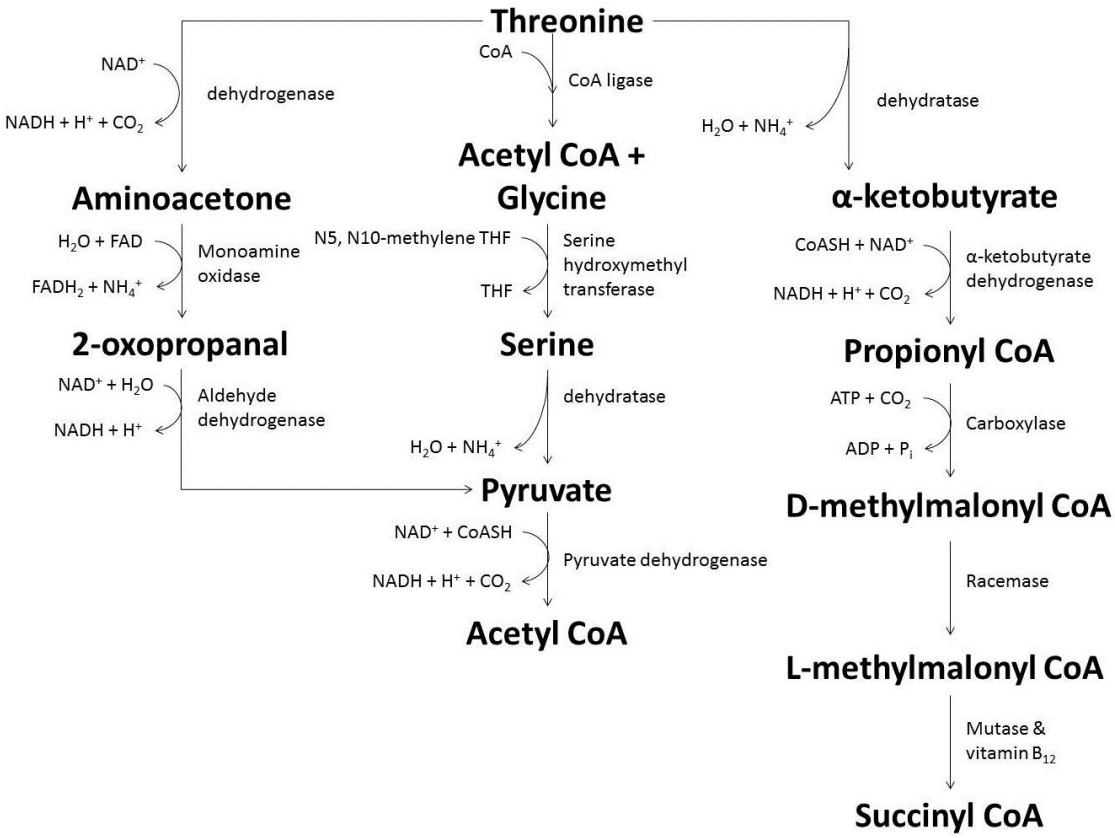
Intestinal mucin synthesis is also influenced by other diet constituents. In particular, increased fiber in the diet has been associated with increased mucin production in rats (Ito et al., 2009; Satchithanandam et al., 1990). In swine, feedstuffs containing greater amounts of hemicellulose are associated with greater endogenous threonine losses (Myrie et al., 2008).

Therefore, increased dietary fiber would be expected to increase threonine requirements in animals; however, this was not the case when fiber addition to chick diets actually reduced threonine requirements compared to controls (Wils-Plotz and Dilger, 2013). Horses can be fed diets with forages that have a range of fiber content (Pagan, 1997), but the effect of fiber inclusion in equine diets on threonine use or requirement has not been investigated.

*Overview of threonine metabolic pathways in mammals*

Threonine catabolism is known to take place through several pathways in mammals (Bird and Nunn, 1983; Dale, 1978; Salway, 2004) (Figure 2.2). Two pathways create acetyl-CoA. One pathway for threonine degradation is through glycine and serine, while the other has α-ketobutyrate as an intermediate (Darling et al., 1999), which is also part of the pathway for the catabolism of methionine (Finkelstein, 1990; Salway, 2004).

**Figure 2.2: Threonine catabolism pathways adapted from Salway (2004) and Darling et al. (1999)**




***Methionine as a limiting amino acid***

Methionine has been identified as a first limiting amino acid in poultry diets (Bornstein and Lipstein, 1975; Davidson and Boyne, 1962), and ruminant diets (Kanjanapruthipong, 1998; Kudrna et al., 2009). It has also been identified as limiting in rat diets (Tujioka et al., 2005), human vegetarian diets (Hegsted et al., 1955) and human AIDS patients (Laurichesse et al., 1998). Methionine is considered to be one of the most toxic amino acids when excess is supplemented (Edmonds and Baker, 1987a; Fau et al., 1987; Harper et al., 1970; Katz and Baker, 1975). In horses, supplementation of methionine (total methionine intake of 54 mg/kg BW/d) to a basal diet providing

methionine at 42 mg/kg BW/d had no effect on nitrogen balance or growth of weanlings (Winsco et al., 2011).

**Common protein sources for equine feeds**

Protein in equine diets comes from forages, concentrates and, in the case of foals, milk. Milk is regarded as a high quality protein due to its high degree of digestibility (Hintz et al., 1971). On a fluid basis, equine milk ranges from 1.8-3.1% protein with protein content decreasing as length of lactation progresses (NRC, 2007). Amino acid profile of milk is fairly similar to that of other species (Davis et al., 1994a).

Legume forages (~18% CP) generally have higher crude protein than grasses and cool season grasses (~13% CP) generally have higher crude protein content than warm season grasses (~8% CP) (Ball et al., 2007a). Stage of maturity also plays a role in the nutrient and therefore protein content of the forage with immature plants having greater protein content than more mature plants (Pagan, 1997). For example, crude protein content of alfalfa decreased from 15% to 9% after 20 additional days of growth from June 3$^{rd}$ to 23$^{rd}$ of 1966 (Darlington and Hershberger, 1968).

Digestibility of CP in forages is also dependent on type of forage and maturity. Early harvest alfalfa has an apparent CP digestibility of 74% compared to 65% for early harvest timothy in horses (Darlington and Hershberger, 1968). Legumes were found to have higher CP digestibilities than grasses, when comparing apparent digestibility of alfalfa (73%) and tall fescue (67%) (Crozier et al., 1997) and when comparing alfalfa (83%) and coastal bermudagrass (64%) (LaCasha et al., 1999). Late harvest apparent digestibilities of alfalfa and timothy were found to be ~55% (Darlington and

Hershberger, 1968). Apparent protein digestibility is known to increase linearly with crude protein content of the diet (Slade and Robinson, 1970) making it difficult to compare digestibilities of different proteins in feedstuffs with different crude protein contents.

Concentrates commonly fed to horses generally have greater crude protein contents than forages. Concentrates commonly fed to horses include cereal grains, grain by-products, and seed meals. Crude protein content of concentrates within type of grain does not vary nearly as much as with forages. The crude protein content of common cereal grains ranges from 9% on a DM basis for corn to 14% for wheat (NRC, 2007). However, crude protein contents can differ between specific cultivars. Wheat cultivars have ranged from 13-15% protein (Kolev et al., 2011). Oilseed meals typically have greater crude protein content. For example, cottonseed meal is 45% CP on a DM basis and linseed meal is 33% CP on a DM basis (NRC, 2007). Some by-products, such as soybean meal, are purposefully created with a specific level of crude protein (USDA, 1944), usually 44% or 48% (NRC, 2007).

Few studies measure digestibility of concentrates independently of forage, as forage generally makes up the basis of equine diets. In one study using a mobile bag technique, horses were intubated with bags containing cereal grains which were then collected for analysis at a cecal cannula (Rosenfeld and Austbø, 2009a). Prececal protein digestibilities were found to be 68%, 59%, and 62% for oats, barley, and corn, respectively (Rosenfeld and Austbø, 2009a). Studies have subtracted the previously determined digestibility of the forage and found relatively high digestibilities for grains. Apparent total tract digestibility of protein for corn, oats, and sorghum was found to be

98, 89, and 93% respectively (Gibbs et al., 1996). Digestibility can also be affected by processing. Heat damaged soybean meal has been shown to have decreased digestibility of lysine in chicks (Fernandez and Parsons, 1996).

### *Amino acid content of equine feedstuffs*

Whether or not a diet containing a certain feedstuff is limiting in an amino acid is dependent on several factors, including the amount of protein in the feedstuff, the amount of the feedstuff fed, other feedstuffs in the diet, and the amino acid content of the feedstuff. The amino acid profile of a feed is generally different than the profile of amino acids required by the animal. Amino acid profiles were not included in the nutrient composition tables of the most recent equine NRC (2007). However, amino acid compositions of feeds commonly fed to horses can be found in databases for feed composition (NRC, 1982), particularly dairy feeds (NRC, 2001).

Equine milk has an amino acid composition similar to that of other mammalian milk (Davis et al., 1994a) and has been quantified by a number of researchers (Bryden, 1991; Csapo-Kiss et al., 1995; Wickens et al., 2002). Protein content on a fluid basis decreases over time and amino acid content changes in milk in several species (Davis et al., 1994b), including horses (Tanner et al., 2011). Of particular interest is the fact that threonine content decreases as lactation length increases (Tanner et al., 2011), as threonine has been proposed to be limiting in a young horse diet (Graham et al., 1994).

The amino acid profiles of grasses are similar, even when comparing C3 and C4 grasses (NRC, 2001). Legume forages tend to have a higher proportion of lysine compared to grass forages (NRC, 2001). Amino acid profile of alfalfa may change with

stage of maturity (Garcia et al., 1995), but red clover and grass amino acid profiles may not (Vanhatalo et al., 2009). Table 2.1 details the amino acid profile of selected forages (C4 grass, C3 grass, and a legume).

**Table 2.1: Amino acid profiles of selected C-3, C-4, and legume forages, adapted from the dairy NRC (2001)**

| Feed Name | Bermudagrass hay, coastal (C-4) | Grass hay, mid-mat. (C-3) | Legume forage hay, mid-mat. |
|---|---|---|---|
| DM (%AF) | 87.1 | 83.8 | 83.9 |
| CP (%DM) | 10.4 | 13.3 | 20.8 |
| Arg (%CP) | 3.88 | 3.88 | 5.14 |
| His (%CP) | 1.63 | 1.63 | 1.95 |
| Ile (%CP) | 3.32 | 3.32 | 4.23 |
| Leu (%CP) | 6.22 | 6.22 | 7.36 |
| Lys (%CP) | 3.49 | 3.49 | 5.09 |
| Met (%CP) | 1.3 | 1.3 | 1.56 |
| Cys (%CP) | 1.16 | 1.16 | 1.42 |
| Phe (%CP) | 3.92 | 3.92 | 4.76 |
| Thr (%CP) | 3.6 | 3.6 | 4.38 |
| Trp (%CP) | 1.24 | 1.24 | 1.5 |
| Val (%CP) | 4.51 | 4.51 | 5.24 |

Particular grain cultivars may have altered amino acid characteristics. As plant breeders breed for specific characteristics the relative proportions of proteins present in the plant can shift and cause the amino acid profile to change. This is the case with a high fat oat cultivar (Yu et al., 2008). High lysine corn cultivars have been developed specifically to improve the feed value of corn (Hournard et al., 2007; Huang et al., 2005). Ultimately, the amino acid bioavailability of the diet as a whole is more important than the amino acid profile of a single feedstuff. Table 2.2 gives the amino acid profiles of selected concentrates.

**Table 2.2: Amino acid profiles of selected concentrates, adapted from the dairy NRC (2001)**

| Feed Name | Wheat Middlings | Barley Grain, rolled | Corn Grain, cracked, dry | Oats, Grain, rolled | Soybean, Meal, solv. 44% CP |
|---|---|---|---|---|---|
| DM (%AF) | 89.5 | 91 | 88.1 | 90 | 89.1 |
| CP (%DM) | 18.5 | 12.4 | 9.4 | 13.2 | 49.9 |
| Arg (%CP) | 5.86 | 5.07 | 4.61 | 6.82 | 7.38 |
| His (%CP) | 2.75 | 2.3 | 3.13 | 2.44 | 2.77 |
| Ile (%CP) | 3.44 | 3.47 | 3.31 | 3.75 | 4.56 |
| Leu (%CP) | 6.65 | 6.97 | 11.2 | 7.3 | 7.81 |
| Lys (%CP) | 3.63 | 3.63 | 2.84 | 4.18 | 6.28 |
| Met (%CP) | 1.6 | 1.7 | 2.13 | 1.71 | 1.45 |
| Cys (%CP) | 2.04 | 2.28 | 2.13 | 2.85 | 1.52 |
| Phe (%CP) | 4.43 | 5.11 | 4.62 | 5.16 | 5.26 |
| Thr (%CP) | 3.11 | 3.42 | 3.55 | 3.46 | 3.98 |
| Trp (%CP) | 1.28 | 1.17 | 0.72 | 1.19 | 1.27 |
| Val (%CP) | 4.63 | 4.9 | 4.02 | 5.19 | 4.69 |

***Availability of crystalline amino acids***

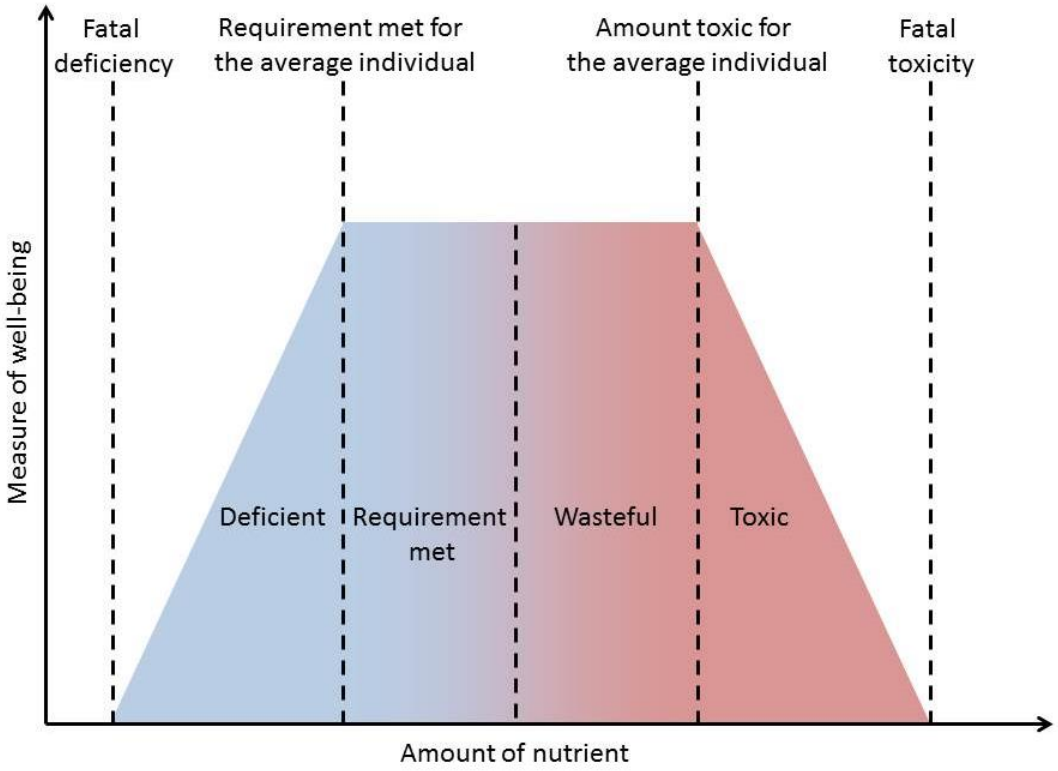
Crystalline amino acids are considered very bioavailable due to the fact that absorption from the intestinal lumen occurs rapidly (Fox et al., 2009), also they generally have higher true digestibilities than casein in swine and cockerels (Chung and Baker, 1992). They have been used in the diets of other agricultural species, such as swine (Colina et al., 2003; Myer and Gorbet, 2004; Sawadogo et al., 1997), fish (Murai et al., 1981; Segovia-Quintero and Reigh, 2004; Whiteman and Gatlin, 2005), and poultry (Applegate et al., 2008; Bornstein and Lipstein, 1975). Products, such as bread (Ericson, 1960), and vegetarian diets (Hegsted et al., 1955) for human consumption have also been supplemented with crystalline amino acids. With demand for crystalline amino acids increasing, processing methods have been improved driving cost down. For example, free lysine has become more available recently with the use of microbes to produce it cheaply

as reviewed by Wittmann and J.Becker (2007). Free amino acids could be a useful tool in formulating equine diets if the knowledge of how to apply them effectively existed.

**Methods of determining amino acid requirements**

As defined by the World Health Organization (WHO), "dietary requirement is the amount of protein or its constituent amino acids, or both, that must be supplied in the diet in order to satisfy the metabolic demand and achieve nitrogen equilibrium" (WHO, 2007). Generally, required levels of nutrients have been determined by feeding graded levels of that nutrient, both below and above the expected requirement, and then measuring some indicator of well-being, such as growth rate, protein synthesis, or nitrogen retention. In most cases, the indicator reaches a maximal value and if the study has a high enough amount of the nutrient there may be a point where toxicity begins and the indicator begins to decrease in value (Figure 2.3).

**Figure 2.3: Generalized nutrient requirement curve relative to increasing amounts of nutrient intake**




Amino acid requirements can be considered in an additive fashion. Some basal amount is needed for maintenance activities and then additional amounts are required for growth, pregnancy, lactation, and exercise. This is known as the factorial approach. Arguably, the maintenance portion of the requirement is the most important as this makes up a large portion of the requirement, especially in a relatively slow growing animal, such as the horse. However, it is easier to make a deficient diet for an animal that would be expected to have greater requirements, such as one that is growing.

Methods used in determining amino acid requirements include nitrogen balance, obligatory nitrogen loss, growth assays, plasma metabolite concentrations, and methods using isotope tracers. In 1971, the World Health Organization's protein and amino acid

requirements for humans were based on nitrogen balance studies, but due to concerns about nitrogen balance studies discussed in the "Nitrogen retention" section, the requirements were increased in 2002 (WHO, 2007). However, in 2006, the human Dietary Reference Intakes for protein were still based on nitrogen balance studies, but isotopic tracer methods were utilized in setting individual amino acid requirements (Food and Nutrition Board, 2006). Protein and lysine requirements in horses are based on nitrogen balance and growth studies, respectively (NRC, 2007).

### *Factors to consider when designing experiments to determine amino acid requirements*

Several factors must be considered in designing experiments to determine amino acid requirements. First, the number and range of intakes must be great enough to accurately detect a requirement and encompass the expected requirement; at least six levels are recommended as reviewed by Baker (1986). The test diets should be both isoenergetic and isonitrogenous, as the measure of well-being could be affected by both the energy content and/or the total nitrogen provided in the diet. For example, horses fed diets limiting in energy lost weight despite protein supplementation (Sticker et al., 1995). In order to make the test diets isonitrogenous, a source of nitrogen must be added to the diets to offset the lower amounts of the test amino acid in all but the diet with the highest concentration of the test amino acid. This source of nitrogen should be as metabolically unrelated as possible. For example, glycine has been used to make treatments isonitrogenous when lysine is being studied in humans (Brewer et al., 1978; Rice et al., 1970), as these amino acids do not share many metabolic pathways. An example of a poor choice of a nitrogen source to offset the test amino acid would be tyrosine when studying phenylalanine as the first step in phenylalanine oxidation is conversion to

tyrosine. Additional considerations for nutrient requirement studies are reviewed by Baker (1986).

***Plasma amino acid concentrations***

The theory behind using plasma amino acid concentrations to determine requirement is that amino acids are removed from the blood for the purpose of building protein in the tissues. A limiting amino acid will therefore have a relatively low concentration within the plasma as much of it will have been removed for protein synthesis. At a certain dietary level of this limiting amino acid there begins to be a greater rate of increase in the plasma amino acid concentrations creating a broken line, this is assumed to be the intake of the limiting amino acid where the requirements have been met (Figure 2.5). This method has been used to determine a lysine requirement in mature Thoroughbred horses (Ohta et al., 2007).

As more of the limiting amino acid is added to the diet, other indispensable amino acid plasma concentrations begin to decrease as additional amounts are used for protein synthesis. At the point where the amino acid requirement of the previously limiting amino acid is met the plasma concentrations of the other indispensable amino acids will begin to plateau (Figure 2.5). This method has been used in horses to determine lysine requirements from threonine plasma concentrations (Fisher et al., 1989).

This method is relatively noninvasive and inexpensive. However, there are some difficulties with this method. First plasma amino acid concentrations are affected by numerous variables. In addition to the amino acid composition of the diet (Graham-Thiers and Bowen, 2009; Longenecker and Hause, 1959), plasma amino acid

concentrations are affected by postprandial state, some concentrations increasing more than 200% of their fasting values in yearling horses fed a concentrate meal (Hackl et al., 2006). Serum amino acid concentrations also increase post-suckle in foals (O'Connor-Robison et al., 2009). Plasma amino acid concentrations in horses do not fall back to baseline at even six hours post-feeding (Hackl et al., 2006; Johnson and Hart, 1974). Circadian rhythms have also been suggested to affect plasma amino acid concentrations in humans (Fernstrom et al., 1979; Tsai et al., 2000; Wurtman et al., 1968) and this phenomenon has been reviewed elsewhere (Feigin et al., 1971). Gender (Milsom et al., 1979) and disease state (McGorum and Kirk, 2001) may also play a role. Given the number of variables that affect plasma amino acid concentrations, the range of amino acid concentrations in equine plasma can be large. Plasma threonine concentrations are given in Table 2.3 to demonstrate the variability.

**Table 2.3: Equine plasma threonine concentrations vary with age, diet, and sampling time relative to feeding.**

| Feeding state | Age | Diet | Plasma Thr (µmol/L) | Source |
|---|---|---|---|---|
| Steady state | Weanling | Variety of lysine levels in the diets | 160-215 | (Fisher et al., 1989) |
| Pre-feeding | Yearling | Amino acid content of diet provided | 40.1 | (Hackl et al., 2006) |
| Post-feeding (2h) | Yearling | Amino acid content of diet provided | 101.1 | (Hackl et al., 2006) |
| Pre-feeding | Adult | Amino acid content of diet provided | 74.0-86.3 | (Graham-Thiers and Bowen, 2011) |
| Post-feeding (1h) | Adult | Amino acid content of diet provided | 128.8-137.0 | (Graham-Thiers and Bowen, 2011) |
| Post-feeding (2h) | Adult | Amino acid content of diet provided | 129.4-147.1 | (Graham-Thiers and Bowen, 2011) |
| Pre-feeding | Adult | Amino acid content of diet not provided | 100 | (Johnson and Hart, 1974) |
| Post-feeding (2h) | Adult | Amino acid content of diet not provided | 141 | (Johnson and Hart, 1974) |

Second, plasma amino acid concentrations may not be indicative of what is occurring in other tissues. Not all tissues metabolize amino acids in the same manner and the blood is used to "ship" amino acids from one organ to another. As a result the plasma amino acid concentrations only provide a snapshot of a dynamic system. They also do not provide information on the source of the amino acids that are entering the plasma pool (diet or tissue protein degradation). Changes in serum (Bergero et al., 2005; Trottier et al., 2002) and plasma amino acids (Graham-Thiers and Bowen, 2011; Wimbush, 2009)

due to exercise are indicative of muscle metabolism in addition to providing information about the diet and amino acid adequacy. Using plasma amino acid concentrations is not considered a sensitive enough method by itself to determine amino acid requirements (Pencharz and Ball, 2003).

***Plasma/serum urea concentrations***

Urea is a product of amino acid catabolism. Amino acids are catabolized at some basal level, but are additionally catabolized when more amino acids are provided in the diet than can be used or if a limiting amino acid causes the other indispensable amino acids to not be able to be used. As an amino acid in the diet goes from deficient to excess the plasma urea concentrations decrease to some basal level and then begin to increase again (Figure 2.5). The requirement is reached when urea levels reach some basal level. Urea concentrations are elevated at deficient intakes of the amino acid because the other amino acids are not used for protein synthesis and are catabolized. The elevated urea concentrations at excess intakes of the amino acid being tested are from the test amino acid being catabolized.

The relationship between blood urea concentration and biological value of protein has been studied using rats and 42 different feedstuffs, finding negative correlation and an r value of 0.95 (Eggum, 1970). Adding lysine to a diet limiting in lysine or tryptophan to a diet limiting in tryptophan has resulted in decreases in plasma urea concentrations in pigs (Brown and Cline, 1974). This technique has been successfully used in pigs to determine lysine requirements (Cameron et al., 2003; Knowles et al., 1997; Sparks, 1998). Serum urea concentrations have been measured in horses as an indicator of amino acid adequacy (Graham-Thiers and Kronfeld, 2005a; Graham et al., 1994) and

concentrations have been shown to decrease with increasing lysine intake (0.37% - 0.87% of diet) (Fisher et al., 1989).

The benefits and pitfalls of using this method are similar to those of using plasma amino acids. One additional benefit of this method is that a meta-analysis found relationships between blood urea concentrations, nitrogen excretion, and dietary nitrogen intake across several species (Kohn et al., 2005). Blood urea nitrogen concentrations in horses specifically have been correlated to nitrogen intake and urinary nitrogen excretion (Fonnesbeck and Symons, 1969). These relationships make blood urea concentration directly relatable to other methods that determine protein and amino acid adequacy, including nitrogen balance and nitrogen excretion. Determining blood urea concentrations is much easier and cost effective than conducting nitrogen balance studies or determining nitrogen excretion.

Similarly to plasma amino acid concentrations, urea blood concentrations are affected by many factors, including the amount of protein in the diet and time after feeding (Eggum, 1970). Equine plasma urea nitrogen concentrations under different conditions are presented in Table 2.4. Increased protein intake was correlated with increased blood urea nitrogen (Kohn et al., 2005). Time after feeding affects the blood concentration of urea for several hours in birds (Kolmstetter and Ramsay, 2000) and swine (Eggum, 1970). Exercise also affects blood urea concentrations. In patients receiving dialysis, exercise decreased blood urea concentrations (Kong et al., 1999). This could be because the rate of urea nitrogen being reincorporated into protein during exercise and recovery is increased in humans exercised on a treadmill at 40 and 70% of $VO_{2max}$ for 180 minutes (Carraro et al., 1993). However, urea production is increased in

prolonged exercise causing elevated serum urea levels (Haralambie and Berg, 1976) to approximately twice that of pre-exercise levels in swimmers (Lemon et al., 1989). Blood urea concentrations have also been used to quantify acute muscle wasting (Thomas and Bishop, 2007). Finally, circadian rhythms or diurnal variation also have been implicated in affecting blood urea concentrations (Castroviejo et al., 1987; Piccione et al., 2006; Piccione et al., 2007), which could be due to circadian rhythmicity of liver activity and/or feeding schedule, which is reviewed by Davidson et al. (2004). Using blood urea nitrogen concentrations is not considered a sensitive enough method by itself to determine amino acid requirements (Pencharz and Ball, 2003).

**Table 2.4: Reported equine blood urea concentration ranges.**

| Feeding or exercise state | Horses | Diet | Plasma or serum | Concentration (mmol/L) range | Source |
|---|---|---|---|---|---|
| Pre-feeding | Lactating French drafts | Forage: concentrate ratios of 95:5 or 50:50 | Plasma | 5.20-6.37 | (Doreau et al., 1992) |
| 1 hr post feeding | Quarter Horse weanlings | Supplemented with graded levels of crystalline lysine | Plasma | 5.5-4.75 | (Fisher et al., 1989) |
| Pre-feeding | Light horse mares | Energy and/or protein restricted diets | Plasma | 3.5-6.5 | (Sticker et al., 1995) |
| Pre-exercise & recovery | Standardbreds | Control vs BCAA supplemented | Plasma | 4.0-6.5 | (Casini et al., 2000) |
| 6 hrs post-feeding | Adult ponies | Two levels of digestible CP | Plasma | 1.15-3.32 | (Olsman et al., 2003) |
| ~3.5 hrs post-feeding | ≤ 10 yrs & ≥20 yrs | Unsupplemented compared to lysine & threonine supplemented | Plasma | 5.6-7.4 | (Graham-Thiers and Kronfeld, 2005a) |
| 4 hrs post-feeding | Thoroughbreds | 7.5% or 12% CP | Plasma | 16.3-22.8 | (Graham-Thiers and Kronfeld, 2005b) |
| Pre-feeding | Welsh ponies | Ad libitum compared to diet restricted to 1% of BW | Plasma | 3.3-4.7 | (Dugdale et al., 2010) |
| ~3.5 hrs post-feeding | Maintenance, pregnant, or lactating | Dependent on physiological state | Plasma | 3.8-7.3 | (Graham-Thiers et al., 2010) |
| Pre-feeding & 1-6 hrs post-feeding | Light horses | Hay diet compared to a hay & grain diet | Plasma | 5.7-7.3 | (Graham-Thiers and Bowen, 2011) |
| Not stated | Brazilian warmbloods 18 - 20 mos | Increasing levels of yeast | Serum | 19.8-22.8 | (Winkler et al., 2011) |
| Pre-feeding | Weanling Quarter Horses | Methionine supplemented | Plasma | 3.2-3.8 | (Winsco et al., 2011) |

***Growth***

The theory with the growth method to study amino acid requirements is that when an animal is deficient in an amino acid, growth is slowed as protein formation is necessary for the tissue accretion that occurs during growth. Response variables used to measure growth include body weight, height, circumference, lean gain, and average daily gain. The response variable must be indicative of success in a given system. Two measures of growth may result in two different requirements. For example, using muscle yield found a lower lysine requirement than overall growth for broilers (Ng'ambi et al., 2009). The requirement is determined at the minimum amount of the test amino acid that supports maximal growth response in the chosen response variable (Figure 2.5).

Equine growth rates have been characterized (Hintz et al., 1979; Staniar et al., 2004b) and reviewed (Green, 1963), establishing norms within breed. Growth rate in horses has been influenced by amount of protein in the diet (Jordan and Myers, 1972; Ott and Kivipelto, 2002), protein type (Hintz et al., 1971; Potter and Huchton, 1975), and amino acid content of the diet (Breuer and Golden, 1971; Graham et al., 1994; Potter and Huchton, 1975).

This method directly ties amino acid adequacy to a tangible measure of production. As production in many cases is the end goal, growth is a very useful measure that can be used to translate whether or not an amino acid requirement is met to an economic consequence. Unfortunately, this method is both time consuming and expensive. These detractors are magnified with horses as they are large and relatively slow growing animals. Study periods must be long enough to accurately track growth rate. Most agricultural species have well-defined measures of productivity that directly

translate to product value or profit. As the horse is generally an athletic species that competes in a variety of sports at a variety of levels, quantifying athleticism or ability to succeed is difficult. Whether or not maximal growth for horses is optimal growth is debatable. Therefore, measures of growth are less of a direct link to profit. Another pitfall of this method is that requirements can only be determined for the growing animal. Requirements for maintenance, exercise, and immune response cannot be determined in this manner. Using growth as a method to determine amino acid requirements in horses is extremely limited.

***Nitrogen retention***

The equation for nitrogen retention is:

nitrogen retention = nitrogen intake - nitrogen excreted

Nitrogenous excretions include urine, feces, sweat, hair, nails/hooves/claws, and other secretions (Food and Nutrition Board, 2006). Nitrogen intake is used to replace nitrogen loss. When the mature animal at maintenance is deficient more nitrogen is lost than is taken in, resulting in a negative nitrogen retention. Animals in positive nitrogen retention are accreting tissue for activities, such as growth or hypertrophy. Nitrogen intake and loss are measured, but the variable that responds to the change in the test amino acid intake is the calculated value of nitrogen retention. Nitrogen retention increases as the test amino acid increases until the requirement is met, at which point nitrogen retention plateaus (Figure 2.5). Nitrogen retention studies have been used to determine both nitrogen (Tao et al., 1979) and amino acid requirements (Wang and Fuller, 1989) in other species.

Granted endogenous losses are relatively small, but not measuring these losses contributes to the measurement error of nitrogen balance studies. Measuring feed intake in animal studies either assumes the animal has eaten everything that has been put in the feed bucket minus orts. It does not capture any of the feed that has been trampled into the bedding, which may overestimate intake. Capturing absolutely all urine and feces is also difficult as some small portion may be trapped in the containers or bags used to collect the excretions. Therefore, overestimation of nitrogen intake coupled with underestimation of nitrogen excretion can lead to artificially high values for nitrogen retention. Similar errors and their subsequent overestimation of nitrogen in human studies has been reviewed by Kopple (1987).

Nitrogen retention studies are the basis for the current equine NRC (2007) protein requirements for horses. They have also been used to evaluate protein bioavailability of nitrogen sources (Reitnour, 1978) and amino acid adequacy (Antilley et al., 2007) in horses. Protein intake has been positively correlated with fecal and urinary nitrogen output in adult ponies with a correlation coefficient of 0.96 (Olsman et al., 2003). Many equine studies calculating nitrogen retention only quantify fecal and urinary nitrogen excretion (Antilley et al., 2007; Freeman et al., 1988; Hintz and Schryver, 1972; Hintz et al., 1971; Olsman et al., 2004; Reitnour and Salsbury, 1972; Slade et al., 1970; Wall et al., 1998) and do not measure other nitrogenous losses, such as shedding hair, sweat, etc. Cutaneous nitrogen losses have been estimated for horses in one paper (Meyer, 1983) and some researchers use this estimate in their calculations of nitrogen retention for horses (Olsman et al., 2003), but cutaneous nitrogen losses would be expected to change with season (Takahashi et al., 1985) and level of activity (Muramatsu, 2002), as horse sweat

(Jirka and Kotas, 1959; Smith, 1890) and hair (Samata and Matsuda, 1988) contain protein.

One benefit of this method is that it measures nitrogen excretion, which can be a measure of interest in planning nutrient management protocols. There are however, several other criticisms to arriving at a requirement utilizing nitrogen balance studies. First, many of the designs are associated with high energy intake as reviewed by Young and Marchini (1990) and energy intake affects nitrogen balance (Rao et al., 1975). To test different nitrogen or amino acid intakes, another nutrient must change conversely and changing this nutrient often affects energy intake. Additionally, since losses such as hair, sweat and other secretions are hard to quantify (Baker et al., 2009; Shirreffs and Maughan, 1998), the technique often yields a balance that is too positive and results in an underestimation of the requirement (Elango et al., 2010; Young et al., 1989). Furthermore, the efficiency of protein utilization decreases near zero balance, as reviewed by Elango et al. (2010), meaning the response curve for nitrogen is nonlinear (Rand et al., 2003). Most nitrogen balance studies are analyzed with linear regression, but two-phase linear regression may be more biologically relevant because at some point the adult will no longer accrete protein regardless of how high the dietary amount (Elango et al., 2010). Furthermore, the urea pool takes several days to stabilize after changes in diets for humans (Rand et al., 1976), which adds to the time needed for the adaptation to each treatment diet and the cost.

***Obligatory loss***

Studies utilizing obligatory nitrogen loss to determine requirements utilized diets with minimal nitrogen (Atinmo et al., 1985; Scrimshaw et al., 1972; Scrimshaw et al.,

1976; Uauy et al., 1978). Obligatory nitrogen loss is the nitrogen loss that occurs despite there being no nitrogen coming into the body (Young et al., 1989). The oxidation of tracer amino acids have also been used to determine amino acid oxidation levels on protein-free diets (Raguso et al., 1999). The response variables measured are nitrogen losses or amino acid oxidation on protein-free diets. The idea is that eliminating nitrogen intake reveals basal metabolism of protein and amino acids. The losses are taken to be the minimum the body requires.

The benefit of this method is that it determines requirements independently of protein digestibility. However, when obligatory loss is determined by nitrogenous excretions, the same problems with nitrogen collections are also present in obligatory nitrogen loss studies. In some cases, no attempts are made to quantify nail or hair loss (Atinmo et al., 1985) or sweat (Scrimshaw et al., 1972; Scrimshaw et al., 1976; Uauy et al., 1978). When amino acid tracers are used, their introduction adds exogenous nitrogen into the system, albeit not through dietary intake. Despite adding nitrogen to the system estimations of obligatory oxidative amino acid loss are similar using the tracer method or the estimations from nitrogen loss (Raguso et al., 1999).

Further pitfalls of this type of study relate to practicality the artificial physiological conditions produced by this approach. Protein-free diets may not be palatable, weight loss and other symptoms of protein deficiency may occur, and the study conditions do not mirror a natural diet limiting the applicability of the findings. No studies of obligatory loss in horses have been conducted to date, likely due to issues of palatability.

***Isotope methodologies***

Finally, isotopic tracers can be used in several ways to determine requirements. They can be used to measure direct amino acid oxidation, indicator amino acid oxidation, or direct amino acid balance. In these studies, the tracers are now generally labeled with a stable isotope, although radioactive tracers have been used, as reviewed by Stellaard and Elzinga (2005) the methods are based on the principle that amino acids that are not used in protein synthesis or to synthesize other metabolites are oxidized as reviewed by Allison (1955) and the tracers are labeled with isotopic carbon such that the labeled carbon is exhaled as carbon dioxide (Spahr et al., 2003), $^{14}CO_2$ or $^{13}CO_2$, for radioactive and stable labeled isotope tracers, respectively. When an amino acid is fed under the requirement only a basal amount is oxidized, but when it is fed in excess of what is used for protein synthesis, the excess is oxidized. Both indicator amino acid oxidation and direct amino acid oxidation utilize this concept, and each of these methods will be described below in greater detail.

Isotopic methods were first employed to determine requirements of growing pigs (Kim et al., 1983) and now are used extensively in the determination of human amino acid requirements (WHO, 2007). They have also been used to determine total protein requirements (Elango et al., 2011; Humayun et al., 2007a), metabolic availability of amino acids (Humayun et al., 2007b; Moehn et al., 2005), and determining limiting amino acids (Brunton et al., 2007), in addition to determining individual amino acid requirements (Elango et al., 2007; Kim et al., 1983; Kurpad et al., 2003; Lazaris-Brunner et al., 1998; Moehn et al., 2008; Wilson et al., 2000; Zello et al., 1993).

There are several positives common to all three of these isotope methods. Adaptation time to a particular diet can be relatively short due to the quick response of amino acid pools to dietary changes (Watanabe et al., 1998), decreasing the length of time needed for a study. Plasma amino acid concentrations respond to dietary changes within 2 days in rats (Watanabe et al., 1998) and 1 day in pigs (Moehn et al., 2004b) and horses (Ohta et al., 2007). Isotope methodologies can detect relatively small differences in amino acid requirements, they have even been able to detect an increase in the lysine requirement of 2.7 mg/kg/d (8%) during the luteal phase of menstruation from the follicular phase in women (Kriengsinyos et al., 2004).

There are also several considerations to take into account for isotope studies. With the exception of 24 hr tracer studies, the isotope tracer studies may be influenced by circadian rhythms or other transient factors. However, when subjects are detained for 24 hr infusions their basal level of activity would be expected to be less than an average individual in the population at large for whom the study is attempting to predict a requirement. This could be an important factor as it is known that bed rest can have a negative impact on both muscle and whole body protein synthesis (Ferrando et al., 1996). Originally, these studies were carried out with radioactive isotopes (Ball and Bayley, 1985), allowing for very small amounts of the tracers to be used. However, larger amounts must be used for the safer stable-labeled isotopes due to the natural occurrence of these isotopes as reviewed by D'Mello (2012). These larger amounts may be relevant in calculating the amount of an amino acid entering the body pools and should be taken into account within the design of the study and calculations.

*Direct amino acid oxidation*

In direct amino acid oxidation, the amino acid for which the requirement is being tested is the isotope. The oxidation of the isotopic tracer is measured. As the test amino acid increases in the diet the oxidation of the test amino acid remains at a basal level until the requirement is reached (Figure 2.5). After the requirement is reached oxidation of the test amino acid increases as more is available than can be used for protein synthesis.

The benefit of this method is that it measures the amino acid of interest directly. This is also a downside as introducing the tracer increases the amount of the test amino acid entering the system, which then has to be taken into account as reviewed by Kurpad and Thomas (2011). Also, the isotopic tracer changes according to which amino acid is being studied. Some amino acids lend themselves to being better tracers than others due to their basal oxidation levels and catabolism pathways. Criteria for amino acid tracers used to determine amino acid requirements are enumerated by Hsu et al. (2006b).

*Indicator amino acid oxidation*

In indicator amino acid oxidation, an "indicator" amino acid is the isotope and the amount of test amino acid varies to obtain changes in oxidation of the indicator amino acid (Di Buono et al., 2001b; Pencharz et al., 2007; Zello et al., 1995). The indicator amino acid is an indispensable amino acid other than the test amino acid. As the test amino acid intake increases in the diet, from a deficient to adequate amount and beyond, more of the indicator amino acid can be used for protein synthesis (as the test amino acid is less limiting) and its oxidation, and subsequent production of $^{13}CO_2$, decreases until the requirement is met, at which point the oxidation plateaus (Figure 2.5). Using the indicator amino acid method allows the researcher to use the same isotopic tracer while studying

different amino acid requirements, as some amino acids work better as tracers than others (Hsu et al., 2006b).

### *Tissue composition*

The amino acid composition of tissue has been used as a proxy for determining requirements empirically when there is insufficient data from other methods. The thinking is that the amino acid composition of the tissue is representative of the amino acids needed by the organism for tissue accretion and repair. Block and Bolling (1944) were the first to suggest that feedstuffs with amino acid compositions similar to the carcass were of higher biological value. The idea was further refined with experiments with chicks, rats, and pigs (Williams et al., 1954). The correlation between fat free mass composition and amino acid requirements for the chick has been suggested to have a correlation coefficient as high as 0.99 (Fisher and Scott, 1954). However, measuring the amino acid composition of an entire horse is logistically problematic due to the animal's size.

In order to estimate amino acid requirements from tissue composition, the requirement for lysine is measured with another method and then the rest of the requirements are estimated from the ratio of those amino acids to lysine in the tissue. Due to the lack of work on individual amino acid requirements in horses, this method allows at least an estimate of all the essential amino acids in horses. Whole body tissue amino acid composition can be more easily determined for smaller species, especially those that are used for human consumption. Amino acid composition of the whole horse has not been determined, but amino acid composition of equine muscle (Badiani et al., 1997; Lorenzo and Pateiro, 2013; Wickens et al., 2002) and milk (Davis et al., 1994a; Davis et

al., 1994b; Wickens et al., 2002) has been determined. Bryden (1991) was the first to use tissue composition in horses to suggest amino acid requirement estimates. Since then the idea has been expanded to the lactating mare (Wickens et al., 2002).

Muscle is a popular proxy for whole body amino acid composition because muscle makes up 53% of live weight in Thoroughbreds and 44% of live weight in other horses (Gunn, 1987). This percentage could be even greater in halter horses, especially those with hyperkalemic period paralysis as reviewed by Gordon et al. (2005). Amino acid requirement ratios based on equine muscle tissue are given in Table 2.5. In order to use this table to estimate amino acid requirements a lysine requirement for the physiological state must be identified. Lysine requirement estimates are listed in the tables and online software provided by the NRC (2007). If the lysine requirement is 100 mg/kg BW/d, then the threonine requirement, according to the ratios set forth by Bryden (1991), is 62 mg/kg BW/d.

**Table 2.5: Amino acid requirement estimate ratios as based on the amino acid composition of equine muscle**

| | Requirement estimates based on muscle | | | |
|---|---|---|---|---|
| | Gluteal (Bryden, 1991) | Gluteus and gastrocnemius (Wickens et al., 2002) | "Thigh" (Badiani et al., 1997) | Average of various muscles (Lorenzo and Pateiro, 2013) |
| Arginine | 74 | 75 | 74 | 62 |
| Histidine | 65 | 75 | 57 | 48 |
| Isoleucine | 56 | 58 | 58 | 56 |
| Leucine | 109 | 95 | 97 | 94 |
| Lysine* | 100 | 100 | 100 | 100 |
| Methionine | 29 | 32 | 31 | 16 |
| Phenylalanine | 60 | 47 | 52 | 48 |
| Threonine | 62 | 51 | 54 | 57 |
| Valine | 63 | 59 | 61 | 57 |

* Lysine has been set to 100.

The amino acid composition of muscle has been shown to change somewhat as the horse is growing (Manso Filho et al., 2009). For example, lysine concentrations were found to be greater at birth than at 14 days of age, while threonine and methionine concentrations within the muscle were not affected by age up to 12 months (Manso Filho et al., 2009). As age may affect amino acid composition of muscle, care may need to be taken in generalizing requirements across ages.

Milk can be used in addition to body composition in the factorial method to estimate amino acid requirements for lactating mares (Wickens et al., 2002). Milk amino acid composition has also been used to estimate the amino acid requirements of growing horses as milk is the only nutrition foals receive at first. It would make sense if the mare's milk provided amino acids in the ratio of the ideal protein required by the offspring. For

older foals and those transitioning from milk to other feeds, milk may be less accurate in predicting amino acid requirements as the gut is changing to adapt to other feedstuffs and sources of protein.

**Table 2.6: Amino acid requirement estimate ratios as based on the amino acid composition of milk.**

| | Requirement estimates based on milk | | | | |
|---|---|---|---|---|---|
| | (Bryden, 1991) | (Wickens et al., 2002) | Intermediate milk (Davis et al., 1994b) | Mature milk (Davis et al., 1994a) | Late lactation (Tanner et al., 2011) |
| Arginine | 79 | 69 | 73 | 82 | 68 |
| Histidine | 36 | 33 | 29 | 29 | 30 |
| Isoleucine | 64 | 78 | 53 | 53 | 75 |
| Leucine | 129 | 147 | 121 | 127 | 133 |
| Lysine* | 100 | 100 | 100 | 100 | 100 |
| Methionine | 30 | 32 | 29 | 30 | 26 |
| Phenylalanine | 56 | 53 | 58 | 59 | 53 |
| Threonine | 53 | 72 | 46 | 53 | 45 |
| Valine | 77 | 97 | 60 | 64 | 98 |

* Lysine has been set to 100.

The benefits of this method is that it provides requirements for all indispensable amino acids and it is relatively inexpensive to measure compared to the other methods which must determine amino acid requirements separately. However, there is a lack of data on the amino acid composition of the whole body of the horse. Estimates using muscle and milk, but not other tissues do not account for the distribution of amino acids elsewhere in the body or the metabolism and use of the amino acids in other organs of the body. For example, in humans muscle only accounts for ~30% of whole body protein catabolism, while non-muscle lean tissue accounts for ~70% (Morais et al., 2000).

Another example would be that threonine usage is particularly great in the gut of other non-ruminant species, such as pigs (Law et al., 2007; van der Schoor et al., 2007), and using the ratio of lysine to threonine in the muscle tissue to estimate whole-body threonine requirements may result in an underestimated requirement.

There are also some amino acids which have inordinate concentrations within the muscle. For example, histidine content of equine muscle is particularly variable (Table 2.5). This variability is likely due to the carnosine content of the muscle. Carnosine is present in equine muscle at inordinately high levels as compared to other species (Harris et al., 2012) and the content varies with muscle fiber type (Dunnett and Harris, 1995; Sewell et al., 1992). Although this approach is currently used to suggest amino acid requirements for horses, it is important to recognize the shortcomings of this method when estimating the requirements of amino acids where muscle or milk compositions may not be reflective of whole-body protein content or whole-body use of that amino acid.

### *Concluding remarks on methods of determining amino acid requirements and implications for equine research*

Our current understanding of nitrogen metabolism in the horse is diagrammed in (Figure 2.4). Each of the methodologies discussed has pros and cons and they are related to how and which portions of the horse's nitrogen metabolism are measured. The expected response curves to an increasing amount of the test amino acid for these measures are shown in Figure 2.5. The exceptions are obligatory nitrogen loss, as this approach requires a protein-free diet, and the use of tissue composition as a proxy because it is not dependent on diet.

**Figure 2.4: Generalized schematic of equine nitrogen metabolism**

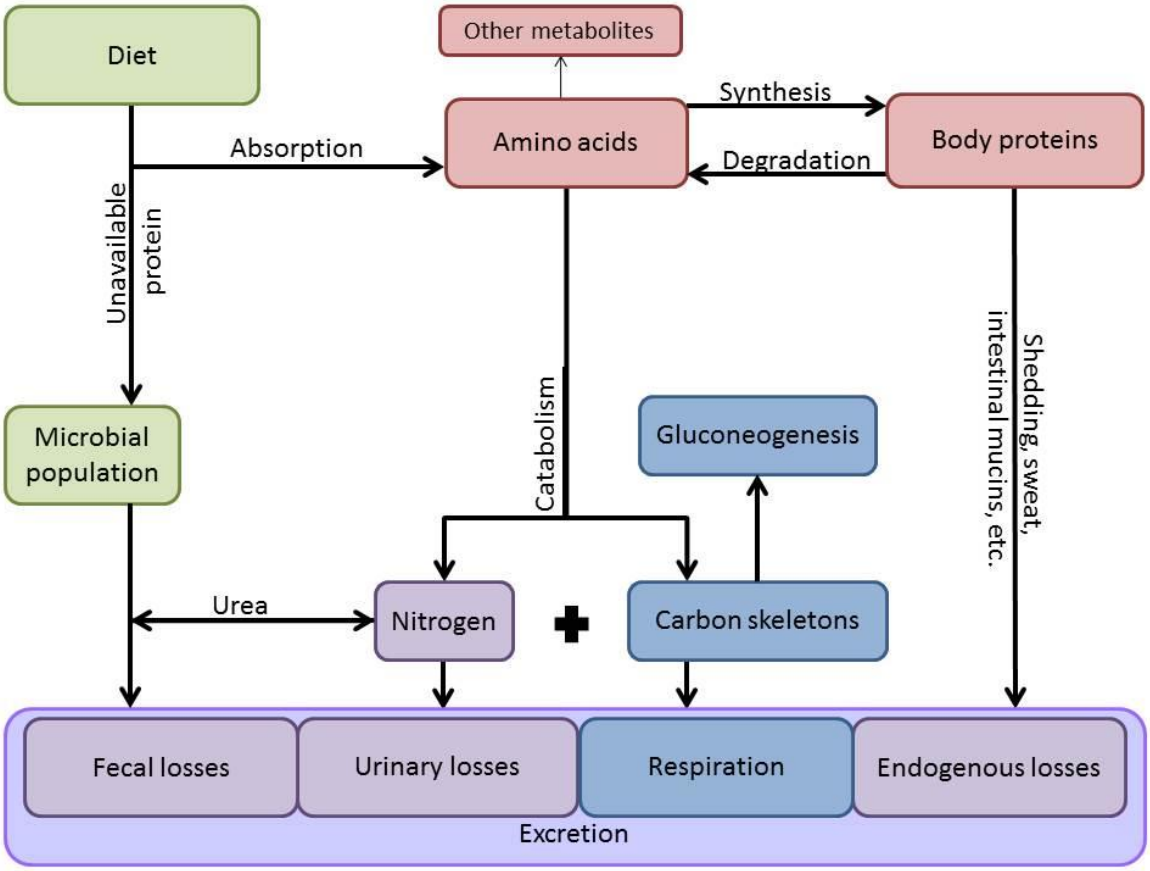
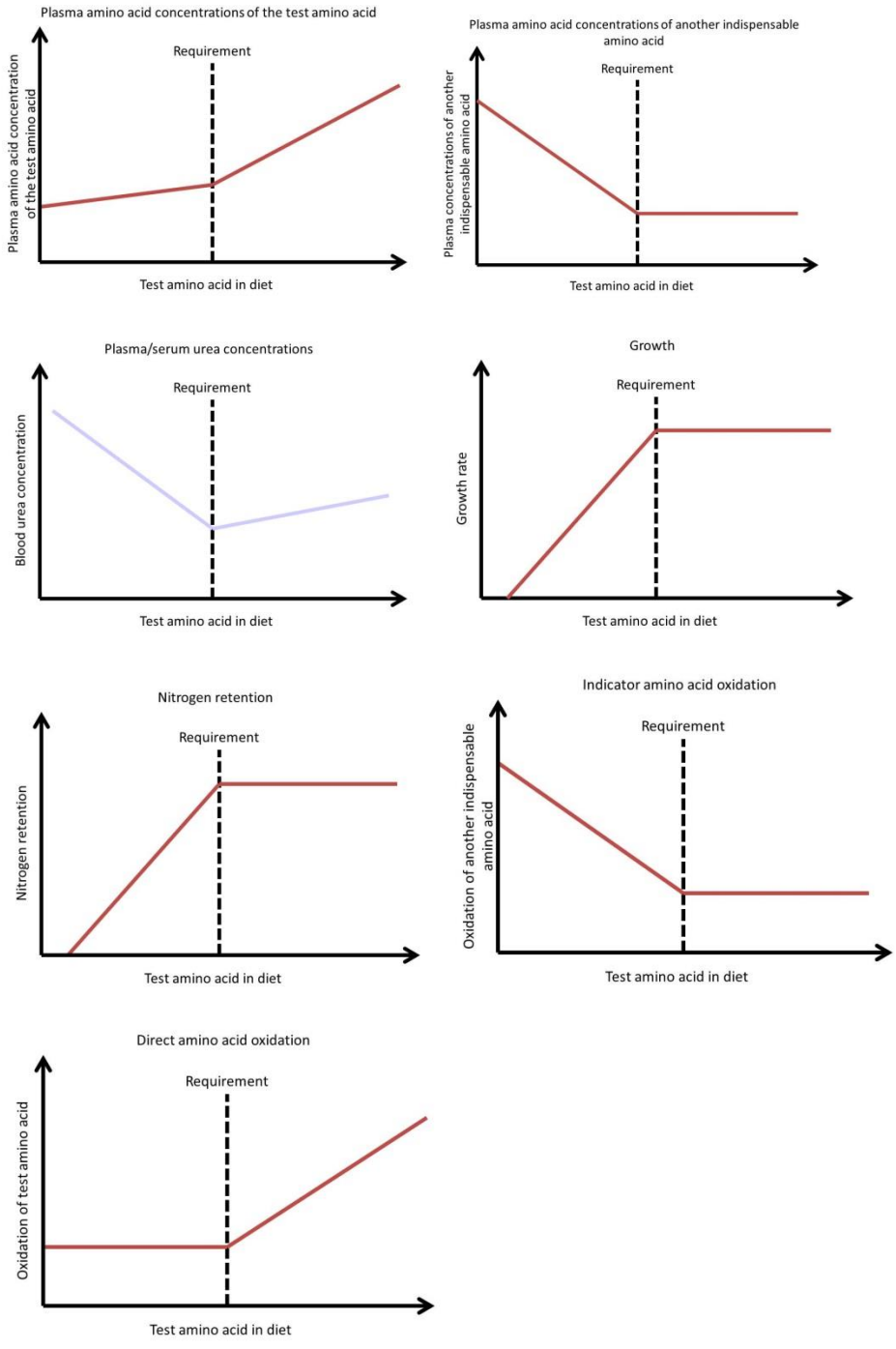


**Figure 2.5: Metabolic response curves in response to increasing amounts of the test amino acid in the diet**



As amino acid requirements have been largely uninvestigated in the horse, there is an opportunity to select an appropriate methodology with which to determine amino acid requirements. Lysine is the most studied amino acid in equine diets. Table 2.7 summarizes the results of studies that sought to determine lysine requirements for horses and whether or not they meet the criterion for amino acid requirement studies as discussed in the "Factors to consider when designing experiments to determine amino acid requirements" section. Currently, the NRC (2007) lysine requirement for horses is based on the crude protein requirement and lysine content of common horse feeds, resulting in a lysine requirement of 4.3% of the crude protein requirement.

**Table 2.7: Summary of lysine requirements for horses determined by various methods**

| Method | Age, gender and/or physiological state | Lysine requirement | Levels of intake | Isonitrogenous? | Isoenergetic? | Source |
|---|---|---|---|---|---|---|
| Plasma amino acid concentration of lysine | Mature males | 0.47% of diet | 4 | Yes | Yes | (Ohta et al., 2007) |
| Plasma amino acid concentrations threonine | Weanlings, mixed gender | 36.5 g/d | 5 | No | No | (Fisher et al., 1989) |
| Growth | Weanlings, gender not stated | 29 g/d or 0.6% of diet | 6 | No | No | (Breuer and Golden, 1971) |
| Meta-analysis | Weanlings (6 mo) | 29.1 g/d* | N/A | N/A | N/A | (NRC, 2007) |
| Meta-analysis | Yearlings | 36.4 g/d* | N/A | N/A | N/A | (NRC, 2007) |
| Meta-analysis | Mature | 27.1 g/d* | N/A | N/A | N/A | (NRC, 2007) |

* NRC lysine requirements are based on a mature weight of 500 kg. The meta-analysis included 7 studies that reported diet composition, intake, and retention of nitrogen. These studies were fit to a broken-line analysis to determine a lysine requirement, which is 4.3% of the crude protein requirement.

**Conclusions drawn from the literature**

The equine industry could benefit from additional knowledge of amino acid requirements to decrease waste, improve equine health and athletic performance, and optimize growth. Unfortunately, the research regarding equine amino acid requirements lags behind that of other species. However, this presents an opportunity in this field for equine researchers as the methodologies have been pioneered in other species and need only to be adapted to horses. Isotopic measurements are considered the gold standard for human studies. The isotopic methods have the advantage of being able to study a variety of physiological states.

## Chapter 3. Rationale and objectives of the reported studies

Given the importance of protein nutrition to the athletic and growing horse and lack of empirical data defining amino acid requirements and bioavailability in the horse there are many opportunities for research in this area. Particularly, the amino acid adequacy of low protein diets for horses, effects of amino acid supplementation, and tailoring methods used in other species to the horse. The studies presented in this dissertation are the first to investigate amino acid adequacy and requirements using isotopic methods in horses.

Objectives of Chapter 4:

1. Use stable isotope kinetics to estimate whole-body protein synthesis in growing horses for the first time.
2. Compare whole-body protein kinetics between horses receiving two different diets: one that included a commercial concentrate for growing horses in combination with forage, which provided CP above the current NRC recommendations and a second that provided a lower protein concentrate in combination with the same forage and provided a level of CP similar to the current NRC recommendations for growing horses.

Objectives of Chapter 5:

1. Investigate the response of whole-body and muscle tissue protein synthesis and plasma and tissue amino acid concentrations in yearling horses receiving graded levels of lysine, with levels both above and below the current NRC (2007) recommendations.

2. Empirically determine a lysine requirement for the yearling horse.

Objectives of Chapter 6:

1. Investigate the effect of threonine supplementation on whole body protein synthesis of weanlings fed a commercial concentrate and grass forage.
2. Investigate the effect of threonine supplementation on whole body protein synthesis in mature horses fed a low threonine concentrate and high fiber forage.

Objectives of Chapter 7:

1. Determine whether the oral infusion of [1-$^{13}$C]phenylalanine allowed for measureable amounts of isotope to be detected in the collected breath and plasma samples.
2. Determine splanchnic extraction of phenylalanine at two different levels of threonine intake.

# Chapter 4. Dietary crude protein intake influences rates of whole-body protein synthesis in weanling horses

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## Introduction

Similar to other animals, horses are believed to have a dietary requirement for each of the individual indispensable amino acids. Unlike other species (NRC, 2012; WHO, 2007), equine amino acid requirements have not yet been well defined and therefore feeding recommendations are primarily for a certain level of total protein intake. Around the world, there are several different systems to express protein requirements, including crude protein (CP) (NRC, 2007), horse digestible CP (Martin-Rosset and Tisserand, 2004), apparent digestible CP (Ellis, 2004), and prececally digested protein (Coenen et al., 2011). A limiting amino acid is the dietary indispensable amino acid that is provided the most below its requirement and therefore protein synthesis is limited to the rate at which that amino acid is available.

Several studies have identified lysine as a commonly limiting amino acid in equine diets (Breuer and Golden, 1971; Graham et al., 1994; Ott et al., 1981). These studies are the basis of lysine requirements listed in the National Research Council (NRC) (2007) and the French feeding recommendations (Martin-Rosset and Tisserand, 2004). In North America and Europe, CP values are readily available on feed labels and in ration formulation software, whereas the amino acid contents of equine feeds are not as readily available.

In the United States, horses are often fed CP well above the NRC recommendations (Harper et al., 2009), which for 6 month old weanlings is 3.1 g/kg BW/d (NRC, 2007). Although, little is known about the indispensable amino acid requirements of growing horses, lysine has been identified as a first limiting amino acid in some equine diets (Breuer and Golden, 1971; Graham et al., 1994), and the NRC recommends a lysine requirement of 32 g per day for a 6 month old weanling with an estimated mature weight of 550 kg (NRC, 2007). Threonine (Graham et al., 1994) and methionine (Graham-Thiers et al., 2012; Winsco et al., 2011) have also been suggested as potentially limiting amino acids in yearling and weanling horse diets, respectively. The NRC suggests that the other amino acid requirements for horses could be estimated from the tissue composition of the amino acids relative to lysine based on the work of Bryden (1991), but these rough estimates are not currently included in either the requirement tables or software of the NRC (2007). Although there has been some *in vivo* evaluation of these estimates (Graham-Thiers and Bowen, 2013), amino acid requirements have not been evaluated individually or over a variety of life stages. High protein diets could have detrimental effects for the horse itself. They could affect acid-base balance (Graham-Thiers et al., 2001), heat production (Kronfeld, 1996), water requirements (Graham-Thiers et al., 2001), and potentially respiratory health (Whittaker et al., 2009). A refined understanding of dietary limiting amino acids for growing horses could potentially result in improved diet formulation by enabling total CP intake to be reduced whilst meeting requirements of the amino acids most likely to be limiting with crystalline amino acids. In support of this, reducing the CP of a pasture supplement from 14% to 9% did not affect growth of Thoroughbreds, when fortified with 0.6% lysine and 0.4% threonine

(Staniar et al., 2001). Formulating diets in this manner has also been shown to reduce nitrogenous waste excretion in other species, including livestock and poultry (Applegate et al., 2008; Jongbloed and Lenis, 1992). Nitrogenous waste can have a negative effect on the environment (Fenn et al., 2003) therefore extending the value of improved diet formulation in regards to protein beyond the equine industry.

Amino acid intakes above requirements will not be used for protein synthesis, and must be metabolized to urea and $CO_2$. The conversion of an amino acid to $CO_2$ can be measured by infusing a 1-$^{13}$C-labeled amino acid and measuring $^{13}CO_2$ expired in the breath. Once amino acid oxidation is known, non-oxidative disposal, an estimate of whole-body protein synthesis, can be calculated (Waterlow et al., 1978). Such isotopic methods have been used extensively in other species, such as humans (Kurpad and Thomas, 2011), swine (Moehn et al., 2005), and chickens (Coleman et al., 2003), to determine amino acid requirement and to identify limiting amino acids (Brunton et al., 2007). The concept and application of isotopic methods has been reviewed elsewhere (Elango et al., 2008a) and thoroughly compared to other methods of determining amino acid requirements (WHO, 2007). Our recently validated amino acid isotope infusion technique for use in horses (Urschel et al., 2012) can now be used to improve our understanding of protein metabolism in horses.

The objective of this study was to compare whole-body protein kinetics between horses receiving two different diets: one that included a commercial concentrate for growing horses in combination with forage, which provided CP above the current NRC recommendations and a second that provided a lower protein concentrate in combination with the same forage and provided a level of CP similar to the current NRC

recommendations for growing horses. Both rations provided the recommended intake of lysine. Not only does this study investigate the effects of CP intake on whole-body protein kinetics in weanling horses, but it is also the first time that stable isotope kinetics have been used to estimate whole-body protein synthesis in growing horses.

## Materials and methods

### *Animals, housing, and feeding*

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC 2010−0709). Six light breed weanling colts of similar age and weight were obtained from University of Kentucky's Maine Chance Farm. At the time of sampling, the weanlings were on average across treatments 182 ± 8 days and 236 ± 20 kg.

During the 2-week adaptation period and the week between sampling periods, the weanlings were kept in individual dry lot pens and were brought into individual sawdust bedded stalls (3.7 m × 3.7 m) twice daily for concentrate feeding. Forage was fed in the dry lots. After catheterization and during all sample collection and isotope infusion procedures, horses remained in the stalls. Body weights were taken weekly through the adaptation and study periods on a livestock scale (TI-500, Transcell Technology Inc.).

During the 2-week adaptation period, when the horses were adapting to the research environment, diets were designed to meet or exceed all the 2007 NRC recommendations (NRC, 2007). The concentrate to forage ratio of 58:42 in the present study was similar to that of other growing horse diets (Gibbs and Potter, 2002; Ott et al., 1981). Concentrate and alfalfa hay cubes (10 g cubes/kg BW/d; Table 4.1) were fed twice daily at 0700 and 1400 h, and the horses had free access to salt and water at all times. The

concentrate consisted of a commercial pellet designed for growing horses (Growth Pellet, Buckeye Nutrition; 11.6 g/kg BW/d) in addition to a ration balancer pellet (Gro ‘N Win Alfa, Buckeye Nutrition; 1.9 g/kg BW/d). Feed was collected throughout the study into plastic bags and stored at room temperature before being sent to Dairy One Cooperative Inc. for proximate analysis via wet chemistry. The concentrates and the forage were also analyzed for amino acid content as described below.

**Table 4.1: As-fed nutrient composition of the feeds used in the commercial crude protein (CCP) and recommended crude protein (RCP) treatments.**

| | Alfalfa Hay Cubes | Ration Balancer Pellet[a] | CCP pellet[b] | RCP Pellet[c] |
|---|---|---|---|---|
| ***Overall nutrient composition*** | | | | |
| Dry matter (%) | 90 ± 0.1 | 90.6 ± 0.3 | 91.2 ± 0.2 | 90.8 ± 0.7 |
| Crude protein (%) | 17.8 ± 0.5 | 12.9 ± 0.1 | 18.3 ± 0.2 | 9.0 ± 0.1 |
| Lignin (%) | 7.4 ± 0.6 | 2.3 ± 0.4 | 2.2 ± 0.5 | 1.2 ± 0.3 |
| ADF (%) | 30.3 ± 2.7 | 25.1 ± 0.1 | 10.8 ± 0.9 | 13.9 ± 0.5 |
| NDF (%) | 38.6 ± 2.2 | 39.5 ± 1.0 | 22.3 ± 1.0 | 22.2 ± 0.5 |
| Water soluble carbohydrates (%) | 8.4 ± 0.6 | 4.4 ± 1.0 | 6.5 ± 0.4 | 4.8 ± 0.4 |
| Ethanol soluble carbohydrates (%) | 5.7 ± 0.8 | 2.8 ± 0.4 | 3.9 ± 0.3 | 1.4 ± 0.4 |
| Starch (%) | 1 ± 0.3 | 10.1 ± 1.8 | 23 ± 0.4 | 33.3 ± 0.8 |
| Crude fat (%) | 1.5 ± 0.2 | 5.1 ± 0.5 | 8.6 ± 0.1 | 6.1 ± 0.1 |
| Calcium (%) | 1.84 ± 0.22 | 1.28 ± 0.05 | 0.91 ± 0.02 | 1.22 ± 0.01 |
| Phosphorus (%) | 0.22 ± 0.01 | 1.48 ± 0.01 | 0.75 ± 0.01 | 0.72 ± 0.01 |
| Iron (mg/kg) | 674 ± 61 | 1037 ± 15 | 342 ± 20 | 623 ± 60 |
| Zinc (mg/kg) | 22 ± 2 | 436 ± 10 | 168 ± 3 | 193 ± 13 |
| ***Amino acid composition (g/100 g feed)*** | | | | |
| Alanine (%) | 0.62 ± 0.08 | 0.49 ± 0.03 | 0.80 ± 0.06 | 0.42 ± 0.04 |
| Arginine (%) | 0.74 ± 0.14 | 0.70 ± 0.03 | 1.26 ± 0.06 | 0.46 ± 0.05 |
| Aspartate + Asparagine (%) | 1.22 ± 0.11 | 0.75 ± 0.04 | 1.46 ± 0.16 | 0.32 ± 0.03 |
| Glutamate + Glutamine (%) | 1.13 ± 0.14 | 1.66 ± 0.09 | 3.22 ± 0.19 | 1.14 ± 0.10 |
| Glycine (%) | 0.49 ± 0.09 | 0.56 ± 0.04 | 0.70 ± 0.04 | 0.35 ± 0.05 |
| Histidine (%) | 0.24 ± 0.03 | 0.17 ± 0.01 | 0.31 ± 0.04 | 0.13 ± 0.02 |
| Isoleucine (%) | 0.60 ± 0.11 | 0.39 ± 0.03 | 0.76 ± 0.05 | 0.32 ± 0.04 |
| Leucine (%) | 0.94 ± 0.15 | 0.75 ± 0.05 | 1.36 ± 0.08 | 0.70 ± 0.06 |
| Lysine (%) | 0.62 ± 0.10 | 1.16 ± 0.12 | 1.00 ± 0.18 | 0.97 ± 0.09 |
| Methionine (%) | 0.02 ± 0.01 | 0.13 ± 0.02 | 0.18 ± 0.03 | 0.21 ± 0.02 |
| Phenylalanine (%) | 0.64 ± 0.09 | 0.45 ± 0.02 | 0.83 ± 0.04 | 0.35 ± 0.03 |
| Proline (%) | 1.08 ± 0.08 | 0.74 ± 0.10 | 1.10 ± 0.06 | 0.54 ± 0.05 |
| Serine (%) | 0.43 ± 0.02 | 0.52 ± 0.04 | 0.44 ± 0.03 | 0.24 ± 0.06 |
| Threonine (%) | 0.40 ± 0.06 | 0.30 ± 0.03 | 0.38 ± 0.04 | 0.19 ± 0.03 |
| Tyrosine (%) | 0.36 ± 0.05 | 0.37 ± 0.02 | 0.51 ± 0.04 | 0.26 ± 0.03 |
| Valine (%) | 0.71 ± 0.12 | 0.47 ± 0.03 | 0.86 ± 0.06 | 0.38 ± 0.03 |

[a] Gro 'N Win Alfa, Buckeye Nutrition
[b] Growth Pellet, Buckeye Nutrition
[c] Ingredient composition listed in Table 4.2

**Table 4.2: Composition of recommended crude protein (RCP) pellet on an as-fed basis.**

| Ingredient | Amount, % |
|---|---|
| Ground corn | 44.40 |
| Soybean hulls | 24.76 |
| Wheat middlings | 6.12 |
| Soybean oil | 5.58 |
| Shredded beet pulp | 5.00 |
| Oatmill byproduct | 5.00 |
| Monocalcium phosphate (21%) | 2.16 |
| Dynamate[a] | 1.47 |
| Alfalfa meal | 1.00 |
| Salt | 1.00 |
| Vitamin E | 0.82 |
| Lysine hydrochloride | 0.76 |
| Calcium carbonate | 0.63 |
| Dyna K[b] | 0.27 |
| Corn distillers dried grains | 0.25 |
| Soybean meal | 0.25 |
| DL methionine | 0.20 |
| Proprietary vitamin/mineral premix[c] | 0.12 |
| Proprietary trace mineral premix[d] | 0.09 |
| Binder protein | 0.08 |
| Magnesium oxide | 0.03 |
| Choline | 0.01 |
| Copper sulfate | 0.009 |
| Vitamin A | 0.002 |
| Vitamin D3 | 0.0003 |

[a] Dynamate is a potassium and magnesium sulfate product made by The Mosaic Company

[b] Dyna K is a potassium chloride product made by The Mosaic Company

[c] The proprietary vitamin/mineral premix was designed specifically for inclusion in Buckeye Nutrition feeds and is manufactured by Trouw Nutrition

[d] The proprietary trace mineral premix was designed specifically for inclusion in Buckeye Nutrition feeds and is manufactured by Trouw Nutrition

***Study design and procedures***

After the 2-week adaptation period, this study was conducted as a crossover design, where each colt was studied while receiving each of 2 dietary treatments, in a randomly determined order. The 2 treatments were the commercial crude protein diet (CCP), where horses continued to receive the commercial concentrate, as described in the previous section and the recommended crude protein diet (RCP), where horses received a concentrate containing ~50% of the CP contained in the commercial pellet (Table 4.1 & Table 4.2). The CCP concentrate in combination with the forage cube provided protein well above requirements as is typical in the equine industry (Harper et al., 2009; Honore and Uhlinger, 1994), whereas the RCP treatment provided protein at a level consistent with the current NRC recommendations (NRC, 2007). The commercial and the RCP concentrate were formulated to contain similar amounts of lysine and micronutrients (Table 4.1). Orts were collected and weighed prior to each meal feeding, although after the adaptation period, feed refusals were minimal. To standardize tyrosine and phenylalanine intakes between the 2 treatments the RCP concentrate was top dressed with tyrosine (15.3 mg/kg BW/d) and phenylalanine (51 mg/kg BW/d). Amino acid intakes from the treatment diets are provided in Table 4.3. Standardization of phenylalanine and tyrosine intake is common practice for studies evaluating different levels of protein and/or different feedstuffs using phenylalanine isotope kinetics (Humayun et al., 2007b; Moehn et al., 2005). Phenylalanine is a common choice for an indicator amino acid and the criteria for an appropriate indicator amino acid are enumerated by Hsu et al. (2006b). All horses received each treatment diet for 3 days before sampling. The short adaptation period required for this method (Elango et al., 2009; Moehn et al., 2004b) makes it ideal

for studying growing animals, as the animals can be studied while receiving potentially deficient diets and the risk of detrimental effects associated with prolonged nutrient restriction is minimized. Any effects of the diet before receiving the treatment diet is likely minimal and commentary on length of adaptation and the use of the indicator amino acid oxidation method can be found elsewhere (Elango et al., 2012b).

**Table 4.3: Intake of crude protein (CP) and amino acids from the total dietary treatment on a daily basis and current NRC recommendations.**

| Amino acid (mg/kg BW/d) | CCP | | RCP | | NRC |
|---|---|---|---|---|---|
| | Average | SD | Average | SD | requirement |
| Alanine | 162.75 | 0.61 | 118.74 | 0.71 | - |
| Arginine | 231.62 | 0.87 | 138.18 | 0.88 | - |
| Aspartate + Asparagine | 301.88 | 1.12 | 169.74 | 1.16 | - |
| Glutamate + Glutamine | 516.13 | 2.01 | 273.36 | 1.73 | - |
| Glycine | 139.03 | 0.52 | 98.58 | 0.64 | - |
| Histidine | 62.10 | 0.23 | 41.83 | 0.26 | - |
| Isoleucine | 154.07 | 0.57 | 102.48 | 0.63 | - |
| Leucine | 262.93 | 0.98 | 186.66 | 1.10 | - |
| Lysine | 188.93 | 0.70 | 193.89 | 1.16 | 133.30 |
| Methionine | 70.39 | 0.26 | 83.39 | 0.44 | - |
| Phenylalanine | 166.37 | 0.62 | 111.34 | 0.68 | - |
| Proline | 246.58 | 0.92 | 181.71 | 1.13 | - |
| Serine | 101.97 | 0.38 | 79.48 | 0.55 | - |
| Threonine | 88.47 | 0.33 | 66.55 | 0.43 | - |
| Tyrosine | 101.24 | 0.38 | 72.10 | 0.46 | - |
| Valine | 178.53 | 0.66 | 122.06 | 0.75 | - |
| CP (g/kg BW/d) | 4.1 | 0.1 | 3.1 | 0.1 | 3.1 |

On the afternoon of day 3 for each treatment, 2 jugular vein catheters were inserted, 1 for blood sampling and 1 for isotope infusion (Urschel et al., 2012). In order to determine the effect of concentrate CP on plasma amino acid concentrations, baseline

blood samples (7 mL) were taken 15 min and immediately before feeding the 1400 h allocation of concentrate. Another sample was taken at 120 min post-feeding.

The next day, whole-body phenylalanine kinetics were determined using a 2 h primed (7.1 μmol/kg BW), constant (6 μmol/kg BW/h) infusion of [$^{13}$C]sodium bicarbonate, to measure whole-body $CO_2$ production (Coggan et al., 1993), followed immediately by a 4 h primed (8.4 μmol/kg BW), constant infusion of [1-$^{13}$C]phenylalanine (6 μmol/kg BW/h), to measure phenylalanine oxidation to $CO_2$ and phenylalanine flux. The isotope doses and lengths of infusion were previously validated in mature horses and resulted in a rapid rise to plateau isotopic enrichment in the plasma phenylalanine (Urschel et al., 2012). In order to infuse the isotope and allow the weanling horses to move freely in their stalls, cordless infusion pumps (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc.) were attached to surcingles that were worn by the weanlings. Beginning 90 min before the start of isotope infusion procedures 1/24 of the morning meal, including both the hay cube and the concentrates, was given every half hour during infusion in order to reduce fluctuations in phenylalanine oxidation (Moehn et al., 2004a; Mohn et al., 2003). Breath samples were collected every 30 min beginning 30 min prior to the start of the [$^{13}$C]sodium bicarbonate infusion until the end of the [1-$^{13}$C]phenylalanine infusion into gas impermeable bags using a modified equine Aeromask (Urschel et al., 2009). Blood samples were collected every 30 min beginning 30 min prior to the start of the [1-$^{13}$C]phenylalanine infusion until the end of the infusion procedures.

At the end of the isotope infusion procedures, catheters were removed and the weanlings had a 4 day washout period, during which they were fed and managed as described during the adaptation period, before being allocated to the other treatment. At

the end of the study, the weanlings were returned to University of Kentucky's Maine Chance Farm research herd.

***Sample analyses***

*Blood Sample Processing.* All blood samples were collected into heparinized vacutainers (Vacutainer; Becton-Dickinson) and promptly centrifuged at 1,500 × *g* for 10 min at 4 ºC. Plasma was removed and frozen at -20 ºC until the time of analysis.

*Amino Acid Analyses.* Plasma free amino acid concentrations and total feed amino acid concentrations were measured using reverse phase HPLC (3.9 × 300 mm PICO-TAG reverse phase column) of phenylisothiocyanate derivatives as previously described (Urschel et al., 2011). Performic acid oxidation was performed to obtain values for methionine content (AOAC International, 2005). Three samples of each feedstuff collected over the course of the study were used for amino acid analyses and each sample was analyzed in duplicate.

*Plasma Urea.* Plasma urea nitrogen was determined using a colorimetric spectrophotometric assay as previously described (Urschel et al., 2007), with an intra-assay variation of 5.5% and an inter-assay variation of < 10%.

*Isotope Enrichment.* The amount of $^{13}CO_2$ relative to $^{12}CO_2$ in breath samples collected during the phenylalanine and bicarbonate infusions was determined immediately after sampling using an infrared isotope analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH).

The relative concentrations of [1-$^{13}$C]phenylalanine to unlabeled phenylalanine, known as the phenylalanine isotopic enrichment, in the plasma samples was determined

by Metabolic Solutions, Inc. using a previously described method (Matthews et al., 1990). In brief, the isotopic enrichment of plasma samples was determined by negative chemical ionization GC-MS analysis of a heptafluorobutyric, n-propyl derivative. [1-$^{13}$C]phenylalanine enrichment was measured using methane negative chemical ionization GC-MS (Agilent 5973 EI/CI MSD with an Agilent 6890 GC). A Phenomenex ZB-1MS capillary column was used to separate the derivative of phenylalanine. Selected ion chromatograms were obtained by monitoring ions *m*/*z* 383 and 384 for phenylalanine and [1-$^{13}$C]phenylalanine, respectively.

***Calculations***

*Whole-body Phenylalanine Kinetics.* For both blood and breath sample enrichment, the average enrichment at plateau was determined. A plateau was defined as at least 3 points with a slope that was not different from 0 ($P$>0.05) using linear regression analyses (GraphPad Prism 4 Software, GraphPad Prism Inc.).

Whole-body phenylalanine flux was calculated via the following formula, where Q is flux (μmol/kg BW/h), i is the isotope infusion rate (μmol/kg BW/h), Ei is the enrichment of infused isotope expressed as a mole fraction, and Ep is the plateau plasma enrichment also expressed as a mole fraction calculated as previously described (Hsu et al., 2006a):

$$Q = i \times [(Ei/Ep) - 1]$$

Once flux was known, stochastic analysis was used to determine phenylalanine entering the free amino acid pool from dietary intake (I), *de novo* synthesis (N), and protein breakdown (B), or leaving the pool through protein synthesis (Z), oxidation (E), or the conversion to other metabolites (M) are all included in flux (Q).

$$Q = I + N + B = Z + E + M$$

Total dietary phenylalanine intake was multiplied by 0.5 to account for prececal phenylalanine digestibility (Almeida et al., 1999) and the extraction of phenylalanine by the splanchnic tissues as estimated in other species (Beelen et al., 2011; Shoveller et al., 2007). Although splanchnic extraction does not need to be accounted for in the fasted state and was not accounted for in studies that occurred before values of splanchnic extraction were determined, other studies have taken it into account (Beelen et al., 2011; Castillo et al., 1994). As horses do not synthesize phenylalanine de novo, the phenylalanine entering the free amino acid pool from protein breakdown was determined from the following equation, where I is phenylalanine intake corrected for splanchnic extraction:

$$B = Q - I$$

[1-$^{13}$C]phenylalanine oxidation was calculated using the equations described by Hsu et al. (2006a). The difference between phenylalanine flux and oxidation is the non-oxidative phenylalanine disposal. This value can be used as an indicator of whole-body protein synthesis. Phenylalanine conversion to tyrosine was considered to be minimal and any that did occur should be the same for both diets as both phenylalanine and tyrosine intakes were similar in both treatments. As such, changes in non-oxidative phenylalanine were taken to indicate changes in phenylalanine utilization for whole-body protein synthesis.

### *Statistical analysis*

All data were analyzed using SAS (version 9.1.3, SAS Institute Inc.), using the MIXED procedure unless otherwise noted. In all cases, differences were considered

significant at $P<0.05$. The phenylalanine kinetics parameters were analyzed using a one-way analysis of variance with treatment as the fixed effect and horse as the random effect. Plasma amino acid and urea concentration data were analyzed using a repeated measures analysis, with treatment, time, and time × treatment interaction as fixed effects and horse as the random effect. In all cases, when differences were significant, means were separated using the pdiff option with the Tukey-Kramer adjustment.

## Results

### *Plasma amino acid concentrations*

The effects of time, diet, and the interaction of time by diet on plasma amino acid concentrations were significant ($P<0.05$; Table 4.4). Horses on the CCP diet showed an increase ($P<0.05$) in the plasma concentration of isoleucine, leucine, lysine, threonine, valine, alanine, arginine, asparagine, glutamine, ornithine, proline, serine, and tyrosine at 120 min post-feeding, whereas the horses on the RCP diet did not achieve a similar increase ($P>0.05$). Baseline plasma amino acid concentrations were greater on the CCP diet for histidine, isoleucine, leucine, threonine, valine, asparagine, proline, and serine ($P<0.05$). Notable exceptions were the amino acids where intakes were similar between the diets (phenylalanine, tyrosine, lysine, and methionine; Table 4.1). Phenylalanine, tyrosine, lysine, and methionine were greater ($P<0.05$) in the plasma of horses receiving the RCP treatment at both the 0 and 120 min time points, with the exception of tyrosine, which was not different between the two treatments ($P>0.05$) at the 120 min time point (Table 4.4).

**Table 4.4: The effects of feeding a commercial crude protein (CCP; 18.3 ± 0.2% CP) or recommended crude protein (RCP; 9.0 ± 0.1% CP) concentrate meal to 6 month old weanling colts on plasma amino acid and urea concentrations at baseline and 120 min post-feeding[1,2]**

| | CCP Concentrate | | RCP Concentrate | | SEM | *P*-values | | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | 120 min | Baseline | 120 min | | Treatment | Time | treatment x time |
| **Indispensable** | | | | | | | | |
| Histidine | 53[a] | 79[b] | 37[*] | 36[*] | 4 | < 0.0001 | 0.01 | 0.003 |
| Isoleucine | 47[a] | 75[b] | 30[*] | 27[*] | 4 | < 0.0001 | 0.01 | 0.003 |
| Leucine | 78[a] | 123[b] | 53[*] | 48[*] | 6 | < 0.0001 | 0.01 | 0.001 |
| Lysine | 87[a] | 64[b] | 157[*] | 174[*] | 15 | 0.0008 | 0.05 | NS |
| Methionine | 33 | 44 | 65[*] | 75[*] | 4 | < 0.0001 | NS | NS |
| Phenylalanine | 63 | 77 | 100[*] | 111[*] | 6 | < 0.0001 | NS | NS |
| Threonine | 90 | 119 | 61[*] | 59[*] | 8 | < 0.0001 | NS | NS |
| Tryptophan | 5 | 5 | 4 | 4 | 1 | 0.03 | NS | NS |
| Valine | 165[a] | 202[b] | 101[*] | 97[*] | 8 | < 0.0001 | NS | 0.10 |
| **Dispensable** | | | | | | | | |
| Alanine | 237[a] | 290[b] | 214 | 225[*] | 14 | < 0.0001 | 0.05 | NS |
| Arginine | 114[a] | 160[b] | 95 | 97[*] | 8 | < 0.0001 | 0.04 | 0.04 |
| Asparagine | 76[a] | 100[b] | 66[*] | 57[*] | 12 | < 0.0001 | NS | 0.05 |
| Aspartate | 17 | 20 | 14 | 17 | 2 | 0.05 | NS | NS |
| Citrulline | 107 | 108 | 101 | 96 | 7 | 0.01 | NS | NS |
| Glutamate | 43 | 47 | 41 | 41 | 4 | NS | NS | NS |
| Glutamine | 561[a] | 679[b] | 511 | 494[*] | 24 | < 0.0001 | NS | 0.01 |
| Glycine | 628 | 679 | 546 | 519[*] | 29 | < 0.0001 | NS | NS |
| Ornithine | 52[a] | 64[b] | 47 | 41[*] | 3 | < 0.0001 | 0.07 | 0.02 |
| Proline | 151[a] | 183[b] | 114[*] | 102[*] | 8 | < 0.0001 | NS | 0.07 |
| Serine | 280[a] | 348[b] | 244[*] | 235[*] | 12 | < 0.0001 | 0.06 | 0.005 |
| Taurine | 34 | 41 | 36 | 37 | 3 | NS | NS | NS |
| Tyrosine | 83[a] | 108[b] | 115[*] | 114 | 5 | < 0.0001 | 0.07 | 0.05 |
| **Urea** | 0.82 | 0.84 | 0.59 | 0.55 | 0.14 | NS | NS | NS |

[1] Plasma amino acid concentrations are presented as least squares mean values (μmol/L)
[2] Plasma urea concentrations are presented as least squares mean values (mmol/L)
[*] Significantly different ($P<0.05$) from the CCP treatment at the same time point
[a,b] Different superscripts within a row indicate a significant effect of time ($P<0.05$)

### *Plasma urea nitrogen concentrations*

Plasma urea nitrogen concentrations were not significantly different due to treatment ($P$=0.22), time ($P$=0.78) or the interaction of treatment and time ($P$=0.40; Table 4.4).

### *Phenylalanine kinetics*

Phenylalanine flux was not different between treatments (Table 4.5). Phenylalanine intake was slightly (~6%) greater for horses receiving the CCP treatment ($P$<0.0001; Table 4.5). Horses receiving the RCP diet had greater rates of phenylalanine oxidation ($P$=0.02) and lower rates of non-oxidative phenylalanine disposal ($P$=0.04), indicating reduced whole-body protein synthesis (Table 4.5). Rates of phenylalanine release from protein breakdown were not different between the two treatments (Table 4.5). $CO_2$ production was also lower ($P$=0.04) in horses receiving the CCP diet (Table 4.5), suggesting less oxidation of unused amino acids compared to the RCP diet.

**Table 4.5: Whole-body phenylalanine kinetics in 6 month old Thoroughbred colts that were receiving alfalfa hay cubes and either a commercial crude protein (CCP; 18.3 ± 0.2% CP) or recommended crude protein (RCP; 9.0 ± 0.1% CP) concentrate[a]**

| | CCP treatment | RCP treatment | Pooled SE | *P*-value |
|---|---|---|---|---|
| ***Phenylalanine flux [µmol/kg BW/h]*** | | | | |
| | 73 | 81 | 5 | 0.16 |
| ***Carbon dioxide production [µmol/kg BW/h]*** | | | | |
| | 16372 | 21909 | 2326 | 0.04 |
| ***Phenylalanine entering the free phenylalanine pool [µmol/kg BW/h][b]*** | | | | |
| Phenylalanine from dietary intake | 23.6 | 22.2 | 0.1 | < 0.0001 |
| Phenylalanine from protein breakdown | 49.5 | 58.8 | 5.1 | 0.11 |
| ***Phenylalanine leaving the free phenylalanine pool [µmol/kg BW/h][b]*** | | | | |
| Phenylalanine oxidation | 9.1 | 29.3 | 6.8 | 0.02 |
| Non-oxidative phenylalanine disposal | 64.0 | 51.7 | 5.1 | 0.04 |

[a] Values are least squares means ± SE, as determined using a one-way analysis of variance

[b] The following stochastic model of phenylalanine kinetics was used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal; a 50% rate of splanchnic phenylalanine extraction was assumed when calculating the amount of dietary phenylalanine reaching general circulation

## Discussion

This study was the first to evaluate whole-body protein synthesis in growing horses using isotope infusion methodology. This methodology can greatly expand our understanding of dietary amino acid needs in animals during growth. In weanling Thoroughbreds, we found that whole-body protein synthesis was lower in horses receiving the RCP diet in comparison to the CCP diet, potentially as a result of a limiting amino acid.

Plasma and serum amino acid concentrations peak 1 to 2 h post-feeding (Hackl et al., 2006; Woodward et al., 2011), which was the response seen in the horses receiving the CCP treatment. The lack of a similar increase of plasma amino acid concentrations in horses receiving the RCP diet was likely due to the lower intake of amino acids. This finding agrees with Woodward et al. (2011), who found greater serum amino acid concentration increases in horses fed alfalfa (CP 25%) compared to timothy (CP 8%).

When an amino acid is reduced in a diet to a point where it becomes limiting, there is an increase in the plasma concentrations of other amino acids (Almquist, 1954). The increased amino acid concentrations result from the inability to use these other amino acids for protein synthesis because of the limiting amino acid provided below requirement. For the amino acids where there were similar intakes in both diets, particularly phenylalanine, tyrosine, lysine, and methionine, horses receiving the RCP concentrate had greater plasma concentrations both pre and post-feeding, suggesting that these amino acids were not utilized for protein synthesis due to another amino acid being limiting in the RCP diet (Table 4.4). The greater plasma concentrations of these amino acids when horses received the RCP diet could also be due to differing digestibilities in protein bound versus free amino acids (Rolls et al., 1972). In order to ensure similar total intakes of phenylalanine, tyrosine, lysine, and methionine between treatments, additional amounts of these amino acids were added in crystalline form to the RCP diet concentrate. This resulted in dietary amino acid profiles that may not have been equally bioavailable. Unfortunately, the digestibility of amino acids remains largely unexplored in the horse (Almeida et al., 1999).

The timing of the post-feeding blood sampling was based on maximal post-feeding amino acid concentrations (Hackl et al., 2006), while maximal urea response to feeding occurs later as amino acids must be metabolized to urea, as has been demonstrated in stallions (DePew et al., 1994). The sampling protocol in the present study may not have been able to detect a treatment effect on plasma urea concentrations. Lower plasma urea nitrogen concentrations were found to occur in horses when a limiting amino acid is supplemented (Graham et al., 1994). Blood urea nitrogen also increases with increasing protein intake in horses (Sticker et al., 1995). Despite the phenylalanine kinetics data indicating a limiting amino acid in the RCP diet, the plasma urea nitrogen concentrations in horses receiving this treatment were only numerically, but not significantly, greater than those receiving the CCP diet. This lack of treatment difference in plasma urea nitrogen concentration may have been due to the fact that the CCP diet provided substantially more CP than the RCP diet. Therefore, any increase in plasma urea nitrogen concentrations in horses receiving the RCP treatment may have been eclipsed by the greater total nitrogen intake and its effects on plasma urea nitrogen concentrations in horses receiving the CCP treatment.

The phenylalanine kinetics data show that whole-body protein synthesis was lower when weanlings received the RCP compared to the CCP diet, because this treatment resulted in lower rates of non-oxidative phenylalanine disposal. Non-oxidative disposal of phenylalanine is mostly through protein synthesis, especially when the diet contains adequate tyrosine (Thorpe et al., 2000). In agreement, the rate of phenylalanine oxidation was greater in horses receiving the RCP diet, indicating that the horses metabolized more phenylalanine, despite the phenylalanine intake being similar between

treatments, because it was not being used for protein synthesis. There is an inverse relationship between the oxidation of an indicator amino acid and protein synthesis (Elango et al., 2008a).

Phenylalanine flux and non-oxidative disposal (Table 4.5) were also found to be greater in the weanling horses in the current study than in the mature horses of a previous study (Urschel et al., 2012). These values reflect greater protein accretion during growth compared to maintenance. Similarly, flux values in children, ~89 μmol/kg BW/h (Pillai et al., 2010) are greater than in adults, ~52 μmol/kg BW/h (Elango et al., 2009).

Phenylalanine and tyrosine intakes are commonly standardized in studies evaluating different levels of protein and/or feedstuffs with phenylalanine isotope kinetics (Humayun et al., 2007b; Moehn et al., 2005). Although the phenylalanine intakes were similar for the two treatments, the sources were different, possibly resulting in differing bioavailabilities of phenylalanine. When horses consumed a diet with supplemental phenylalanine, both phenylalanine flux and phenylalanine oxidation were elevated, compared to when horses received an unsupplemented diet (Urschel et al., 2012). However, because there was no difference in phenylalanine flux between the two treatments in the current study, this potential difference in phenylalanine bioavailability between the RCP and CCP treatments would not be expected to affect the values for either phenylalanine oxidation or non-oxidative phenylalanine disposal, because these parameters are not dependent on phenylalanine intake for their calculation (Waterlow et al., 1978). If horses receiving the RCP treatment did in fact absorb more phenylalanine than when receiving the CCP diet, we would have underestimated phenylalanine intake and subsequently overestimated protein breakdown for the RCP treatment. Therefore, the

protein breakdown data from the present study must be interpreted with caution as it is possible that the relative differences between groups were not as large because they may have been influenced by differences in dietary phenylalanine bioavailability.

CP is a relatively crude way of measuring a feedstuff's suitability in meeting the nutrient requirements of the horse, because it is a measure of nitrogen content and not bioavailable nitrogen. Although the NRC does not take into account differences in availability/digestibility between feedstuffs (NRC, 2007), other systems have attempted to take these factors into account. There is a dearth of knowledge relating to amino acid availability/digestibility in the horse (Almeida et al., 1999), making it difficult to implement other more descriptive systems to express amino acid requirements, such as on a standardized ileal digestible basis (NRC, 2012). In light of this knowledge gap, protein digestibility data from ruminants has been used as a proxy (Austbo, 2004; Coenen et al., 2011). These adjusted values are linked to the CP content of the feed. In the case of the present study, arbitrarily reducing the CP to the NRC requirement has resulted in a limiting amino acid confirming that a blanket protein requirement does not adequately describe the growing horse's nitrogen needs.

The NRC requirement for the weanlings studied was 3.1 g CP/kg BW/d. Back calculating using a protein digestibility of 66%, the German protein requirement for these weanlings was 4.1 g CP/kg BW/d (Coenen, 2004). Also working backwards using protein digestibilities of 75% and 60% and k factors (correction factors used in the Matières Azotées Digestibles Cheval system) of 1 and 0.85 for the concentrates and dehydrated alfalfa, respectively, the French requirement for these weanlings would be 3.8 g CP/kg BW/d (Martin-Rosset and Tisserand, 2004). Our CCP diet met or exceeded the CP

requirements proposed by all three systems, while the RCP diet met only the NRC requirement (Table 4.3), given the aforementioned assumptions.

Although the NRC (2007), French standards (Martin-Rosset and Tisserand, 2004), and German standards (Coenen, 2004) have a lysine requirement, only the German and French standards have a requirement for threonine. The German threonine requirement was adapted from swine (Coenen, 2004), while the French requirement (Martin-Rosset and Tisserand, 2004) is based on a review of available equine data by Ott (2001). It has been suggested that tissue composition of amino acids and their ratios to lysine could be used to estimate other amino acid requirements (Bryden, 1991; Wickens et al., 2002). However, empirical work on individual equine amino acid requirements, other than for lysine, has not yet been performed.

Because the RCP diet met the current NRC requirements for both CP and lysine, the lower rate of whole-body protein synthesis implies that one or more of the other amino acids supplied in the RCP diet did not meet the weanlings' requirements for maximal protein accretion. Lysine (Breuer and Golden, 1971; Ott et al., 1981) and threonine (Graham et al., 1994) have been shown to be limiting in equine diets and methionine has been shown to be limiting in diets for other species (Phillips and Walker, 1979; Tujioka et al., 2005). Growing horses fed graded amounts of methionine had no differences in growth characteristics, nitrogen balance, or urinary urea (Winsco et al., 2011). However for the present study, lysine and methionine intakes were equivalent in both diets, whereas threonine intake was approximately 25% lower in the RCP diet compared to the CCP diet.

When using muscle tissue composition to estimate amino acid requirements, as suggested by the NRC (2007), threonine intake (requirement estimate of 81 mg/kg BW/d) was limiting in the RCP diet, but not the CCP diet (Table 4.3). However, in using this approach to estimate amino acid requirements, histidine (requirement estimate of 77 mg/kg BW/d) also appears to be limiting in both diets, which to the best of the authors' knowledge has not been described in other species, with the exception of lactating dairy cattle (Vanhatalo et al., 1999). A study using diets deficient in histidine based on a requirement determined from equine muscle tissue reported no deficiency symptoms while the horses maintained nitrogen balance (Graham-Thiers and Bowen, 2011). Interestingly, the Lys: His requirement ratio is ~3 in many growing species (NRC, 1993, 1994, 1998; WHO, 2007). If the Lys:His ratios are calculated based on equine muscle tissue (Bryden, 1991) or equine milk (Wickens et al., 2002), the ratios are 1.73 and 3.02, respectively. Using muscle tissue in horses likely overestimates histidine requirements. Using the ratio of 3:1, both the RCP and CCP diets provided adequate dietary histidine. Given that threonine is a commonly limiting amino acid in other species, histidine was likely adequately supplied, and other amino acids were supplied above the requirement as estimated from equine muscle, threonine is a likely candidate for the limiting amino acid in the RCP diet.

**Conclusions**

This research is important because a thorough knowledge of amino acid requirements of horses has the potential to allow for the formulation of diets that reduce nitrogenous waste through more efficient feeding and because limiting amino acids have an adverse effect on growth. Amino acid intakes that were similar between diets

generally resulted in greater plasma amino acid concentrations on the RCP diet, possibly indicating that a limiting amino acid kept these amino acids from being used for protein synthesis. Greater rates of phenylalanine oxidation and lower rates of non-oxidative phenylalanine disposal indicate that less protein synthesis occurred when weanlings received the RCP diet. This is suggestive of a limiting amino acid in the RCP treatment, likely threonine, since it was provided above the estimate based on muscle tissue in the CCP diet and below in the RCP diet, in addition to being indicated as a second limiting amino acid in the literature. Additional research is needed to further elucidate the amino acid requirements of growing horses.

# Chapter 5. Evaluation of whole-body protein synthesis and plasma amino acid concentrations in yearling horses fed graded amounts of lysine

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## Introduction

Compared to humans and swine, relatively little work has been done to elucidate amino acid requirements for horses. Currently, the NRC (2007) lists only a crude protein requirement and a lysine requirement in the requirement tables and software. Identifying amino acid requirements for various physiological states (NRC, 2012, 1998) has helped the swine industry to minimize nitrogenous waste (Panetta et al., 2006) without compromising pig growth (Tuitoek et al., 1997). A study of equine rations in the Chesapeake Bay area found that horses were provided with 157± 21.6% of the 2007 NRC requirement in protein (Harper et al., 2009) presumably resulting in unnecessary nitrogenous waste. Other consequences of overfeeding protein to horses could be compromised respiratory health from increased ammonia output (Whittaker et al., 2009), disrupted acid-base balance (Graham-Thiers and Kronfeld, 2005b; Graham-Thiers et al., 1999), and decreased bone mineralization from increased calcium excretion through urine (Hashimoto et al., 1996). Determining amino acid requirements in horses will allow diet formulation to more closely match equine needs.

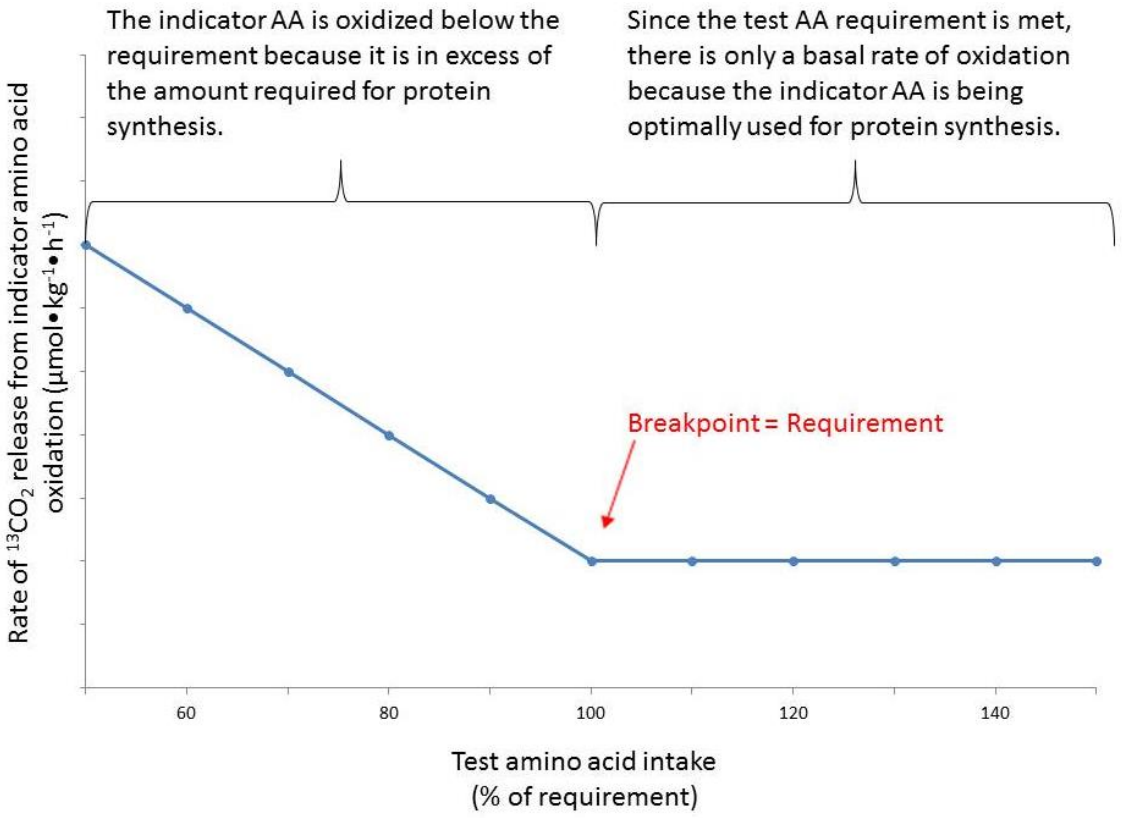
Lysine is a limiting amino acid for many species (Brown and Cline, 1974; Fox et al., 1995; Metges et al., 2005; Tome and Bos, 2007; Tujioka et al., 2005). Particularly, it has been found to be limiting in some diets for growing horses (Breuer and Golden, 1971;

Fisher et al., 1989; Graham et al., 1994; Hintz et al., 1971; Ott et al., 1981; Potter and Huchton, 1975). However, these studies in horses were generally not designed to measure specific lysine requirements and they did not use isonitrogenous treatments, but rather used treatments with different protein sources, and/or did not include many levels of lysine intake. The NRC (2007) applied broken line analysis to 7 studies that reported diet composition, intake, and nitrogen retention to estimate a lysine requirement at 4.3% of the crude protein requirement (NRC, 2007). The lysine requirement as a percentage of crude protein is in line with other species as reviewed by Ball et al. (2007b), but has not been experimentally confirmed in horses of any age. For yearling horses, the current NRC (2007) recommendation for lysine is 113 mg/kg/d.

Isotopic methods have been widely used in other species to determine amino acid requirements (Bertolo et al., 2005; Kurpad and Thomas, 2011; Tabiri et al., 2002). These methods are based on the concept that amino acids that cannot be used for protein synthesis are oxidized to $CO_2$. The production of $CO_2$ from amino acid metabolism can be determined from an infusion of a 1-$^{13}$C-labeled amino acid and measuring $^{13}CO_2$ expelled in the breath. Using an isotopic essential amino acid other than the amino acid being tested as an indicator of amino acid metabolism is known as the indicator amino acid oxidation (IAAO) method. As the dietary intake of a test amino acid, such as lysine, increases, less of the indicator or 1-$^{13}$C-labeled amino acid is oxidized until a plateau is reached (Figure 5.1). The intersection of this decline and plateau defines the requirement of the test amino acid. There are several reviews on the use and application IAAO (Brunton et al., 1998; Elango et al., 2008a). Isotopic methods are also explained in Chapter 2 in the section titled “Isotope methodologies”. The isotope infusion technique

necessary to determine requirements via the IAAO method has been adapted to horses and recently validated (Urschel et al., 2012). In addition to calculating amino acid requirements, the isotopic data can also yield estimates of rates of whole-body protein synthesis and breakdown (Waterlow et al., 1978).

**Figure 5.1: Indicator amino acid oxidation methodology concept summary adapted from Urschel (2009)**




Although isotope methods investigate whole-body protein synthesis, they do not give insight as to where in the body protein synthesis is taking place. Measuring phosphorylation of signaling proteins in the mechanistic target of rapamycin (mTOR) pathway can indicate protein synthesis at the tissue level and has been extensively reviewed elsewhere (Hay and Sonenberg, 2004; Yang et al., 2008). Protein (Atherton et al., 2010), amino acids as a group (Tremblay and Marette, 2001), leucine independently

(Rachdi et al., 2012; Suryawan et al., 2012), and arginine independently (Yao et al., 2008) have been shown to stimulate the mTOR pathway. Lysine has not been investigated as an independent activator of mTOR.

This study investigates the response of whole-body and muscle tissue protein synthesis and plasma and tissue amino acid concentrations in yearling horses receiving graded levels of lysine, with levels both above and below the current NRC (2007) recommendations.

## Materials and Methods

### *Animals, housing, and feeding*

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC 2008−0385). Six yearling (401±5 d) Thoroughbred colts (362 ± 19 kg) obtained from University of Kentucky's Maine Chance Farm were used in this study.

A 2-wk adaptation period to diet and housing was followed by six 1-wk treatment periods. Yearlings were housed in individual dry lot pens and all sampling procedures were conducted in individual sawdust bedded stalls (3.7 m × 3.7 m) except muscle biopsies, which were conducted in equine stocks (Priefert® Rodeo & Ranch Equipment, Mount Pleasant, TX). A livestock scale (TI-500, Transcell Technology Inc., Buffalo Grove, IL) was used to obtain daily body weights.

Diets were designed to meet or exceed the 2007 Nutrient Requirements of Horses recommendation, with the exception of lysine (NRC, 2007). Two isonitrogenous and isocaloric versions of the same concentrate pellets were formulated for this study (Table 5.1 & Table 5.2). The only difference between the two concentrates was that one

contained free lysine and the other an isonitrogenous amount of free glycine, and a small amount of rolled oats to account for the difference in weight of the added glycine versus lysine. By mixing the two concentrates at different ratios and feeding timothy hay cubes at the same rate for all treatments, 6 treatment diets were created with the following lysine intakes: 76, 90, 104, 118, 127, & 136 mg/kg/d. Three of the lysine intakes were above the estimated lysine requirement of 113 mg/kg/d and three of the intakes were below. Glycine was selected because it is a dispensable amino acid and not metabolically related to lysine. The concentrate was provided at 1.11% of BW/d with timothy hay cubes at 1.37% of BW/d (Table 5.1). Timothy was used as the forage component of the diets because of its relatively low lysine content (Woodward et al., 2011). Concentrate and hay cubes were fed twice daily (0700 and 1500 h) with water and salt available at all times. Feed was collected throughout the study and four samples of each feedstuff were sent to Dairy One Cooperative Inc. (Ithaca, NY) for proximate analysis at the conclusion of the study. Weekly feed samples were analyzed for amino acid content as described below.

**Table 5.1: As-fed nutrient composition of the feeds (average ± SE) used in creating the treatments with 6 levels of lysine intake.**

| | Timothy Hay Cubes[1] | Low Lysine Concentrate[2] | High Lysine Concentrate[2] |
|---|---|---|---|
| ***Overall nutrient composition*** | | | |
| Dry matter (%) | 91.2 ± 0.2 | 89.3 ± 0.1 | 89.6 ± 0.5 |
| DE (Mcal/kg)[3] | 1.69 ± 0.11 | 2.97 ± 0.02 | 3.06 ± 0.06 |
| Crude protein (%) | 7.1 ± 0.1 | 13.9 ± 0.1 | 13.7 ± 0.3 |
| Lignin (%) | 4.9 ± 0.1 | 0.8 ± 0.3 | 0.9 ± 0.1 |
| ADF (%) | 39.4 ± 0.6 | 10.1 ± 0.5 | 9.3 ± 1.1 |
| NDF (%) | 60.4 ± 0.5 | 19.9 ± 0.7 | 17.5 ± 2.0 |
| Water soluble carbohydrates (%) | 10.9 ± 0.3 | 3.9 ± 0.1 | 4.1 ± 0.1 |
| Ethanol soluble carbohydrates (%) | 5.6 ± 0.2 | 2.3 ± 0.8 | 2.8 ± 0.3 |
| Starch (%) | 1.0 ± 0.1 | 32.3 ± 0.4 | 33.4 ± 1.9 |
| Crude fat (%) | 1.6 ± 0.1 | 6.7 ± 0.1 | 7.1 ± 0.1 |
| Calcium (%) | 0.37 ± 0.01 | 1.86 ± 0.10 | 1.94 ± 0.17 |
| Phosphorus (%) | 0.20 ± 0.01 | 0.30 ± 0.01 | 0.32 ± 0.02 |
| Iron (mg/kg) | 149 ± 6 | 175 ± 9 | 215 ± 61 |
| Zinc (mg/kg) | 101 ± 16 | 114 ± 6 | 126 ± 34 |
| ***Amino acid composition (g/100 g feed)*** | | | |
| Alanine (%) | 0.34 ± 0.03 | 0.62 ± 0.02 | 0.61 ± 0.01 |
| Arginine (%) | 0.35 ± 0.04 | 0.61 ± 0.03 | 0.60 ± 0.01 |
| Aspartate + Asparagine (%) | 0.51 ± 0.04 | 0.61 ± 0.06 | 0.53 ± 0.05 |
| Glutamate + Glutamine (%) | 0.64 ± 0.07 | 2.50 ± 0.09 | 2.57 ± 0.08 |
| Glycine (%) | 0.25 ± 0.02 | 1.06 ± 0.02 | 0.40 ± 0.01 |
| Histidine (%) | 0.05 ± 0.01 | 0.16 ± 0.01 | 0.16 ± 0.01 |
| Isoleucine (%) | 0.28 ± 0.03 | 0.51 ± 0.02 | 0.47 ± 0.02 |
| Leucine (%) | 0.47 ± 0.04 | 1.19 ± 0.02 | 1.19 ± 0.02 |
| Lysine (%) | 0.25 ± 0.02 | 0.37 ± 0.02 | 0.92 ± 0.05 |
| Methionine (%) | 0.02 ± 0.01 | 0.59 ± 0.06 | 0.70 ± 0.05 |
| Phenylalanine (%) | 0.32 ± 0.02 | 0.60 ± 0.01 | 0.60 ± 0.02 |
| Proline (%) | 0.36 ± 0.03 | 0.98 ± 0.03 | 0.98 ± 0.01 |
| Serine (%) | 0.19 ± 0.01 | 0.32 ± 0.04 | 0.45 ± 0.03 |
| Threonine (%) | 0.18 ± 0.01 | 0.38 ± 0.01 | 0.38 ± 0.01 |
| Tyrosine (%) | 0.14 ± 0.02 | 0.39 ± 0.03 | 0.39 ± 0.02 |
| Valine (%) | 0.35 ± 0.02 | 0.56 ± 0.02 | 0.53 ± 0.01 |

[1]Timothy Balance Cube™ (Ontario Dehy Inc., Goderich, ON, Canada).
[2] Mixed for this experiment by Buckeye Nutrition (Dalton, OH). For ingredient composition see Table 5.2.
[3]Diets were top-dressed with 0.43 mL/kg·d canola oil to increase the DE of the diets.

**Table 5.2: Composition of high and low lysine concentrates[1].**

| Ingredient | Amount, % | |
|---|---|---|
| | Low Lysine Concentrate | High Lysine Concentrate |
| Wheat | 46.558 | 46.551 |
| Soy hulls | 16.251 | 16.264 |
| Oatmill byproduct | 14.109 | 14.105 |
| Corn gluten | 7.221 | 7.22 |
| Calcium carbonate | 4.95 | 4.949 |
| Soybean oil | 4.967 | 4.966 |
| Cane molasses | 2.458 | 2.456 |
| Corn distiller's dry grain | 1.264 | 1.262 |
| DL-Methionine | 0.833 | 0.833 |
| Glycine | 0.669 | - |
| Lysine | - | 0.846 |
| Rolled oats | 0.171 | - |
| Threonine | 0.168 | 0.168 |
| Gro 'N Win®[2] | 0.13 | 0.13 |
| Selenium premix | 0.093 | 0.093 |
| Zinc sulfate | 0.029 | 0.03 |
| Magnesium oxide | 0.027 | 0.027 |
| 21% Monoc | 0.028 | 0.028 |
| Dyna K[3] | 0.028 | 0.028 |
| Dynamate[4] | 0.028 | 0.028 |
| Copper II Sulfate | 0.018 | 0.018 |

[1]Information provided by Buckeye Nutrition (Dalton, OH)

[2]Gro 'N Win® is a ration balancer pellet produced by Buckeye Nutrition (Dalton, OH)

[3]Dyna K is a potassium chloride product made by The Mosaic Company (Plymouth MN, USA)

[4]Dynamate is a potassium and magnesium sulfate product made by The Mosaic Company (Plymouth MN, USA)

***Study design and procedures***

Horses were studied in two groups in order to study the horses all the horses at similar ages. Diets were fed in a random order within the groups with the stipulation that no two horses were on the same treatment diet at the same time. Following the initial adaptation, treatment diets were fed for 7 d each. This length of adaptation was chosen

because less than 2 d of adaptation has been found to be sufficient for humans (Elango et al., 2009) and swine (Moehn et al., 2004b), but adaptation time's effect on IAAO analyses has not yet been ascertained in horses. On d 6 of treatment, venipuncture blood samples (10 mL) were taken immediately before and 90 min post the morning concentrate meal into an evacuated glass tube containing a heparin anti-coagulant to measure plasma glucose, insulin, urea nitrogen, and amino acid concentrations. Horses were then sedated with 100 mg/mL AnaSed® (Lloyd Laboratories, Shenandoah, IN) administered intravenously at a dose of 0.5 mg/kg. Muscle biopsies (~500 mg) were taken after sedation took effect (~100 min post-feeding) from the *gluteus medius* at a depth of ~6 cm with a Bergstrom needle (Lindholm and Piehl, 1974). A portion of the muscle tissue was immediately processed for western blots and the remainder was flash frozen in liquid nitrogen. While the horses were still sedated, both jugular veins were catheterized, one side for isotope infusion and the other for blood sampling (Urschel et al., 2012) in preparation for the following day's procedures.

On d 7 of treatment, whole body phenylalanine kinetics in response to lysine intake were determined. Whole-body $CO_2$ production was measured using a 2h primed (7.1 µmol/kg), constant (5.9 µmol/kg/h) infusion of [$^{13}$C]sodium bicarbonate (Coggan et al., 1993). Immediately following the bicarbonate infusion was a 6h primed (12.6 µmol/kg), constant infusion of [1-$^{13}$C]phenylalanine (9.0 µmol/kg/h), which measured phenylalanine oxidation to $CO_2$ and phenylalanine flux. Prime to constant rate isotope ratios and infusion lengths have been validated in mature horses (Urschel et al., 2012). Cordless infusion pumps (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc., Loveland, CO) were fastened to surcingles that were worn by the yearlings. This

approach allowed the yearlings freedom of movement within their stalls. Starting 90 min prior to the start of isotope infusion procedures 1/24 of the morning meal was given every half hour during infusion for the reduction of fluctuations in phenylalanine oxidation (Moehn et al., 2004a; Mohn et al., 2003). Collection of breath samples occurred every 30 min beginning 30 min prior to the start of the [$^{13}$C]sodium bicarbonate infusion until the completion of the [1-$^{13}$C]phenylalanine infusion into gas impermeable bags using a modified equine Aeromask (Urschel et al., 2009). Blood samples (10 mL) were collected from a jugular vein catheter every 30 min beginning 30 min prior to the start of the [1-$^{13}$C]phenylalanine infusion until the completion of infusion procedures.

Upon completion of the isotope infusion procedures, catheters were removed and the yearlings were allocated to their next treatment beginning with their afternoon concentrate meal for that day. Yearlings were returned to University of Kentucky Department of Animal and Food Sciences' Maine Chance Farm research herd at the end of the study.

***Sample analyses***

*Blood sample processing*

Heparinized vacutainers (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ) were used for blood collection. Samples were inverted and then promptly centrifuged at 1,500 × g for 10 min at 4ºC. Plasma was taken off and frozen at -20ºC until analyses.

*Amino acid analyses*

Plasma free, muscle free, and total feed amino acid concentrations were measured using reverse phase HPLC (3.9 × 300 mm PICO-TAG reverse phase column; Waters,

Milford, MA) of phenylisothiocyanate derivatives as previously described (Urschel et al., 2011). Muscle samples were freeze dried (Miller-Graber et al., 1990) and homogenized in 6 μL/μg of 0.4 mM norleucine made in 0.1 N HCl prior to deproteination. Both muscle homogenates and plasma samples were deproteinated by centrifugation at 14,000 × g for 30 min at 4°C using 10-kd cutoff centrifugation filters (Urschel et al., 2011). Six samples of each feedstuff collected throughout the study were used for amino acid analyses (AOAC International, 2005), with each sample analyzed in duplicate.

*Plasma metabolite concentrations*

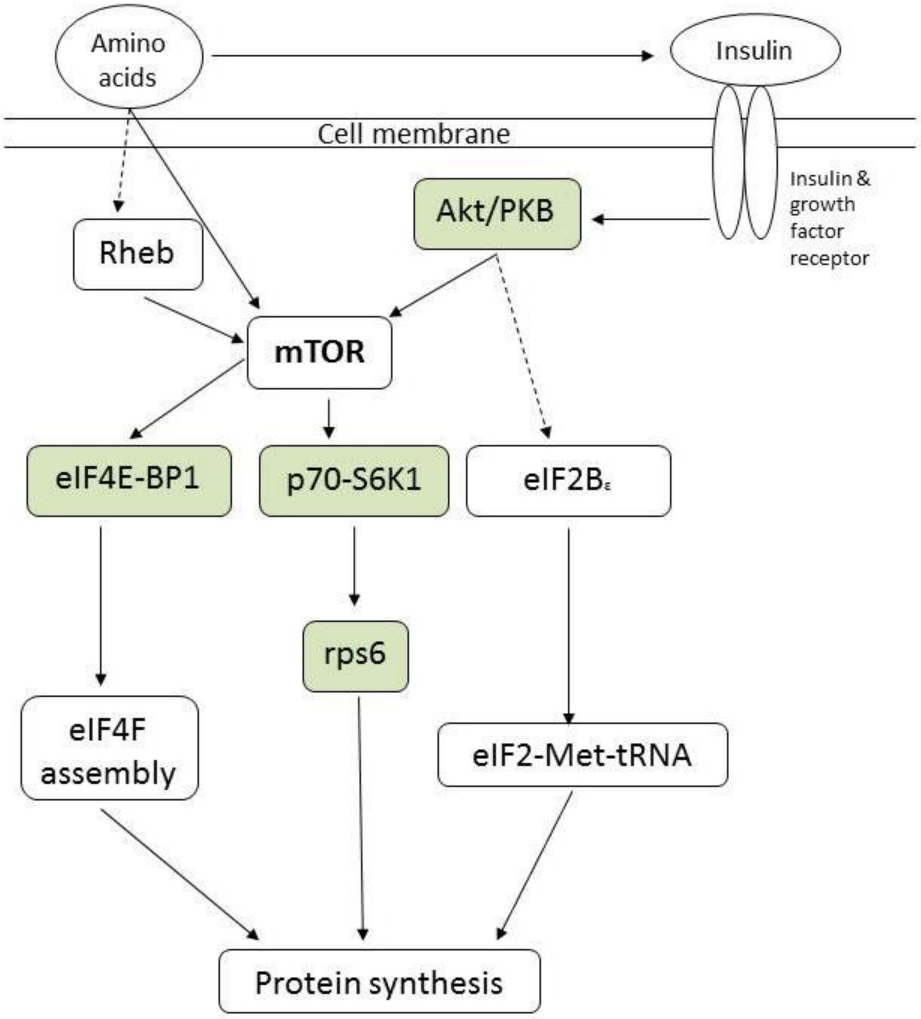
A YSI 2300 STAT Plus Glucose and Lactate Analyzer (YSI Inc., Life Sciences, Yellow Springs, OH) was used to enzymatically determine plasma glucose concentrations. The intra-assay variation was 1.5% with all samples being run in a single batch. As previously described (Urschel et al., 2007), a colorimetric spectrophotometric assay was used to determine plasma urea nitrogen, with an intra-assay variation of 3.5% and an inter-assay variation of < 10%. A Coat-A-Count RIA kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL) was used to determine plasma insulin concentrations that was previously validated for use in horses (Reimers et al., 1982; Tinworth et al., 2011).

*Western blotting*

Homogenized muscle tissue was used for western blotting of the following mTOR pathway proteins: Protein kinase B (Akt), eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1), riboprotein s6 (rps6), and S6 kinase (p70). The relationship of these translation initiation factors to mTOR signaling is depicted in Figure 5.2. Muscle tissue was homogenized and prepared for western blotting as previously described (Urschel et al., 2011; Wagner and Urschel, 2012). Protein concentrations of the samples were

determined via Bradford assay (Thermo Scientific, Rockford, IL) and 1X Laemmli buffer was used to obtain a protein concentration of 1 µg/µL for each sample. The diluted samples were heated at 95°C in a heat block for 5min and then kept in a chilled microcentrifuge rack before 20 µg of protein was loaded per sample for gel electrophoresis. Information regarding the 1° antibodies used is provided in Appendix B. Abundance and phosphorylation of the mTOR signaling factors was conducted as previously described (Urschel et al., 2011; Wagner and Urschel, 2012) with the following modifications. Radiograph films were imaged using the charge coupled device camera on a VersaDoc™ imager (Bio-Rad Laboratories, Inc., Hercules, CA ) (Gassmann et al., 2009) and then band density was quantified using ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/nih-image/), a public domain software (Goodman et al., 2011).

**Figure 5.2: Simplified mTOR schematic showing the relationship of the translation initiation factors studied to mTOR signaling and protein synthesis.**




This figure is based on several review papers (Hay and Sonenberg, 2004; Proud, 2006; Yang et al., 2008). The solid arrows indicate activation favorable for protein synthesis, while the dotted arrows indicate proposed activation.

*Isotope enrichment*

Immediately after sampling, the amount of $^{13}CO_2$ relative to $^{12}CO_2$ in breath samples collected throughout the isotope infusions was determined using an infrared isotope analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany).

Phenylalanine isotopic enrichment, which is the relative concentrations of [1-$^{13}$C]phenylalanine to unlabeled phenylalanine, in the plasma samples was measured by Metabolic Solutions, Inc. (Nashua, NH) using a previously described method (Matthews et al., 1990).

### *Calculations*

#### *Whole-body phenylalanine kinetics*

The average enrichment at plateau was determined for both blood and breath sample enrichments, where a plateau was defined as at least 3 points with a slope that was not different from 0 ($P > 0.05$) using linear regression analyses (GraphPad Prism 4 Software, GraphPad Prism Inc., San Diego, CA).

Whole-body phenylalanine flux, oxidation, non-oxidative disposal, and phenylalanine balance were calculated as previously described in Chapter 4. Non-oxidative phenylalanine disposal was used as an indicator of whole-body protein synthesis. Conversion of phenylalanine to tyrosine was considered negligible and, if any occurred, the intake of phenylalanine and tyrosine was the same across treatments.

#### *Western blot data*

The ratio of phosphorylated to total protein was measured for each initiation factor. The average for the treatment with a lysine intake of 76 mg/kg/d was set to an arbitrary unit of one for each protein.

### *Statistical Analysis*

Data were analyzed using the MIXED procedure in SAS (version 9.1.3, SAS Institute Inc., Cary, NC), unless otherwise noted. Differences were considered significant

at $P < 0.05$ and statistical trends were considered when $0.05 < P < 0.10$. One-way ANOVA with treatment as the fixed effect and horse as the random effect was used to evaluate phenylalanine kinetics parameters and Western blot data. A repeated measures analysis, with treatment, time, and time × treatment interaction as fixed effects and nested within treatment as the random effect was used to analyze plasma amino acid, urea, glucose, and insulin concentration data. Means were separated using orthogonal polynomial contrasts with coefficients for unequally spaced treatments that were generated using SAS proc IML.

A broken line analysis as described by Robbins et al. (2005) using the NLIN procedure in SAS was performed using lysine intake and phenylalanine oxidation. Starting estimates of breakpoint and line slope were determined using a method previously described by Fadel (2004). This method of broken line analysis has been applied to IAAO data for the determination of amino acid requirements in swine (Levesque et al., 2011).

## Results

All horses completed all study treatments and remained healthy throughout the study. Horses were an average of 400 ± 5 d old and had a BW of 358 ± 5 kg on isotope infusion study days.

### *Amino acid intakes*

Amino acid intakes exceeded NRC requirements (NRC, 2007) that were based on muscle and milk profiles determined by Bryden (1991) with the following exceptions for all diets:  histidine and threonine (Table 5.3). Lysine was also below NRC requirements (NRC, 2007) for three of the treatments, as dictated by the study design.

**Table 5.3: Amino acid intakes on the different dietary treatments and requirements based on the tissue composition of equine muscle and milk**

| | Dietary treatments | | | | | | Standard deviation | Requirement estimates[1] Gluteal Muscle | Mare's Milk |
|---|---|---|---|---|---|---|---|---|---|
| | mg/kg/d | | | | | | | | |
| Lysine | 76 | 90 | 104 | 118 | 127 | 136 | 7 | 113 | 113 |
| Alanine | 116 | 116 | 116 | 115 | 115 | 115 | 5 | - | - |
| Arginine | 116 | 115 | 115 | 115 | 114 | 114 | 8 | 84 | 89 |
| Aspartate + Asparagine | 138 | 136 | 134 | 132 | 131 | 130 | 12 | - | - |
| Glutamate + Glutamine | 365 | 367 | 368 | 370 | 371 | 373 | 19 | - | - |
| Glycine | 152 | 135 | 118 | 101 | 89 | 78 | 4 | - | - |
| Histidine | 25 | 25 | 25 | 25 | 25 | 25 | 3 | 73 | 41 |
| Isoleucine | 95 | 94 | 93 | 92 | 92 | 91 | 6 | 63 | 72 |
| Leucine | 195 | 196 | 196 | 196 | 196 | 196 | 8 | 123 | 146 |
| Methionine | 69 | 72 | 74 | 77 | 79 | 81 | 7 | 33 | 34 |
| Phenylalanine | 110 | 110 | 110 | 110 | 110 | 110 | 4 | 68 | 63 |
| Proline | 158 | 158 | 158 | 158 | 159 | 159 | 6 | - | - |
| Serine | 61 | 65 | 68 | 71 | 74 | 76 | 5 | - | - |
| Threonine | 66 | 66 | 66 | 66 | 66 | 66 | 3 | 70 | 60 |
| Tyrosine | 62 | 62 | 62 | 63 | 63 | 63 | 6 | - | - |
| Valine | 110 | 109 | 109 | 108 | 108 | 107 | 5 | 71 | 87 |

[1]Estimates based on Bryden (1991).

***Plasma amino acid concentrations***

Plasma amino acid concentrations increased 90 minutes post feeding ($P<0.001$) compared with baseline values. Plasma lysine and glycine concentrations increased linearly ($P<0.05$) within the 90 min time point with increasing lysine and glycine intakes, respectively (Table 5.5).

**Table 5.4: Plasma metabolites before feeding of the morning concentrate portion of diets providing varying lysine levels.**

| | Dietary lysine content (mg/kg·d) | | | | | | Pooled | p-values | |
|---|---|---|---|---|---|---|---|---|---|
| | **76** | **90** | **104** | **118** | **127** | **136** | **SE** | **Treatment** | **Treatment x time** |
| Urea (mmol/L) | 4.4 | 4.6 | 4.3 | 4.1 | 3.5 | 4.8 | 1.5 | 0.72 | 0.98 |
| Glucose (mmol/L) | 4.93 | 4.72 | 5.15 | 4.2 | 5.27 | 4.94 | 0.43 | 0.68 | 0.44 |
| Insulin (µIU/mL) | 1.89 | 2.18 | 1.52 | 1.73 | 2.25 | 2.52 | 1.24 | 0.82 | 0.84 |
| **Amino acids (µmol/L)** | | | | | | | | | |
| Alanine | 190 | 192 | 194 | 182 | 189 | 181 | 13 | 0.44 | 0.52 |
| Arginine | 41 | 59 | 48 | 51 | 35 | 48 | 13 | 0.17 | 0.56 |
| Asparagine | 19 | 19 | 20 | 17 | 18 | 19 | 4 | 0.67 | 0.77 |
| Aspartate | 40 | 37 | 36 | 36 | 37 | 34 | 3 | 0.35 | 0.8 |
| Citrulline | 104 | 106 | 107 | 105 | 100 | 107 | 11 | 0.84 | 0.62 |
| Glutamate | 345 | 300 | 301 | 316 | 356 | 339 | 57 | 0.27 | 0.89 |
| Glutamine | 223 | 275 | 264 | 230 | 227 | 219 | 42 | 0.42 | 0.18 |
| Glycine [a] | 825 | 697 | 717 | 623 | 670 | 690 | 78 | <0.001 | 0.03 |
| Histidine | 70 | 73 | 72 | 68 | 66 | 74 | 7 | 0.83 | 0.7 |
| Isoleucine | 57 | 59 | 56 | 57 | 53 | 56 | 8 | 0.46 | 0.98 |
| Leucine | 105 | 107 | 105 | 102 | 98 | 100 | 16 | 0.4 | 0.79 |
| Lysine | 95 | 106 | 102 | 110 | 105 | 118 | 19 | <0.001 | <0.001 |
| Methionine | 80 | 78 | 76 | 78 | 81 | 71 | 16 | 0.2 | 0.8 |
| Ornithine | 101 | 89 | 92 | 87 | 95 | 91 | 21 | 0.34 | 0.87 |
| Phenylalanine | 62 | 63 | 63 | 61 | 61 | 64 | 6 | 0.59 | 0.86 |
| Proline | 121 | 117 | 116 | 110 | 113 | 113 | 16 | 0.97 | 0.88 |
| Serine | 271 | 265 | 268 | 241 | 247 | 251 | 28 | 0.32 | 0.91 |
| Taurine | 51 | 44 | 49 | 51 | 50 | 51 | 5 | 0.75 | 0.76 |
| Threonine | 137 | 132 | 133 | 119 | 123 | 139 | 14 | 0.32 | 0.31 |
| Tryptophan | 7 | 6 | 6 | 6 | 6 | 6 | 1 | 0.73 | 0.55 |
| Tyrosine | 91 | 95 | 93 | 97 | 95 | 97 | 6 | 0.67 | 0.97 |
| Valine | 152 | 146 | 140 | 141 | 131 | 140 | 18 | 0.25 | 0.97 |
| **Total amino acids** | **3215** | **3093** | **3086** | **2913** | **2980** | **3034** | **255** | **0.13** | **0.9** |

[a] Quadratic within baseline time point (P<0.05)

**Table 5.5: Plasma metabolites 90 min after feeding of the morning concentrate portion of diets providing varying lysine levels.**

| | Dietary lysine content (mg/kg·d) | | | | | | Pooled | p-values | |
|---|---|---|---|---|---|---|---|---|---|
| | **76** | **90** | **104** | **118** | **127** | **136** | **SE** | **Treatment** | **Treatment x time** |
| Urea (mmol/L) | 4.4 | 4.6 | 4.3 | 4.1 | 3.5 | 4.8 | 1.5 | 0.72 | 0.98 |
| Glucose (mmol/L) | 4.93 | 4.72 | 5.15 | 4.2 | 5.27 | 4.94 | 0.43 | 0.68 | 0.44 |
| Insulin (µIU/mL) | 1.89 | 2.18 | 1.52 | 1.73 | 2.25 | 2.52 | 1.24 | 0.82 | 0.84 |
| **Amino acids (µmol/L)** | | | | | | | | | |
| Alanine | 190 | 192 | 194 | 182 | 189 | 181 | 13 | 0.44 | 0.52 |
| Arginine | 41 | 59 | 48 | 51 | 35 | 48 | 13 | 0.17 | 0.56 |
| Asparagine | 19 | 19 | 20 | 17 | 18 | 19 | 4 | 0.67 | 0.77 |
| Aspartate | 40 | 37 | 36 | 36 | 37 | 34 | 3 | 0.35 | 0.8 |
| Citrulline | 104 | 106 | 107 | 105 | 100 | 107 | 11 | 0.84 | 0.62 |
| Glutamate | 345 | 300 | 301 | 316 | 356 | 339 | 57 | 0.27 | 0.89 |
| Glutamine | 223 | 275 | 264 | 230 | 227 | 219 | 42 | 0.42 | 0.18 |
| Glycine [a] | 825 | 697 | 717 | 623 | 670 | 690 | 78 | <0.001 | 0.03 |
| Histidine | 70 | 73 | 72 | 68 | 66 | 74 | 7 | 0.83 | 0.7 |
| Isoleucine | 57 | 59 | 56 | 57 | 53 | 56 | 8 | 0.46 | 0.98 |
| Leucine | 105 | 107 | 105 | 102 | 98 | 100 | 16 | 0.4 | 0.79 |
| Lysine | 95 | 106 | 102 | 110 | 105 | 118 | 19 | <0.001 | <0.001 |
| Methionine | 80 | 78 | 76 | 78 | 81 | 71 | 16 | 0.2 | 0.8 |
| Ornithine | 101 | 89 | 92 | 87 | 95 | 91 | 21 | 0.34 | 0.87 |
| Phenylalanine | 62 | 63 | 63 | 61 | 61 | 64 | 6 | 0.59 | 0.86 |
| Proline | 121 | 117 | 116 | 110 | 113 | 113 | 16 | 0.97 | 0.88 |
| Serine | 271 | 265 | 268 | 241 | 247 | 251 | 28 | 0.32 | 0.91 |
| Taurine | 51 | 44 | 49 | 51 | 50 | 51 | 5 | 0.75 | 0.76 |
| Threonine | 137 | 132 | 133 | 119 | 123 | 139 | 14 | 0.32 | 0.31 |
| Tryptophan | 7 | 6 | 6 | 6 | 6 | 6 | 1 | 0.73 | 0.55 |
| Tyrosine | 91 | 95 | 93 | 97 | 95 | 97 | 6 | 0.67 | 0.97 |
| Valine | 152 | 146 | 140 | 141 | 131 | 140 | 18 | 0.25 | 0.97 |
| **Total amino acids** | **3215** | **3093** | **3086** | **2913** | **2980** | **3034** | **255** | **0.13** | **0.9** |

[a] Linear within 90 min time point (P<0.05)

### *Plasma metabolite concentrations*

Plasma urea nitrogen, glucose, and insulin concentrations (Table 5.4 & Table 5.5) were greater post-feeding when compared to baseline values ($P < 0.01$), but were not affected by treatment. The interactions between time and treatment were also not significant ($P > 0.05$), as were linear, quadratic, or cubic effects ($P > 0.05$).

### *Muscle free amino acid concentrations*

*Gluteus medius* muscle free amino acid concentrations (Table 5.6) did not respond to changing lysine intakes in the same manner as plasma amino acid concentrations (Table 5.4 & Table 5.5). Free asparagine, aspartate, arginine, glutamine, lysine, taurine, and tryptophan concentrations responded quadratically to lysine intake ($P <0.05$). Free tyrosine concentrations responded cubically to lysine intake ($P = 0.04$).

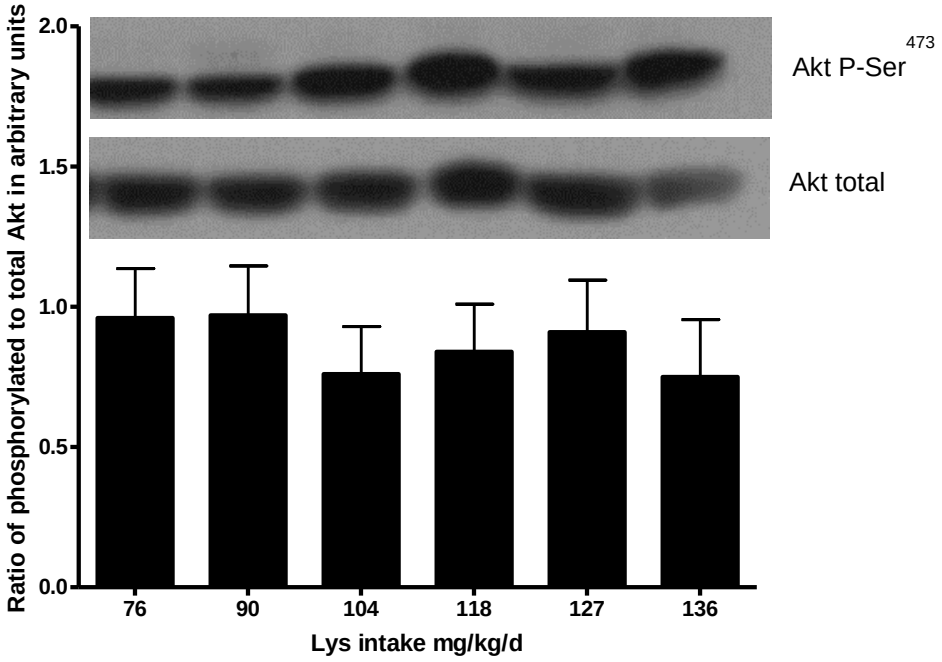
**Table 5.6: Free amino acids µmol/g of freeze dried *gluteus medius* muscle from yearling horses fed varying levels of lysine ~100 min after a morning concentrate meal.**

| | Dietary lysine content (mg/kg/d) | | | | | | Pooled SE | P-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 76 | 90 | 104 | 118 | 127 | 136 | | Treatment | Linear | Quadratic | Cubic |
| 3-methyl histidine | 0.86 | 0.66 | 0.81 | 1.03 | 0.99 | 1.04 | 0.16 | 0.29 | 0.08 | 0.44 | 0.20 |
| Alanine | 3.06 | 2.04 | 2.85 | 2.72 | 3.13 | 3.73 | 0.63 | 0.50 | 0.26 | 0.16 | 0.71 |
| Asparagine | 0.49 | 0.31 | 0.35 | 0.40 | 0.37 | 0.57 | 0.10 | 0.37 | 0.54 | 0.05 | 0.93 |
| Aspartate | 0.83 | 0.57 | 0.53 | 0.66 | 0.64 | 0.90 | 0.13 | 0.31 | 0.66 | 0.03 | 0.84 |
| Arginine | 0.26 | 0.25 | 0.25 | 0.25 | 0.25 | 0.27 | 0.27 | 0.06 | 0.82 | 0.01 | 0.53 |
| Carnosine | 86.42 | 71.64 | 85.90 | 94.23 | 93.47 | 91.31 | 13.68 | 0.72 | 0.34 | 0.76 | 0.27 |
| Citrulline | 0.40 | 0.27 | 0.38 | 0.36 | 0.36 | 0.40 | 0.07 | 0.63 | 0.71 | 0.36 | 0.45 |
| Cysteine | 8.06 | 6.25 | 6.31 | 7.05 | 7.27 | 7.00 | 1.86 | 0.96 | 0.90 | 0.50 | 0.54 |
| Glutamate | 4.75 | 3.09 | 4.19 | 4.10 | 4.46 | 4.57 | 0.83 | 0.67 | 0.69 | 0.30 | 0.34 |
| Glutamine | 5.61 | 3.42 | 4.42 | 4.32 | 4.54 | 6.60 | 1.14 | 0.33 | 0.46 | 0.04 | 0.96 |
| Glycine | 4.42 | 3.81 | 3.54 | 3.30 | 4.43 | 3.37 | 0.79 | 0.74 | 0.54 | 0.55 | 0.53 |
| Histidine | 0.40 | 0.23 | 0.33 | 0.27 | 0.31 | 0.31 | 0.08 | 0.54 | 0.60 | 0.29 | 0.39 |
| Isoleucine | 0.23 | 0.17 | 0.19 | 0.24 | 0.23 | 0.22 | 0.04 | 0.30 | 0.49 | 0.32 | 0.06 |
| Leucine | 0.52 | 0.38 | 0.44 | 0.50 | 0.51 | 0.51 | 0.08 | 0.51 | 0.47 | 0.33 | 0.17 |
| Lysine | 0.50 | 0.37 | 0.20 | 0.38 | 0.54 | 0.78 | 0.13 | 0.12 | 0.11 | 0.01 | 0.78 |
| Methionine | 0.54 | 0.45 | 0.46 | 0.56 | 0.51 | 0.55 | 0.07 | 0.80 | 0.63 | 0.44 | 0.42 |
| Ornithine | 1.02 | 0.39 | 1.01 | 0.77 | 0.64 | 0.79 | 0.39 | 0.48 | 0.79 | 0.67 | 0.42 |
| Phenylalanine | 0.10 | 0.09 | 0.11 | 0.09 | 0.10 | 0.09 | 0.02 | 0.98 | 0.79 | 0.97 | 0.73 |
| Proline | 0.52 | 0.35 | 0.44 | 0.44 | 0.45 | 0.53 | 0.09 | 0.74 | 0.75 | 0.24 | 0.60 |
| Serine | 1.39 | 0.86 | 1.04 | 1.13 | 1.10 | 1.29 | 0.18 | 0.33 | 0.93 | 0.07 | 0.29 |
| Taurine | 3.24 | 3.14 | 3.43 | 4.45 | 3.94 | 9.21 | 1.38 | 0.01 | 0.01 | 0.01 | 0.11 |
| Threonine | 1.25 | 0.86 | 0.86 | 1.05 | 1.54 | 1.44 | 0.45 | 0.61 | 0.38 | 0.19 | 0.53 |
| Tryptophan | 0.19 | 0.12 | 0.12 | 0.13 | 0.13 | 0.19 | 0.03 | 0.24 | 0.98 | 0.02 | 1.00 |
| Tyrosine | 0.41 | 0.29 | 0.39 | 0.42 | 0.45 | 0.39 | 0.06 | 0.21 | 0.34 | 0.65 | 0.04 |
| Valine | 0.51 | 0.38 | 0.42 | 0.48 | 0.46 | 0.46 | 0.07 | 0.61 | 0.94 | 0.35 | 0.18 |
| Total amino acids | 140.43 | 112.11 | 131.27 | 141.15 | 143.14 | 143.29 | 17.61 | 0.71 | 0.43 | 0.44 | 0.27 |

***mTOR pathway signaling***

Lysine intake did not affect activation of Akt (P > 0.1) (Figure 5.3) in the muscle tissue of the *gluteus medius*. However, activation of eukaryotic initiation factor 4E-BP1 increased linearly with increasing lysine intake (P = 0.01) (Figure 5.5). The opposite trend was observed for p70 with increasing lysine intake (P = 0.06)(Figure 5.6). Riboprotein S6 (rpS6) responded cubically to lysine intake (P = 0.01)(Figure 5.4).

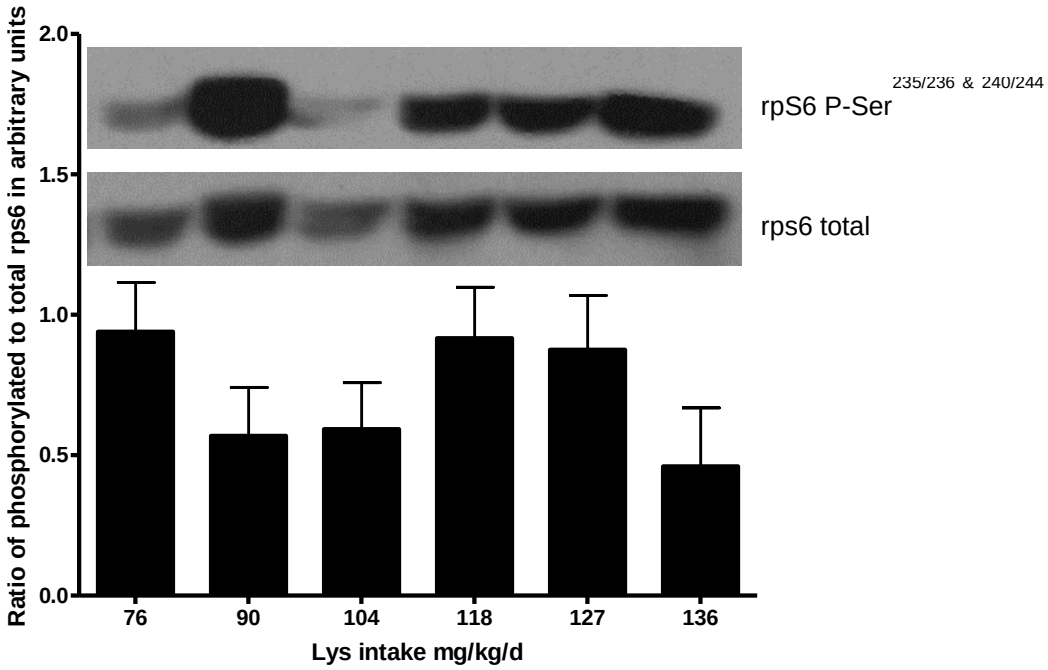
**Figure 5.3: Phosphorylation of post-feeding gluteal muscle Akt.**




| | P-values | | | |
|---|---|---|---|---|
| **Protein** | **Treatment** | **Linear** | **Quadratic** | **Cubic** |
| Akt | 0.77 | 0.38 | 0.81 | 0.78 |

The phosphorylated forms of the translation initiation factors were corrected by the respective abundance of the total form, with the average of the values of this ratio set to 1.0 arbitrary unit when the lysine intake was 75 mg/kg BW/d. Values are least square means ± SE. Outliers as determined by proc univariate were removed. Representative images of immunoblots are shown above the graph.

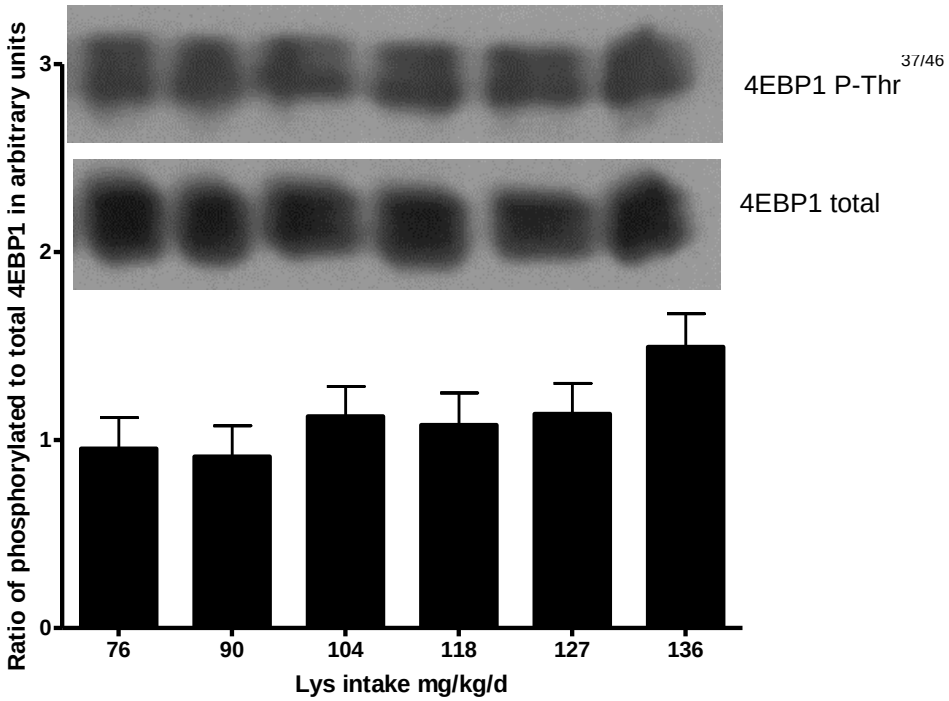
**Figure 5.4: Phosphorylation of post-feeding gluteal muscle rpS6.**




| | P-values | | | |
|---|---|---|---|---|
| **Protein** | **Treatment** | **Linear** | **Quadratic** | **Cubic** |
| rpS6 | 0.11 | 0.49 | 0.95 | 0.01 |

The phosphorylated forms of the translation initiation factors were corrected by the respective abundance of the total form, with the average of the values of this ratio set to 1.0 arbitrary unit when the lysine intake was 75 mg/kg BW/d. Values are least square means ± SE. Outliers as determined by proc univariate were removed. Representative images of immunoblots are shown above the graph.

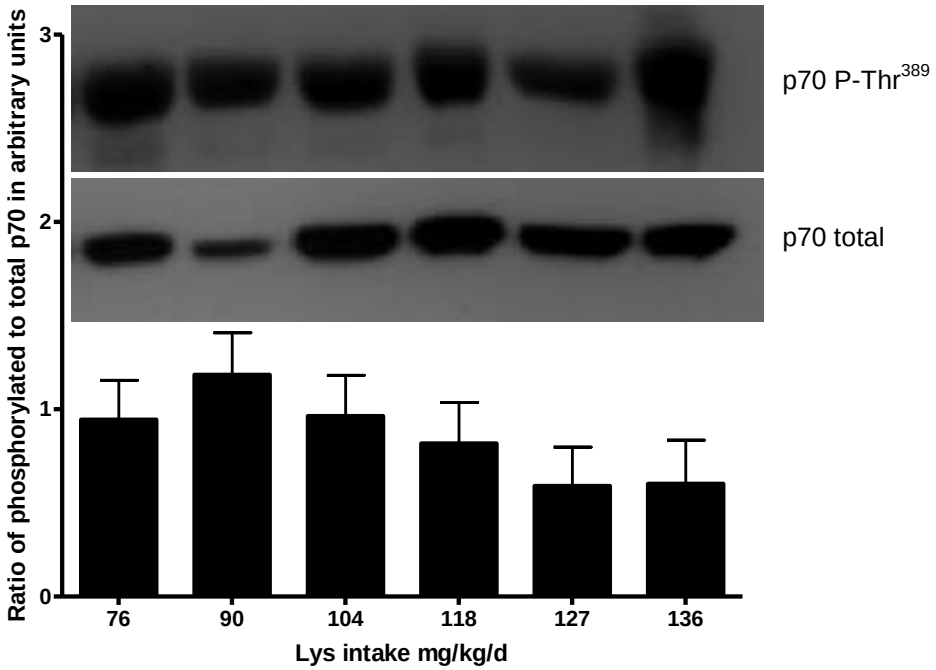
**Figure 5.5: Phosphorylation of post-feeding gluteal muscle 4E-BP1.**




| | P-values | | | |
|---|---|---|---|---|
| **Protein** | **Treatment** | **Linear** | **Quadratic** | **Cubic** |
| 4E-BP1 | 0.07 | 0.01 | 0.17 | 0.38 |

The phosphorylated forms of the translation initiation factors were corrected by the respective abundance of the total form, with the average of the values of this ratio set to 1.0 arbitrary unit when the lysine intake was 75 mg/kg BW/d. Values are least square means ± SE. Outliers as determined by proc univariate were removed. Representative images of immunoblots are shown above the graph.

**Figure 5.6: Phosphorylation of post-feeding gluteal muscle p70.**




| | P-values | | | |
|---|---|---|---|---|
| **Protein** | **Treatment** | **Linear** | **Quadratic** | **Cubic** |
| p70 | 0.31 | 0.06 | 0.30 | 0.35 |

The phosphorylated forms of the translation initiation factors were corrected by the respective abundance of the total form, with the average of the values of this ratio set to 1.0 arbitrary unit when the lysine intake was 75 mg/kg BW/d. Values are least square means ± SE. Outliers as determined by proc univariate were removed. Representative images of immunoblots are shown above the graph.

***Phenylalanine kinetics***

Phenylalanine and tyrosine intakes were equivalent for all treatments. Phenylalanine flux, release from protein breakdown, oxidation, non-oxidative disposal, and balance did not differ between treatments (P>0.10) (Table 5.7). Carbon dioxide production responded cubically to treatment (P = 0.03). A broken line analysis of lysine

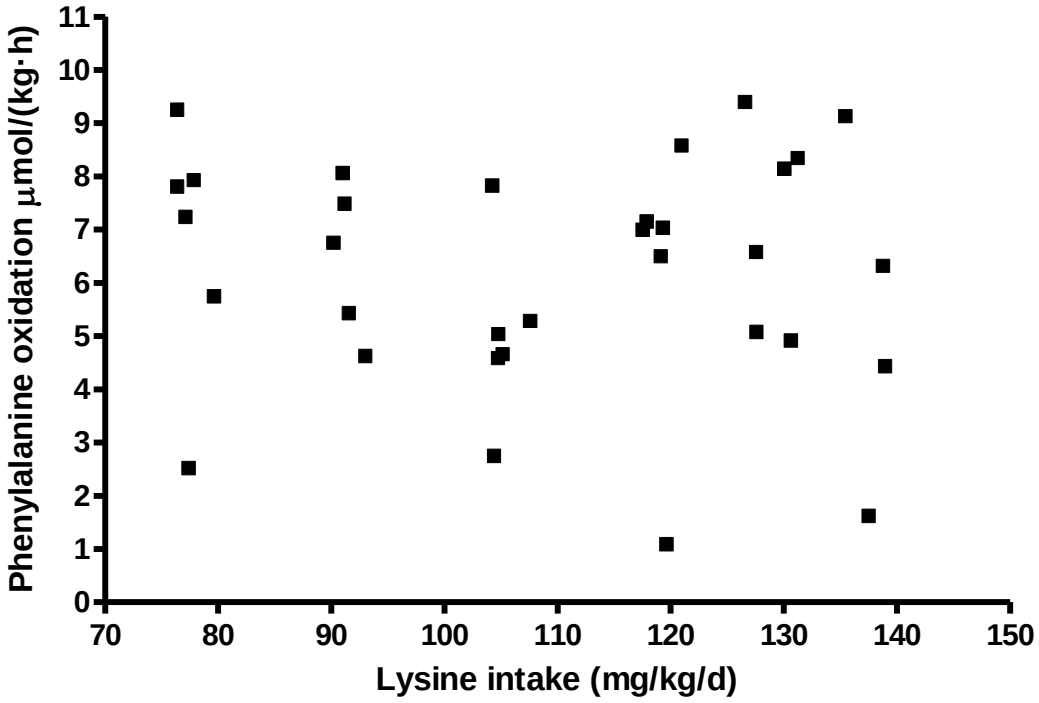
intake and phenylalanine oxidation failed to yield a breakpoint from which to determine a lysine requirement for yearling Thoroughbred colts (Figure 5.7).

**Table 5.7: Whole-body phenylalanine kinetics in yearling Thoroughbred colts that were receiving graded levels of dietary lysine[1]**

| | Graded lysine intakes (mg/kg/d) | | | | | | Pooled SE | P-values | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 76 | 90 | 104 | 118 | 127 | 136 | | Treatment | Linear | Quadratic | Cubic |
| ***Phenylalanine flux [µmol/(kg·h)]*** | | | | | | | | | | | |
| | 51 | 49 | 48 | 47 | 53 | 48 | 2 | 0.10 | 0.55 | 0.28 | 0.24 |
| ***Carbon dioxide production [µmol/(kg·h)]*** | | | | | | | | | | | |
| | 20870 | 18250 | 19017 | 19475 | 19698 | 18614 | 816 | 0.24 | 0.34 | 0.30 | 0.03 |
| ***Phenylalanine entering the free phenylalanine pool [µmol/(kg·h)][2]*** | | | | | | | | | | | |
| Phenylalanine from dietary intake | 15.7 | 15.5 | 16.1 | 16.2 | 16.0 | 15.9 | 0.2 | 0.17 | 0.11 | 0.28 | 0.16 |
| Phenylalanine from protein breakdown | 35.8 | 33.4 | 32.1 | 30.5 | 37.0 | 31.6 | 1.9 | 0.09 | 0.43 | 0.25 | 0.32 |
| ***Phenylalanine leaving the free phenylalanine pool [µmol/(kg·h)][2]*** | | | | | | | | | | | |
| Phenylalanine oxidation | 7.2 | 7.3 | 5.0 | 6.2 | 6.5 | 5.3 | 0.9 | 0.39 | 0.20 | 0.60 | 0.70 |
| Non-oxidative phenylalanine disposal | 44.3 | 41.6 | 43.1 | 40.5 | 46.5 | 42.3 | 2.1 | 0.31 | 0.96 | 0.46 | 0.38 |
| ***Phenylalanine balance [µmol/(kg·h)][3]*** | | | | | | | | | | | |
| | 8.5 | 8.2 | 11.1 | 10.0 | 9.5 | 10.7 | 1.0 | 0.23 | 0.11 | 0.49 | 0.94 |

[1]Values are least squares means ± SE determined using a one-way analysis of variance
[2]Stochastic model of phenylalanine kinetics used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal; a splanchnic phenylalanine extraction rate of 50% was assumed when calculating the amount of dietary phenylalanine reaching general circulation
[3]Phenylalanine balance = non-oxidative phenylalanine disposal - phenylalanine release from protein breakdown

**Figure 5.7: Plotting lysine intake and phenylalanine oxidation fail to yield a breakpoint to indicate the lysine requirement for yearling Thoroughbred colts.**




Six weanling Thoroughbred colts were fed diets containing graded amounts of lysine. Phenylalanine oxidation was not affected by diet (P>0.10).

## Discussion

This study underscores the limitations in our current knowledge regarding equine amino acid requirements and the need for further investigation into amino acids other than lysine that were potentially limiting in our treatment diets.

### *Determination of a lysine requirement for yearling horses*

We hypothesized that a breakpoint would be seen in our phenylalanine kinetics data, such as is shown in Figure 5.1, denoting a lysine requirement for yearling horses. We were unable to determine a breakpoint using the phenylalanine oxidation data (Figure 5.7). Rates of non-oxidative phenylalanine disposal (Table 5.7) were not affected by

treatment, but were less than those measured in weanlings (Table 4.5 & Table 6.6) and only slightly greater than those measured in adult mares (Table 6.7). Phenylalanine balance was positive (Table 5.7), indicating that the yearlings were accreting protein. However, as this is the first time whole-body protein synthesis was measured in yearling horses using this method, there is no data to compare these values with to determine if protein accretion was submaximal for this age of horse. Maximal whole-body protein synthesis for yearlings would be expected to slightly higher than maximal for mature horses as the average daily gain of yearlings is relatively small (Pagan et al., 1996; Staniar et al., 2004a).

The plasma metabolite concentrations also do not indicate a requirement (Table 5.4 & Table 5.5). A previous study by Ohta et al. (2007) found as a broken line in plasma lysine concentrations when feeding graded amounts of lysine were fed, which is unlike the linear increase in plasma concentrations with increasing lysine intake that we found in this study (Table 5.5). Also, our plasma urea measurements also did not differ due to treatment as would be expected as discussed in Chapter 2 in the section titled "Plasma/serum urea concentrations". However, the timing of our blood sampling may not have been optimal to detect changes in plasma urea concentration due to treatment, as another study found significant differences in plasma urea concentrations due to amino acid supplementation in horses sampled 3-4 hrs after meal feeding (Graham-Thiers and Kronfeld, 2005a).

Our data could suggest that the requirement was not included within our ranges of intake, but this is unlikely given the suggested requirements of previous research reviewed in Table 2.7, or that our IAAO data was unable to yield a lysine requirement for

yearling horses receiving these diets (Table 5.1). The ingredient composition of the treatment diets were exactly the same with the exception of lysine and glycine between all treatment diets and therefore the digestibilities of the protein bound amino acids would also be the same between treatment groups (Table 5.2). An amino acid other than lysine that is equally limiting across treatments could explain why a requirement could not be determined.

Protein synthesis rates would not differ if all the diets were equally limiting in some amino acid other than lysine. Protein synthesis rates affect amino acid oxidation, plasma amino acid concentrations, and plasma urea concentrations as discussed in Chapter 2 in the section "Methods of determining amino acid requirements". As there is limited data regarding amino acid requirements in horses, judging diets to contain adequate amounts of particular amino acids other than the test amino acid is inherently difficult. However, both Bryden (1991) and Wickens et al. (2002) have used muscle tissue composition to put forward amino acid ratios relative to lysine to be used for amino acid requirement estimates (Table 2.5 & Table 5.3). Candidates for universally limiting amino acids in the treatment diets include threonine and histidine (Table 5.3). The plasma urea data may be evidence of a universally limiting amino acid across treatments, as these values were relatively high when compared to other data reported in the literature (Table 2.4).

Using ratios for muscle tissue amino acids and the lysine requirement estimate provided by the NRC (2007) the threonine requirement estimate could be 70 (Bryden, 1991) or 58 (Wickens et al., 2002) mg/kg/d for yearling horses. Using ratios from other researchers that reported total equine muscle amino acids the requirement would be ~60

mg/kg/d (Badiani et al., 1997; Lorenzo and Pateiro, 2013). By some of these estimates our rations may have been deficient in threonine, which would explain the lack of response to lysine intake. Threonine digestibility has been shown to vary greatly in swine with feedstuff (Myrie et al., 2008), so in addition to being provided a possibly marginal level in our diets, it may not have been very bioavailable. Further, these estimates of threonine requirements rely on the assumption that threonine incorporation into muscle protein is representative of whole-body threonine use for protein synthesis, and as threonine requirements have not been measured in horses of any age, it is unknown how well these estimates reflect the true threonine requirements. Threonine is heavily used by the gut in other species (Bertolo et al., 1998; Faure et al., 2005; Stoll et al., 1998; van der Schoor et al., 2007; Wang et al., 2010), as much as 91% is believed to be used by the portal drained viscera in neonatal piglets (Schaart et al., 2005), which is not reflected by amino acid requirement estimates from muscle tissue.

If threonine is limiting, as the intake data suggests, it would be expected that threonine plasma concentrations would be lower than what is typical for equine plasma. For our study, the average across treatments was 131 μmol/L threonine before feeding (Table 5.4). However, when compared to the values given in Table 2.3 and Table 4.4 it does not appear to be low, which contradicts the idea that threonine could have been limiting across all treatment diets. Given the lack of data in the literature specifically on yearling plasma amino acid concentrations and differences in analysis methods, it is difficult to determine whether this study's plasma threonine concentrations fell below the normal range.

Using the muscle amino acid ratios, histidine also appears to be a limiting amino acid in our diets, perhaps even more limiting than threonine. Histidine as a potentially limiting amino acid in equine diets has previously been discussed in Chapter 4. Use of muscle amino acid composition as a basis for a histidine requirement likely overestimates the requirement and histidine as the amount of histidine in equine muscle is inordinately high due to the high levels of carnosine compared to other species, as reviewed by Harris et al. (2012). However, the amount of histidine provided in the treatment diets is also below requirement estimates based on milk protein (Table 5.3) as suggested by Wickens et al. (2002). More research is needed to determine the adequacy and bioavailability of the amino acids provided in the treatment diets. Future research should endeavor to supply indispensable amino acids not being studied well above estimated requirements.

Energy limiting across dietary treatments besides amino acids could have caused similar results. The energy cost of protein synthesis has been measured in several species, including chicks (Aoyagi et al., 1988), sheep (Rattray et al., 1974), and caimans (Coulson et al., 1978). The energy cost of protein synthesis has also been reviewed by Reeds et al. (1982). These studies suggest that energy required for protein synthesis is only part of the energy required for maintenance. Diets in this study were top-dressed with canola oil to ensure diets provided adequate energy (Table 5.1). Total diets provided the digestible energy requirement (0.06 Mcal/kg BW/d) for these colts as listed by the NRC (2007). As digestible energy supply was adequate in this study, it is unlikely that these results were caused by an energy deficit.

Although we were not able to determine a lysine requirement in this study, use of the IAAO method for future research into amino acid requirements for horses should not

be discouraged. Phenylalanine kinetics were still able to distinguish between protein synthesis rates in weanling horses fed two different levels of protein in Chapter 4. This method has also been successfully used to determine lysine requirements in swine (Bertolo et al., 2005), humans (Elango et al., 2007), and chickens (Coleman et al., 2003). Future studies in horses should take care to supplement diets with indispensable amino acids other than the test amino acid, especially since little is known about amino acid bioavailability in horses.

### *Effects of lysine intake on muscle parameters*

Our investigations found comparable *gluteus medius* free amino acid concentrations to those previously reported from freeze dried muscle (Miller-Graber et al., 1990). Muscle tissue free amino acids have been studied in the horse in response to exercise (Graham-Thiers et al., 2012; van den Hoven et al., 2010), growth (Graham-Thiers et al., 2012; Manso Filho et al., 2009), and feeding (Urschel et al., 2011). However, to our knowledge this is the first time free tissue amino acids have been studied in response to supplementation of a single amino acid with isonitrogenous treatments in horses.

The underlying reasons for the differences in *gluteus medius* free amino acid concentrations between treatments are not entirely clear, and more research is certainly warranted. Amino acid transporters are regulated in the gut (Wang et al., 2012) and other tissues (Humphrey et al., 2006) by lysine in the diet. It is possible that transporters could be scavenging for lysine at the lower lysine intakes, resulting in greater levels of lysine in the muscle at the lower levels of lysine intake. However, more research would need to be conducted to confirm this mechanism.

Plasma glucose and insulin remained unaffected by lysine intake (Table 5.4 & Table 5.5) indicating that the glycine that replaced lysine for the lower lysine treatments had a similar effect on plasma glucose and insulin as lysine. Administered intravenously, lysine has been found to increase plasma insulin in humans (Floyd et al., 1966). The similar amounts of plasma insulin across treatments separate the effect of lysine as an insulin secretagogue from other mechanisms resulting in mTOR activation. An increase in insulin secretion with increasing lysine intake could have stimulated the mTOR pathway through insulin. Although muscle biopsies were taken when the horses were in a fed state, there was a low insulin response to meal feeding on these diets (Table 5.4 & Table 5.5), so mTOR activation, especially the portion that was driven by insulin, may not have risen above fasted levels. Other amino acids, such as leucine, are thought to act on mTOR in an insulin independent manner (Nobukuni et al., 2005).

Meal feeding is known to greatly upregulate the mTOR pathway in horses (Urschel et al., 2011; Wagner and Urschel, 2012). In this study the net effect of the increasing 4E-BP1 activation and decreasing p70 activation with increasing lysine intake on muscle tissue protein synthesis is unknown as tissue protein synthesis was not measured directly. It would be expected that the activation response pattern would be similar between translation initiation factors. In cell culture these two translation initiation factors respond similarly to insulin and presence of amino acids (Hara et al., 1998). They also have been found to respond similarly when human males ingest whey protein (Atherton et al., 2010). However, arginine supplementation in piglets has been found to increase 4E-BP1 activation and fractional protein synthesis rates as measured with a [$^{3}$H]phenylalanine flooding-dose technique without a concurrent increase in p70

activation (Yao et al., 2008). Lysine has not been shown to be an independent activator of mTOR in other species, unlike leucine (Dreyer et al., 2008; Norton and Layman, 2006) and arginine (Yao et al., 2008).

### *Conclusions*

Supplementation of a single amino acid can affect the metabolism of amino acids on the tissue level even if whole-body protein synthesis is not affected. Our inability to determine a lysine requirement utilizing the IAAO method with these test diets underscores the paucity of data concerning amino acid requirements and amino acid bioavailability data in the horse. Equine amino acid requirements are an area in need of further research.

# Chapter 6. Effects of threonine supplementation on whole-body protein synthesis and plasma metabolites in growing and mature horses

This project involving mature horses was supported by Agriculture and Food Research Initiative Competitive Grant no. 2012-67015-19448 from the USDA National Institute of Food and Agriculture. The research involving growing horses was supported by WALTHAM-Buckeye.

## Introduction

Little is known about limiting amino acids in common equine diets or the indispensable amino acid requirements of horses. In some equine diets lysine has been identified as the first limiting amino acid (Hintz et al., 1971; Ott et al., 1981). Threonine (Graham et al., 1994) and methionine (Graham-Thiers et al., 2012; Winsco et al., 2011) have also been suggested as potentially limiting amino acids in yearling and weanling horse diets, respectively. Supplementation of lysine and threonine together has been shown to increase subjective muscle mass scores and decrease plasma urea nitrogen in mature and aged horses (Graham-Thiers and Kronfeld, 2005a). It has been suggested that the requirements of amino acids for horses may follow the ratio of concentrations of amino acids in the body. Because muscle tissue makes up the majority of the horse (Gunn, 1987), it has been used to suggest amino acid requirement ratios (Bryden, 1991). However, using muscle tissue to estimate requirements does not take into account other uses of amino acids or the fact that muscle protein amino acid composition is not necessarily representative of the composition of other body proteins. For example, use of threonine by the gut in other species is considerable. Threonine use by the gut for mucin

production in other species is detailed in Chapter 2 in the section titled “Threonine incorporation into mucins”.

In Chapter 4, we found that non-oxidative disposal of an indicator amino acid, phenylalanine, was greater for the diet that had a greater crude protein (4.1 g/kg/d) and threonine (88 mg/kg/d) content, even though the diet with less crude protein (3.1 g/kg/d) and threonine (67 mg/kg/d) content met the NRC (2007) requirements for crude protein (3.1 g/kg/d) and lysine (133 mg/kg/d). Those results indicated that whole body protein synthesis was limited in the weanlings receiving the diet with lower protein and threonine content (Chapter 4). The current threonine NRC requirement estimate for the six month old weanling horse is 81 mg/kg/d and it is possible that threonine was the limiting amino acid in the diet with the NRC recommended level of crude protein. Even less research has been conducted on the threonine needs of adult horses than has been done on growing horses. One study has found that horses ≥ 20 years old had improved subjective muscle mass scores and decrease plasma urea nitrogen when supplemented with both lysine (20 g/d) and threonine (15 g/d) (Graham-Thiers and Kronfeld, 2005a). Using the amino acid ratios to lysine put forth by Bryden (1991) the threonine requirement for the average mature horse at maintenance would be 33 mg/kg/d.

As using muscle composition may underestimate threonine requirements and fiber may increase the need for threonine, these studies investigate the effect of dietary threonine supplementation to diets including grass forages on whole-body protein synthesis in horses. The objective of study 1 was to determine the effect of threonine supplementation on whole body protein synthesis of weanlings fed a commercial concentrate and grass forage. The objective of study 2 was to determine the effect of

threonine supplementation on whole body protein synthesis in mature horses fed a low threonine concentrate and high fiber forage.

## Materials & methods

### *Animals, housing, and feeding*

*Study 1*

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC 2012−0991). Six weanling Thoroughbred colts of similar age (176 ± 30 d) and weight (269 ± 24 kg) were obtained from University of Kentucky's Maine Chance Farm. During the 7 day adaptation period and days they were not being sampled, the weanlings were kept in individual dry lot pens and were brought into individual sawdust bedded stalls (3.7 m × 3.7 m) twice daily for concentrate feeding. Horses remained in stalls while catheterized on the days that isotope infusions were performed. Body weights were taken weekly through the adaptation and study periods on a livestock scale (TI-500, Transcell Technology Inc., Buffalo Grove, IL).

Diets were designed to meet or exceed the 2007 Nutrient Requirements of Horses recommendations (NRC, 2007). Each weanling received each of two diets, which consisted of a concentrate (Buckeye Growth, Buckeye Nutrition, Dalton, OH) at 1.7% of body weight, timothy hay cubes (Premium Timothy Hay Cubes, Ontario Dehy, Goderich, ON) at 1% of body weight, and an amino acid supplement. Supplements were isonitrogenous amounts of crystalline threonine (83 mg/kg/d) or glutamate (103 mg/kg/d) top dressed onto the concentrate portion of the feed. Feed was collected weekly

throughout the study and sent to Dairy One Cooperative Inc. (Ithaca, NY) for proximate analysis and was analyzed for amino acid content as described below.

*Study 2*

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (2012-0925). Six mature Thoroughbred mares (12 ± 3 yrs and 564 ± 23 kg) were obtained from University of Kentucky's Maine Chance Farm. During the 7 day adaptation period and days they were not being sampled, the mares were turned out in grass paddocks in two groups of three with muzzles. Feeding and weighing were performed similarly to study 1.

Each mare on study 2 received each of two diets, which consisted of a low threonine formulated concentrate (Table 6.1) at 0.8% of body weight, chopped timothy (Totally Timothy, Lucerne Farms, Fort Fairfield, ME) at 1.2% of body weight, and an amino acid supplement. Supplements were isonitrogenous amounts of threonine (72 mg/kg/d) or glutamate (89 mg/kg/d). Feed collection and analysis were completed as described for study 1.

**Table 6.1: Composition of low threonine concentrate used in study 2 on an as-fed basis[1].**

| Ingredient | Amount, % |
|---|---|
| Soy hulls | 37.50 |
| Corn distiller dried grains | 24.48 |
| Ground corn | 14.40 |
| Oatmill byproduct | 12.50 |
| Wheat middlings | 5.57 |
| Monocalcium phosphate (21%) | 1.92 |
| Calcium carbonate | 1.70 |
| Salt | 0.66 |
| Vitamin E | 0.40 |
| Dynamate[a] | 0.31 |
| Soybean oil | 0.28 |
| Magnesium oxide | 0.20 |
| Proprietary vitamin/mineral premix[b] | 0.04 |
| Proprietary trace mineral premix[c] | 0.03 |
| Choline 60% | 0.005 |
| Copper II sulfate | 0.002 |

[1]The concentrate was mixed by Buckeye Nutrition specifically for this study.
[a] Dynamate is a potassium and magnesium sulfate product made by The Mosaic Company
[b] The proprietary vitamin/mineral premix was designed specifically for inclusion in Buckeye Nutrition feeds and is manufactured by Trouw Nutrition
[c] The proprietary trace mineral premix was designed specifically for inclusion in Buckeye Nutrition feeds and is manufactured by Trouw Nutrition

***Study designs and procedures***

Both studies were conducted as crossover designs, where each horse received both diets. All horses received each treatment diets for 5 days prior to sampling. Growing animals can be studied while receiving potentially deficient diets and the risk of detrimental effects associated with prolonged nutrient restriction is minimized due to the short adaptation period required for this method (Elango et al., 2009; Moehn et al., 2004b).

On the morning of day 6 for each treatment venipuncture samples were taken immediately prior to and 90 minutes post feeding of the morning concentrate meal to investigate the effect of the concentrate meal feeding and amino acid supplements on plasma glucose, urea nitrogen, and amino acid concentrations.

On day 7, two jugular vein catheters were inserted, one for blood sampling and one for isotope infusion (Urschel et al., 2012). Whole-body phenylalanine kinetics were determined using 2 hour primed (7.1 µmol/kg), constant (6 µmol/kg/h) infusions of [$^{13}$C]sodium bicarbonate, to measure whole-body $CO_2$ production (Coggan et al., 1993), followed immediately by 4 hour primed (13 µmol/kg), constant infusions of [1-$^{13}$C]phenylalanine (9 µmol/kg/h), to measure phenylalanine oxidation to $CO_2$ and phenylalanine flux. Validation was previously performed in mature horses and resulted in a rapid rise to plateau isotopic enrichment in the plasma phenylalanine (Urschel et al., 2012). The horses wore cordless infusion pumps (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc., Loveland, CO) that were attached to surcingles to infuse the isotope and allow the horses to move freely in their stalls. Beginning 90 minutes prior to the start of isotope infusion procedures 1/24 of the morning meal was given every half hour during infusion in order to reduce fluctuations in phenylalanine oxidation (Moehn et al., 2004a; Mohn et al., 2003). Meals fed prior to the [1-$^{13}$C]phenylalanine infusion were top-dressed with non-isotopic phenylalanine to ensure that phenylalanine entering the system during the measurement of $CO_2$ production and [1-$^{13}$C]phenylalanine oxidation to $CO_2$ were as equivalent as possible. Breath samples were collected every 30 minutes beginning 30 minutes prior to the start of the [$^{13}$C]sodium bicarbonate infusion until the end of the [1-$^{13}$C]phenylalanine infusion into gas impermeable bags using a modified

equine Aeromask (Urschel et al., 2009). Blood samples were collected every 30 minutes beginning 30 minutes prior to the start of the [1-$^{13}$C]phenylalanine infusion until the end of the infusion procedures.

At the end of the isotope infusion procedures, catheters were removed and the horses were allocated to the next treatment at their afternoon concentrate meal. All horses were returned to University of Kentucky's Maine Chance Farm research herd at the completion of the studies.

***Sample analyses***

*Blood sample processing*

All blood samples were collected into heparinized vacutainers (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ) and promptly centrifuged at 1,500 × g for 10 minutes at 4ºC. Plasma was removed and frozen at -20ºC until the time of analysis.

*Amino acid analyses*

Plasma free amino acid concentrations and total feed amino acid concentrations were measured using reverse phase HPLC (3.9 × 300 mm PICO-TAG reverse phase column; Waters, Milford, MA) of phenylisothiocyanate derivatives as previously described (Urschel et al., 2011). Six samples of each feedstuff were collected over the course of the studies and were used for amino acid analyses.

*Plasma metabolite concentrations*

Plasma glucose concentrations were enzymatically determined using a YSI 2300 STAT Plus Glucose and Lactate Analyzer (YSI Inc., Life Sciences, Yellow Springs, OH). The intra-assay variation was 2.1% for study 1 and 2.5% for study 2, while the inter-

assay variation was < 5% for the glucose analysis. Plasma urea nitrogen was determined using a colorimetric spectrophotometric assay as previously described (Urschel et al., 2007), with an intra-assay variation of 4.4% for study 1 and 5.2% for study 2 and an inter-assay variation of < 10%.

*Isotope enrichment*

The amount of $^{13}CO_2$ relative to $^{12}CO_2$ in breath samples collected during the phenylalanine and bicarbonate infusions was determined immediately after sampling using an infrared isotope analyzer (IRIS-2; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany).

The relative concentrations of [1-$^{13}$C]phenylalanine to unlabeled phenylalanine, known as the phenylalanine isotopic enrichment, in the plasma samples was determined by Metabolic Solutions, Inc. (Nashua, NH) using a previously described method (Matthews et al., 1990), which is described in brief in Chapter 4.

### *Calculations & statistics*

Calculations and statistics were performed as described in Chapter 4.

## Results

All horses completed both treatments in their respective studies. Composition of the diet before the amino acid supplements were added is listed in Table 6.2 for study 1 and Table 6.3 for study 2. For study 1, dietary threonine intake was 162 mg/kg/d on the +Thr treatment and 79 mg/kg/d on the +Glu treatment. The NRC dietary threonine requirement estimate, based on muscle protein composition, is 81 mg/kg/d for 6 mo old weanlings with an expected mature BW of 500 kg. For study 2, dietary threonine intake

was 119 mg/kg/d on the +Thr treatment and 58 mg/kg/d on the +Glu treatment, which is well above the NRC dietary requirement estimate, based on muscle protein composition, of 33 mg/kg/d for adult horses at maintenance. However, these requirements are only estimates based on muscle protein amino acid composition.

**Table 6.2: As-fed nutrient composition of the feeds used in both diets fed to the weanlings.**

| | Timothy Hay Cubes | Buckeye Growth[1] |
|---|---|---|
| ***Overall nutrient composition*** | | |
| Dry matter (%) | 91.7 ± 0.6 | 90.7 ± 0.1 |
| DE (Mcal/kg) | 1.78 ± 0.02 | 3.01 ± 0.03 |
| Crude protein (%) | 10.5 ± 0.4 | 19.5 ± 0.1 |
| Lignin (%) | 6.0 ± 0.2 | 2.5 ± 0.4 |
| ADF (%) | 39.1 ± 0.6 | 10.9 ± 1.0 |
| NDF (%) | 57.9 ± 0.9 | 22.7 ± 0.7 |
| Water soluble carbohydrates (%) | 7.0 ± 0.2 | 6.7 ± 0.6 |
| Ethanol soluble carbohydrates (%) | 6.0 ± 0.3 | 5.4 ± 0.3 |
| Starch (%) | 0.5 ± 0.4 | 21.4 ± 0.2 |
| Crude fat (%) | 2.1 ± 0.4 | 8.9 ± 0.2 |
| Calcium (%) | 0.40 ± 0.02 | 0.88 ± 0.10 |
| Phosphorus (%) | 0.21 ± 0.01 | 0.81 ± 0.09 |
| Iron (mg/kg) | 502 ± 79 | 256 ± 24 |
| Zinc (mg/kg) | 89 ± 13 | 180 ± 3 |
| ***Amino acid composition (g/100g feed)*** | | |
| Alanine (%) | 0.44 ± 0.08 | 0.76 ± 0.07 |
| Arginine (%) | 0.54 ± 0.09 | 1.27 ± 0.12 |
| Aspartate + Asparagine (%) | 0.75 ± 0.13 | 1.53 ± 0.15 |
| Glutamate + Glutamine (%) | 0.85 ± 0.14 | 3.32 ± 0.32 |
| Glycine (%) | 0.33 ± 0.06 | 0.67 ± 0.08 |
| Histidine (%) | 0.17 ± 0.03 | 0.36 ± 0.08 |
| Isoleucine (%) | 0.34 ± 0.06 | 0.69 ± 0.10 |
| Leucine (%) | 0.56 ± 0.10 | 1.41 ± 0.30 |
| Lysine (%) | 0.41 ± 0.08 | 0.86 ± 0.10 |
| Methionine (%) | 0.20 ± 0.02 | 0.16 ± 0.01 |
| Phenylalanine (%) | 0.38 ± 0.08 | 0.79 ± 0.09 |
| Proline (%) | 0.47 ± 0.08 | 1.02 ± 0.10 |
| Serine (%) | 0.30 ± 0.06 | 0.65 ± 0.08 |
| Threonine (%) | 0.24 ± 0.04 | 0.36 ± 0.04 |
| Tyrosine (%) | 0.20 ± 0.04 | 0.49 ± 0.04 |
| Valine (%) | 0.41 ± 0.08 | 0.81 ± 0.12 |

[1]Growth Pellet, Buckeye Nutrition, Dalton, OH

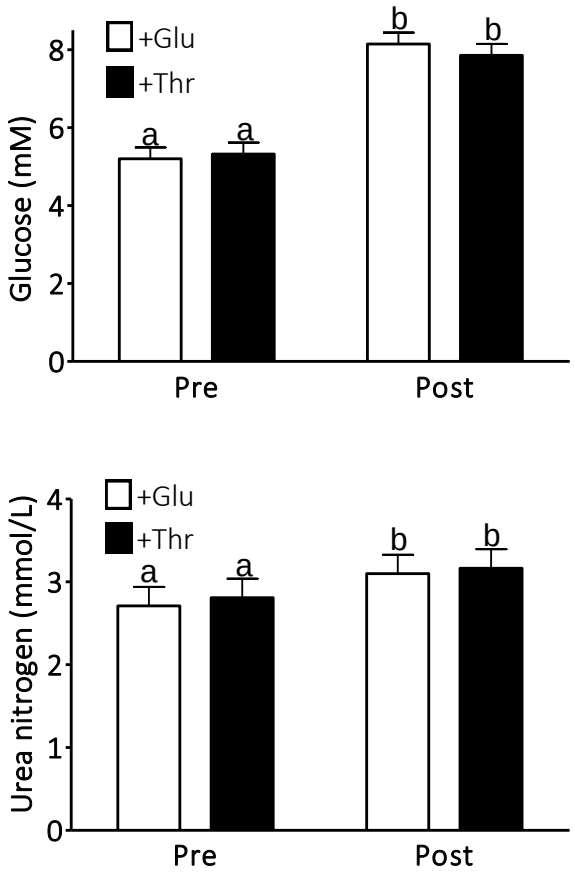
**Table 6.3: As-fed nutrient composition of the feeds used in both diets fed to the adult mares.**

| | Timothy | Low Thr Concentrate |
|---|---|---|
| ***Overall nutrient composition*** | | |
| Dry matter (%) | 90.0 ± 0.8 | 91.3 ± 0.1 |
| DE (Mcal/kg) | 1.97 ± 0.04 | 2.25 ± 0.19 |
| Crude protein (%) | 7.3 ± 0.2 | 15.1 ± 0.1 |
| Lignin (%) | 5.4 ± 0.3 | 3.0 ± 2.1 |
| ADF (%) | 36.3 ± 0.4 | 26.6 ± 7.3 |
| NDF (%) | 53.1 ± 1.0 | 43.0 ± 5.8 |
| Water soluble carbohydrates (%) | 19.6 ± 0.9 | 3.7 ± 0.4 |
| Ethanol soluble carbohydrates (%) | 9.2 ± 1.1 | 5.0 ± 2.5 |
| Starch (%) | 1.2 ± 0.4 | 10.5 ± 1.2 |
| Crude fat (%) | 1.9 ± 0.0 | 4.5 ± 0.2 |
| Calcium (%) | 0.44 ± 0.03 | 1.20 ± 0.19 |
| Phosphorus (%) | 0.23 ± 0.01 | 0.78 ± 0.07 |
| Iron (mg/kg) | 144 ± 6 | 367 ± 17 |
| Zinc (mg/kg) | 17 ± 1 | 139 ± 19 |
| ***Amino acid composition (g/100g feed)*** | | |
| Alanine (%) | 0.30 ± 0.01 | 0.77 ± 0.03 |
| Arginine (%) | 0.25 ± 0.02 | 0.67 ± 0.02 |
| Aspartate + Asparagine (%) | 0.63 ± 0.01 | 0.84 ± 0.03 |
| Glutamate + Glutamine (%) | 0.58 ± 0.04 | 2.08 ± 0.02 |
| Glycine (%) | 0.23 ± 0.02 | 0.59 ± 0.03 |
| Histidine (%) | 0.12 ± 0.01 | 0.34 ± 0.02 |
| Isoleucine (%) | 0.21 ± 0.28 | 0.46 ± 0.33 |
| Leucine (%) | 0.37 ± 0.04 | 1.32 ± 0.04 |
| Lysine (%) | 0.20 ± 0.01 | 0.54 ± 0.01 |
| Methionine (%) | 0.32 ± 0.04 | 0.80 ± 0.06 |
| Phenylalanine (%) | 0.40 ± 0.01 | 0.75 ± 0.01 |
| Proline (%) | 0.36 ± 0.02 | 0.91 ± 0.03 |
| Serine (%) | 0.24 ± 0.01 | 0.67 ± 0.01 |
| Threonine (%) | 0.19 ± 0.02 | 0.47 ± 0.04 |
| Tyrosine (%) | 0.13 ± 0.01 | 0.48 ± 0.02 |
| Valine (%) | 0.27 ± 0.02 | 0.56 ± 0.03 |

***Plasma glucose & urea concentrations***

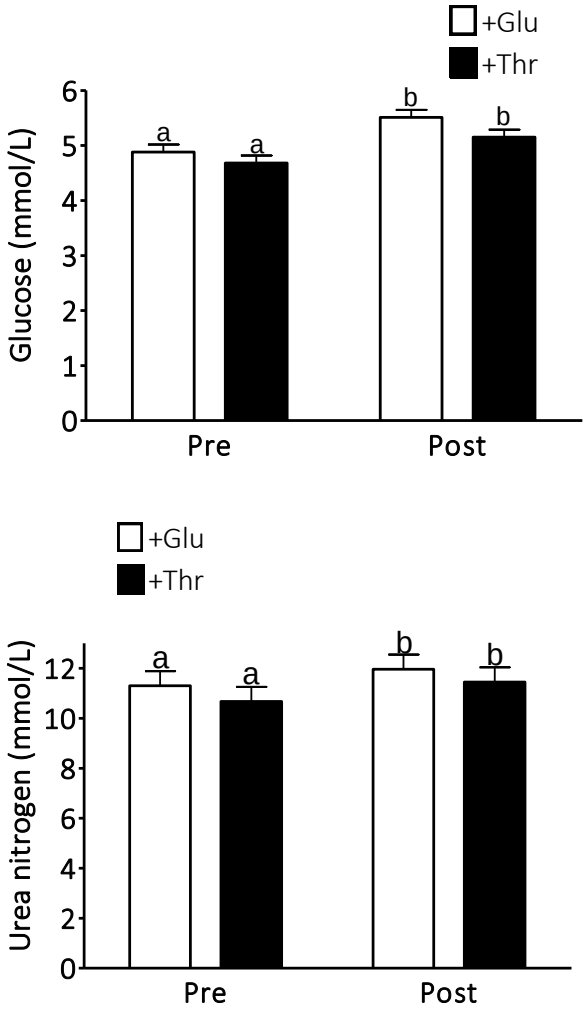
Plasma glucose and urea concentrations were greater at 90 minutes post concentrate meal feeding than pre-feeding (P<0.05). There was no difference due to treatment for study 1(Figure 6.1), but for study 2 there was a tendency for a treatment effect with higher concentrations with the +Glu treatment (P = 0.07)(Figure 6.2).

**Figure 6.1: Plasma glucose (mmol/L) and urea nitrogen (mmol/L) concentrations in weanling Thoroughbred colts before and 90 minutes post concentrate meal feeding when supplemented isonitrogenous amounts of either threonine (+Thr) or glutamate (+Glu) are presented.**




Values are least square means ± pooled SE, n = 6 per treatment group. Bars with different letters indicate a statistical difference (P<0.05).

**Figure 6.2: Plasma glucose (mmol/L) and urea nitrogen (mmol/L) concentrations in adult Thoroughbred mares before and 90 minutes post concentrate meal feeding when supplemented isonitrogenous amounts of either threonine (+Thr) or glutamate (+Glu) are presented.**




Values are least square means ± pooled SE, n = 6 per treatment group. Bars with different letters indicate a statistical difference (P<0.05).

***Plasma amino acid concentrations***

There were significant effects of time, diet, and the interaction of time by diet on plasma amino acid concentrations in both studies (P < 0.05; Table 6.4 & Table 6.5).

Generally, plasma amino acid concentrations were greater at 90 minutes post-feeding when compared to baseline values. Lysine and valine plasma concentrations decreased with threonine supplementation ($P<0.10$), while methionine and threonine plasma concentrations increased ($P<0.10$) in both studies. Notably, glycine plasma concentrations increased with threonine supplementation in both studies and glycine was the only dispensable amino acid to significantly respond to treatment ($P < 0.01$). Threonine plasma concentrations increased with both supplementation and meal feeding and threonine was the only amino acid to have a significant interaction between treatment and time ($P < 0.05$) for the plasma concentrations for both studies.

**Table 6.4: Plasma amino acid concentrations (mmol/L) in weanling Thoroughbred colts at baseline and 90 minutes post concentrate meal feeding when supplemented isonitrogenous amounts of either threonine (+Thr) or glutamate (+Glu)[1]**

| | +Thr | | +Glu | | | P-values | | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | 90 min | Baseline | 90 min | SEM | Treatment | Time | Treatment x time |
| **Indispensable** | | | | | | | | |
| Histidine | 34[a] | 69[b] | 31[a] | 69[b] | 4 | NS | <0.0001 | NS |
| Isoleucine | 56[a] | 100[b] | 59[a] | 121[b] | 6 | 0.07 | <0.0001 | NS |
| Leucine | 87[a] | 161[b] | 95[a] | 199[b,*] | 10 | 0.03 | <0.0001 | NS |
| Lysine | 59[a] | 154[b] | 64[a] | 182[b,*] | 8 | 0.06 | <0.0001 | NS |
| Methionine | 30[a] | 60[b] | 23[a] | 44[b,*] | 3 | 0.0003 | <0.0001 | 0.10 |
| Phenylalanine | 55[a] | 90[b] | 56[a] | 103[b,*] | 4 | 0.09 | <0.0001 | NS |
| Threonine | 178[a] | 351[b] | 77[a,*] | 137[b,*] | 20 | <0.0001 | <0.0001 | 0.01 |
| Tryptophan | 9 | 10 | 2 | 4 | 5 | NS | NS | NS |
| Valine | 161[a] | 243[b] | 166[a] | 278[b] | 12 | 0.10 | <0.0001 | NS |
| **Dispensable** | | | | | | | | |
| Alanine | 136[a] | 259[b] | 118[a] | 250[b] | 8 | NS | <0.0001 | NS |
| Arginine | 68[a] | 166[b] | 69[a] | 189[b] | 8 | NS | <0.0001 | NS |
| Asparagine | 49[a] | 99[b] | 46[a] | 105[b] | 4 | NS | <0.0001 | NS |
| Aspartate | 59 | 58 | 55 | 62 | 4 | NS | NS | NS |
| Citrulline | 80[a] | 105[b] | 77[a] | 103[b] | 4 | NS | <0.0001 | NS |
| Glutamate | 76 | 82 | 79 | 83 | 5 | NS | NS | NS |
| Glutamine | 654[a] | 971[b] | 610[a] | 959[b] | 20 | NS | <0.0001 | NS |
| Glycine | 643[a] | 730[b] | 595[a,*] | 691[b,*] | 11 | 0.002 | <0.0001 | NS |
| Ornithine | 31[a] | 57[b] | 33[a] | 65[b] | 3 | NS | <0.0001 | NS |
| Proline | 90[a] | 191[b] | 90[a] | 207[b] | 7 | NS | <0.0001 | NS |
| Serine | 249[a] | 338[b] | 246[a] | 345[b] | 10 | NS | <0.0001 | NS |
| Taurine | 28[a] | 44[b] | 27[a] | 40[b] | 3 | NS | 0.0002 | NS |
| Tyrosine | 66[a] | 119[b] | 67[a] | 120[b] | 5 | NS | <0.0001 | NS |
| **Total** | **2898** | **4457** | **2685** | **4356** | | | | |

* Significantly different ($P < 0.05$) from the +Thr treatment at the same time point

[a,b] Different superscripts within a row indicate a significant effect of time ($P < 0.05$)

[1]Plasma amino acid concentrations are presented as least squares mean values (μmol/L)

**Table 6.5: Plasma amino acid concentrations (mmol/L) in adult Thoroughbred mares at baseline and 90 minutes post concentrate meal feeding when supplemented isonitrogenous amounts of either threonine (+Thr) or glutamate (+Glu)[1]**

| | +Thr | | +Glu | | | P-values | | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | 90 min | Baseline | 90 min | SEM | Treatment | Time | Treatment x time |
| **Indispensable** | | | | | | | | |
| Histidine | 72[a] | 86[b] | 63[a,*] | 80 [b,*] | 3 | 0.01 | <0.001 | NS |
| Isoleucine | 52[a] | 56[b] | 54[a] | 63 [b] | 2 | NS | 0.01 | NS |
| Leucine | 112[a] | 126[b] | 114[a] | 137 [b] | 6 | NS | 0.01 | NS |
| Lysine | 70[a] | 91[b] | 76[a] | 109 [b,*] | 4 | 0.01 | <0.001 | NS |
| Methionine | 47 | 53 | 31[*] | 40 [*] | 4 | 0.0002 | 0.09 | NS |
| Phenylalanine | 76[a] | 82 [b] | 75[a] | 83 [b] | 2 | NS | <0.001 | NS |
| Threonine | 497[a] | 638[b] | 90[a,*] | 101 [b,*] | 32 | <0.001 | 0.02 | 0.05 |
| Tryptophan | 11 | 12 | 12 | 12 | 0 | NS | 0.06 | NS |
| Valine | 144 | 154 | 149[a] | 168 [b,*] | 5 | 0.05 | 0.0003 | NS |
| **Dispensable** | | | | | | | | |
| Alanine | 220 | 248 | 221[a] | 259 [b] | 9 | NS | 0.0001 | NS |
| Arginine | 83[a] | 106[b] | 83[a] | 120 [b] | 4 | 0.08 | <0.001 | NS |
| Asparagine | 49[a] | 62[b] | 41[a,*] | 57 [b,*] | 3 | 0.02 | <0.001 | NS |
| Aspartate | 22 | 22 | 25 | 29 [*] | 1 | 0.0001 | NS | NS |
| Citrulline | 97[a] | 119[b] | 87[a,*] | 114 [b] | 4 | 0.05 | <0.001 | NS |
| Glutamate | 71 | 70 | 87[*] | 84 [*] | 3 | <0.001 | NS | NS |
| Glutamine | 457[a] | 543[b] | 424[a] | 551 [b] | 19 | NS | <0.001 | NS |
| Glycine | 660 | 676 | 555[*] | 557 [*] | 16 | <0.001 | NS | NS |
| Ornithine | 46[a] | 51[b] | 46[a] | 54 [b] | 2 | NS | 0.01 | NS |
| Proline | 103[a] | 127[b] | 96[a] | 136 [b] | 5 | NS | <0.001 | NS |
| Serine | 286[a] | 319[b] | 224[a,*] | 259 [b,*] | 9 | <0.001 | 0.0004 | NS |
| Taurine | 46 | 48 | 43[*] | 44 [*] | 1 | 0.03 | NS | NS |
| Tyrosine | 87[a] | 104[b] | 76[a,*] | 90 [b,*] | 4 | 0.002 | 0.0004 | NS |
| **Total** | **3308** | **3793** | **2672** | **3147** | | | | |

* Significantly different ($P < 0.05$) from the +Thr treatment at the same time point

[a,b] Different superscripts within a row indicate a significant effect of time within a diet ($P<0.05$)

[1]Plasma amino acid concentrations are presented as least squares mean values (μmol/L)

***Phenylalanine kinetics***

Phenylalanine flux, intake, oxidation and non-oxidative disposal were similar between treatments (Table 6.6 & Table 6.7; P>0.05). Carbon dioxide production was also similar between treatments (P > 0.10) for both studies.

**Table 6.6: Whole-body phenylalanine kinetics in weanling Thoroughbred colts that were receiving either 83 mg/kg/d of threonine supplement (+Thr) or an isonitrogenous amount of glutamate (+Glu; 103 mg/kg/d)[1]**

| | +Glu | +Thr | Pooled SE | P-value |
|---|---|---|---|---|
| *Phenylalanine flux [μmol/(kg·h)]* | | | | |
| | 61 | 62 | 3 | 0.56 |
| *Carbon dioxide production [μmol/(kg·h)]* | | | | |
| | 21238 | 20734 | 1317 | 0.71 |
| *Phenylalanine entering the free phenylalanine pool [μmol/(kg·h)][2]* | | | | |
| Phenylalanine from dietary intake | 24.5 | 23.0 | 1.0 | 0.18 |
| Phenylalanine from protein breakdown | 36.1 | 39.5 | 3.0 | 0.30 |
| *Phenylalanine leaving the free phenylalanine pool [μmol/(kg·h)][2]* | | | | |
| Phenylalanine oxidation | 12.2 | 11.5 | 2.2 | 0.74 |
| Non-oxidative phenylalanine disposal | 48.4 | 51.0 | 3.1 | 0.42 |

[1]Values are least squares means ± SE, as determined using a one-way analysis of variance
[2]The following stochastic model of phenylalanine kinetics was used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal; a 50% rate of splanchnic phenylalanine extraction was assumed when calculating the amount of dietary phenylalanine reaching general circulation

**Table 6.7: Whole-body phenylalanine kinetics in mature Thoroughbred mares that were receiving either 72 mg/kg/d of threonine supplement (+Thr) or an isonitrogenous amount of glutamate (+Glu; 89 mg/kg/d)[1]**

| | +Thr | +Glu | Pooled SE | p-value |
|---|---|---|---|---|
| *Phenylalanine flux [μmol/(kg·h)]* | | | | |
| | 50 | 52 | 3 | 0.96 |
| *Carbon dioxide production [μmol/(kg·h)]* | | | | |
| | 14864 | 14588 | 491 | 0.98 |
| *Phenylalanine entering the free phenylalanine pool [μmol/(kg·h)][2]* | | | | |
| Phenylalanine from dietary intake | 30.3 | 30.4 | 0.1 | 0.73 |
| Phenylalanine from protein breakdown | 20.0 | 21.9 | 3.3 | 0.97 |
| *Phenylalanine leaving the free phenylalanine pool [μmol/(kg·h)][2]* | | | | |
| Phenylalanine oxidation | 11.0 | 11.3 | 1.4 | 0.99 |
| Non-oxidative phenylalanine disposal | 39.2 | 41.0 | 2.6 | 0.96 |

[1]Values are least squares means ± SE, as determined using a one-way analysis of variance
[2]The following stochastic model of phenylalanine kinetics was used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal; a 50% rate of splanchnic phenylalanine extraction was assumed when calculating the amount of dietary phenylalanine reaching general circulation

## Discussion

Although threonine has been indicated to be a limiting amino acid in some equine diets (Graham-Thiers and Kronfeld, 2005a; Graham et al., 1994), our studies did not find that supplementing threonine to the diets we used increased whole body protein synthesis, suggesting that there was no threonine limitation in the unsupplemented (+Glu) diets. We expected that the actual threonine requirement would be greater than the recommendation based on muscle tissue due to threonine's use in the gut, especially if the diet was high fiber. Instead, our findings indicate that the current threonine requirement estimate for weanlings (NRC, 2007) is adequate. The current threonine

requirement for mature horses was not evaluated in study 2 as threonine intakes for both treatment diets were above the NRC recommendation (Table 6.8). Mucin protein compositions in other species contain high percentages of threonine. For example, intracellular mucin in rats is 31% threonine (Fahim et al., 1987) and polymeric colonic mucin in swine is 15% threonine (Fogg et al., 1996) compared to equine muscle protein being only ~5% threonine (Lorenzo and Pateiro, 2013). In humans fed protein-free diets, ileal threonine losses (Fuller et al., 1994) are 28% of the WHO (2007) requirement. It is possible that the +Glu diets provided more threonine than required by the horses, despite expectations.

Threonine intakes in previous studies that suggest that threonine may be limiting in some equine diets are listed in Table 6.8. The lesser threonine intake in study 2 was below than the supplemented intakes of previous studies in the literature that identified effects of threonine supplementation on growth (Graham et al., 1994) and muscle mass (Graham-Thiers and Kronfeld, 2005a). Surprisingly, the study that found differences in subjective muscle mass scores when horses were supplemented with threonine and lysine in mature and aged horses had less of a difference between treatment threonine intakes (Graham-Thiers and Kronfeld, 2005a) than study 2, which did not find differences in whole-body protein synthesis. Perhaps the differences observed in that study were due to differences in lysine intake and not threonine intake (Graham-Thiers and Kronfeld, 2005a). Interestingly, both studies suggesting that threonine may be limiting in some equine diets provided threonine at levels above the NRC (2007) suggested requirements (Graham-Thiers and Kronfeld, 2005a; Graham et al., 1994), indicating that the threonine

requirement may be greater than suggested by using the muscle amino acid ratios put forth by Bryden (1991).

**Table 6.8: Summary of dietary threonine intake levels in literature suggesting threonine could be limiting in equine diets compared to threonine intake levels of the current studies**

| Source/study | Age | Threonine intake (mg/kg/d) | | | Finding when supplemented with threonine |
|---|---|---|---|---|---|
| | | Suggested NRC req't | Low | High | |
| Growth (Graham et al., 1994) | Yearlings | 70 | 94 | 113 | Increased girth circumference |
| Muscle mass (Graham-Thiers and Kronfeld, 2005a) | Mature & aged | 33 | 65 | 102 | Increased subjective muscle mass scores and decreased plasma urea nitrogen |
| Study 1 | Weanlings | 81 | 79 | 162 | No increase in whole-body protein synthesis |
| Study 2 | Mature | 33 | 58 | 119 | No increase in whole-body protein synthesis |

Previous studies that suggest threonine could be limiting in equine diets have used grass hays (coastal bermudagrass and an orchardgrass/timothy mix) and concentrates based heavily on corn and oats (Graham-Thiers and Kronfeld, 2005a; Graham et al., 1994). Our studies used timothy grass hay and the concentrates were based on wheat and soy hulls for studies 1 & 2, respectively. Very little is known about the bioavailability of amino acids to horses in various feedstuffs. Threonine bioavailability is known to vary according to feedstuff for swine (NRC, 2012). Standard ileal digestibilities for corn and oats are greater than those for soy hulls and wheat middlings in swine (Table 6.9). However, an estimation of the availability of threonine in each diet used in the present

study based on swine data is not possible due to the lack of data on forage amino acid digestibility (NRC, 2012). One possible reason that we did not find threonine to be limiting in either of our diets could be due to increased bioavailability of threonine in the forages that were chosen for our diets relative to the forages used in previous studies.

**Table 6.9: Swine threonine standard ileal digestibilities in selected feedstuffs from the NRC (2012)**

| Feedstuff | Standard ileal digestibility of threonine in swine |
|---|---|
| Soybean meal | 85 |
| Corn | 77 |
| Oats | 75 |
| Wheat middlings | 73 |
| Corn dried distillers' grain | 71 |
| Soybean hulls | 62 |

Other reasons that we may not have seen an increase in whole body protein synthesis due to threonine supplementation could be that threonine use in the horse's gut is not as great as we had thought in the ages studied, or fiber may not increase mucin production in the horse as the horse has evolved on forage diets. Studies indicating the highest rates of threonine use in the gut were conducted in neonates (Bertolo et al., 1998; Chapman et al., 2009; Law et al., 2007), which are much younger than the weanling and adult horses that we studied. The effect of dietary fiber in increasing mucin synthesis, which is reviewed by Montagne et al. (2003), has been observed in rats (Ito et al., 2009; Satchithanandam et al., 1990) and swine (Lien et al., 1997), which are omnivores, and evolved to consume lower fiber diets than horses. The effect of dietary fiber on mucin production has not been explored in herbivores and may be unique to omnivores as a way to adapt to their more variable diets. Ileal losses in mucin need to be measured as

opposed to fecal mucin, as gut microbes are known to digest mucins, as reviewed by Derrien et al. (2010).

Interactions between the host and intestinal microbes, including mucin degradation by microbes, have been reviewed and differ according to the diet of the host species (Collinder et al., 2003). Without the presence of mucin degrading bacteria the number and size of goblet cells is decreased in mice (Kandori et al., 1996). Fecal bacterial populations are noted to differ between herbivores, omnivores, and carnivores (Endo et al., 2010). It is possible that the loads of mucin degrading microbes differ between omnivores and herbivores and mucin secretion is lesser in herbivores as a result. With lower amounts of mucin production the need for threonine in the gut would be reduced.

Although threonine supplementation did not increase whole-body synthesis, it did affect plasma concentrations of other amino acids. Some of these differences can be explained if the supplemental threonine was saturating degradation pathways. Threonine catabolism is known to take place through two pathways in mammals (Bird and Nunn, 1983; Dale, 1978). One pathway is known to create glycine, while the other is known to produce α-ketobutyrate (Figure 6.3). Glycine plasma concentrations most likely responded to treatment because glycine can be produced from threonine. Glycine is converted to serine when it is catabolized and although we did not see greater serine plasma concentrations when the colts were supplemented with threonine, we did see an increase in plasma serine concentrations in the adult mares.

The slightly greater ($P = 0.07$) plasma urea concentrations when horses were on the +Glu treatment in study 2 may indicate threonine was limiting in this diet, as plasma

urea concentrations have been used previously to indicate amino acid adequacy in horses (Fisher et al., 1989; Graham et al., 1994; Winsco et al., 2011). The relationship between blood urea nitrogen and amino acid adequacy is discussed in Chapter 2 in the section "Plasma/serum urea concentrations". However, using blood urea nitrogen concentrations is not considered a sensitive enough method by itself to determine amino acid requirements (Pencharz and Ball, 2003).

**Figure 6.3: Relationship between the metabolism of threonine and the metabolism of glycine, serine, and methionine.**

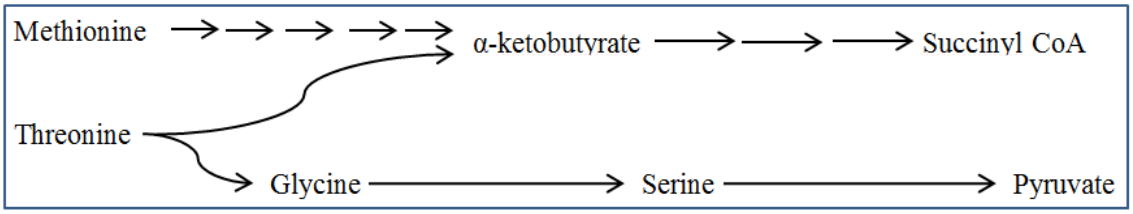



Figure adapted from Finkelstein (1990) and Bird and Nunn (1983).

Methionine catabolism is also known to produce α-ketobutyrate (Finkelstein, 1990). Saturation of the methionine catabolism pathway at this juncture could have resulted in feedback regulation resulting in the elevated methionine plasma concentrations that were found on the +Thr treatments compared to the +Glu treatments. Additional research is needed to confirm the metabolism and determine how these changes in amino acid metabolism affect the horse and any possible methionine sparing action of high dietary threonine levels. These changes may be of particular interest for horses being fed arbitrary amounts of threonine in supplements for muscle gain/maintenance.

Given that our studies found no differences in whole-body protein synthesis when threonine was supplemented, our findings indicate that the threonine supplementation for weanlings receiving this commercial concentrate and a quality grass forage or adult

horses at maintenance on a diet composed of 60% grass forage and 40% concentrate is most likely not warranted. However, threonine supplementation can affect amino acid metabolism and additional research is needed to determine how these changes in metabolism affect long term growth and athletic performance. Additional research is also needed to determine amino acid requirements in horses.

# Chapter 7. Short communication: Splanchnic extraction of phenylalanine in mature mares was not effected by threonine supplementation

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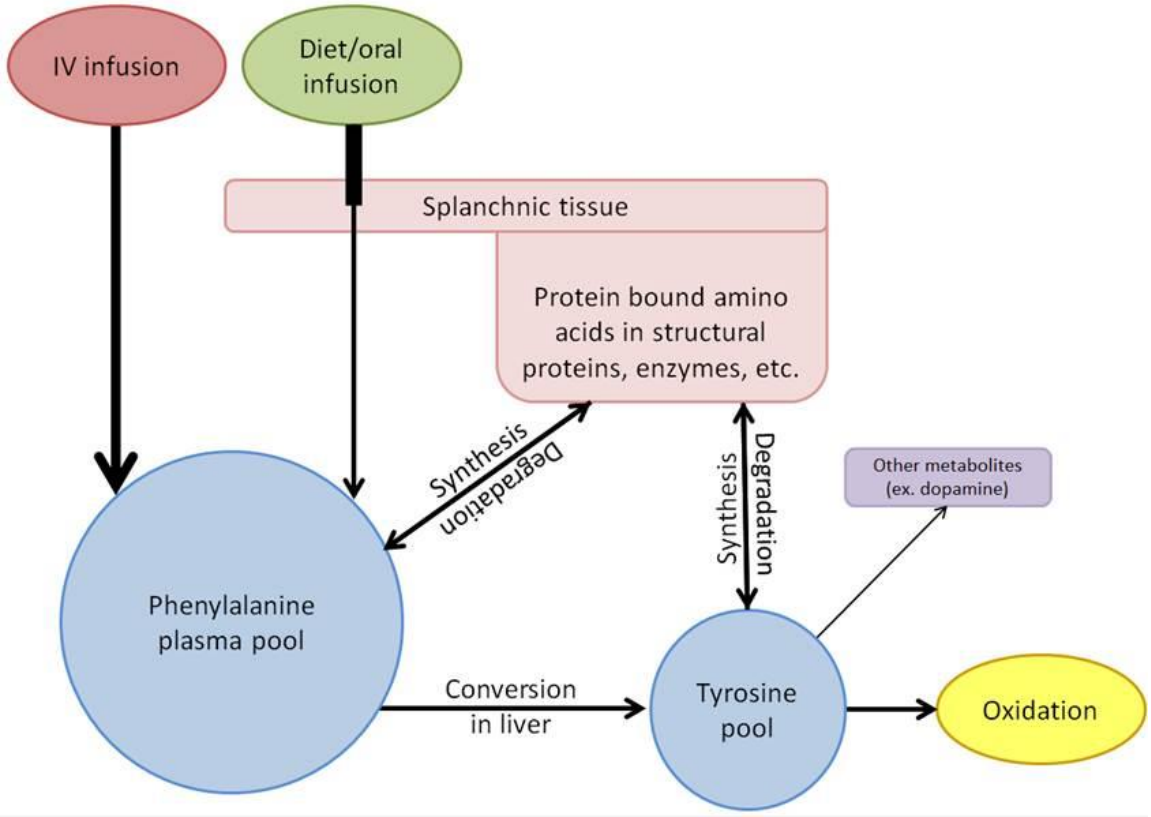
## Introduction

Use of stable label amino acid isotopes for study of whole-body protein metabolism and the determination of amino acid requirements is becoming more common. These studies have shorter experiment durations compared to growth trials and avoid some of the potential pitfalls inherent in nitrogen balance studies, such as not being able to easily recover nitrogen lost in sweat and shedding hair. Isotopic amino acid methodologies are particularly common in human and swine studies and are reviewed elsewhere (Elango et al., 2008b, 2012a; Kurpad and Thomas, 2011).

When using isotope tracers to study whole-body amino acid metabolism, of particular interest is the flux of the amino acid tracer. Flux is the rate of amino acid entrance and exit from a given body pool. Indispensable amino acids enter the free amino acid pool from the diet and from protein breakdown and exit through oxidation, protein synthesis, or conversion to other metabolites. Although dietary intake of the amino acid tracer is generally known in these studies, the dietary intake cannot be used in the formulas to study whole-body amino acid kinetics, as not all of the amino acid consumed enters the free amino acid pool. Specifically, not all protein bound amino acids will be digested in the gastrointestinal tract and available for absorption and not all of the absorbed amino acid will actually reach general circulation. The dietary amino acid must

pass through the splanchnic tissues, including the gastrointestinal tract and the liver, and some of the amino acid will be extracted for use in these splanchnic tissues (Figure 7.1). However, if the splanchnic extraction percentage of a given amino acid is known, then the amount of the amino acid from the diet entering the general plasma pool could be calculated. In a previous equine study using this method, estimates of splanchnic extraction from other species have been substituted (Chapter 4).

**Figure 7.1**: **Orally ingested phenylalanine must pass through the splanchnic tissue before entering the general plasma phenylalanine pool.**




Schematic showing differences in how isotopic phenylalanine used in kinetics studied is metabolized differently based on route of administration.

Splanchnic extraction of phenylalanine has been measured in other species (Table 7.1). The values range from 19% for adult humans (Kriengsinyos et al., 2002) to 47% in elderly humans (Volpi et al., 1999). These studies use a variety of methods to determine the splanchnic extraction of phenylalanine. The majority of the studies, including this study, compared a steady intravenous infusion of isotopic phenylalanine with an oral infusion of the same tracer completed at another time (Cvitkovic et al., 2004; Kriengsinyos et al., 2002; Matthews et al., 1993). Steady dual infusion of two different isotopes of phenylalanine, one intravenously and the other intragastrically/orally, has also been used (Stoll et al., 1997; Volpi et al., 1999). Hounds were studied twice with bolus doses of phenylalanine, once intravenously and once orally (Gooding et al., 2013). Some of the variation in values listed in Table 7.1 could be due to the different methods used in those studies.

**Table 7.1: Values for the splanchnic extraction of phenylalanine as determined in other species**

| Species | Age | Phenylalanine splanchnic extraction% | Source |
|---|---|---|---|
| Dog | 3 yrs | 24 | (Gooding et al., 2013) |
| Pig | 2 wks | 45 | (Stoll et al., 1997) |
| Pig | Neonate | 27 | (Cvitkovic et al., 2004) |
| Human | Elderly | 47 | (Volpi et al., 1999) |
| Human | Young | 29 | (Volpi et al., 1999) |
| Human | Adult | 19 | (Kriengsinyos et al., 2002) |
| Human | Adult | 29 | (Matthews et al., 1993) |

Administration of the isotopic tracer for IAAO studies is commonly through intravenous infusion in several species (Lazaris-Brunner et al., 1998; Moehn et al., 2004b), including horses (Chapter 4). Oral infusion techniques have been developed for

humans to be less invasive, especially for sensitive populations, such as children or the elderly (Elango et al., 2008a). Comparing oral and intravenous infusions to determine splanchnic extraction will also verify the use of oral infusion in horses for the IAAO method. An oral infusion would have the advantage of only requiring one jugular vein catheter instead of the two required for an intravenous infusion.

Knowing the splanchnic extraction of the indicator amino acid, such as phenylalanine, is of limited use if that value changes as the level of the test amino acid changes. Therefore, two different levels of threonine were studied. The objectives of this study were to measure splanchnic extraction of phenylalanine at two different levels of threonine intake and determine whether the oral infusion of [1-$^{13}$C]phenylalanine would allow for measureable amounts of isotope to be detected in the collected breath and plasma samples. This information will help with the development of oral isotope infusion protocols to study amino acid metabolism in horses.

## Materials & methods

### *Animals, housing, and feeding*

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC 2012-0925). Six mature Thoroughbred mares (12 ± 3 yrs and 564 ± 23 kg) were obtained from University of Kentucky's Maine Chance Farm for the study. During the 7 day adaptation period and days they were not being sampled, the mares were turned out in grass paddocks during the day in two groups of three with muzzles. The horses were fed in individual sawdust bedded stalls (3.7 m × 3.7 m) and stayed in overnight to allow enough time to consume

the forage. Horses remained in stalls while catheterized on the days that isotope infusions were performed. Body weights were taken weekly through the adaptation and study periods on a livestock scale (TI-500, Transcell Technology Inc., Buffalo Grove, IL).

Diets were designed to meet or exceed the 2007 Nutrient Requirements of Horses recommendations (NRC, 2007). Each mare received each of two diets, which consisted of a low threonine formulated concentrate (Table 7.2) at 0.8% of body weight, chopped timothy (Totally Timothy, Lucerne Farms, Fort Fairfield, ME) at 1.2% of body weight, and an amino acid supplement. Supplements were isonitrogenous amounts of crystalline threonine (+Thr; 72 mg/kg/d) or glutamate (+Glu; 89 mg/kg/d) that was top dressed onto the concentrate portion of the feed. Feed was collected weekly throughout the study and sent to Dairy One Cooperative Inc. (Ithaca, NY) for proximate analysis and was analyzed for amino acid content as described below.

**Table 7.2: Composition of low threonine concentrate on an as-fed basis[1].**

| Ingredient | Amount, % |
|---|---|
| Soy hulls | 37.50 |
| Corn distiller dried grains | 24.48 |
| Ground corn | 14.40 |
| Oatmill byproduct | 12.50 |
| Wheat middlings | 5.57 |
| Monocalcium phosphate (21%) | 1.92 |
| Calcium carbonate | 1.70 |
| Salt | 0.66 |
| Vitamin E | 0.40 |
| Dynamate[a] | 0.31 |
| Soybean oil | 0.28 |
| Magnesium oxide | 0.20 |
| Proprietary vitamin/mineral premix[b] | 0.04 |
| Proprietary trace mineral premix[c] | 0.03 |
| Choline 60% | 0.005 |
| Copper II sulfate | 0.002 |

[1]The concentrate was mixed by Buckeye Nutrition specifically for this study.
[a] Dynamate is a potassium and magnesium sulfate product made by The Mosaic Company
[b] The proprietary vitamin/mineral premix was designed specifically for inclusion in Buckeye Nutrition feeds and is manufactured by Trouw Nutrition
[c] The proprietary trace mineral premix was designed specifically for inclusion in Buckeye Nutrition feeds and is manufactured by Trouw Nutrition

**Study design and procedures**

Each horse was studied with an intravenous infusion and the oral administration of [1-$^{13}$C]phenylalanine while on each diet. All horses received each diet for 6 days prior to sampling. On day 7, jugular vein catheters were inserted (Urschel et al., 2012). Horses that received the infusion of [1-$^{13}$C]phenylalanine intravenously received two catheters, one for blood sampling and one for infusion. Horses that received the infusion of [1-$^{13}$C]phenylalanine orally only received one jugular vein catheter. Whole-body phenylalanine kinetics were determined using 2 hour primed (7.1 μmol/kg), constant (6 μmol/kg/h) infusions of [$^{13}$C]sodium bicarbonate, to measure whole-body $CO_2$ production (Coggan et al., 1993), followed immediately by 4 hour primed (13 μmol/kg), constant infusions of [1-$^{13}$C]phenylalanine (9 μmol/kg/h), to measure phenylalanine oxidation to $CO_2$ and phenylalanine flux. The horses wore cordless infusion pumps (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc., Loveland, CO) that were attached to surcingles, allowing them to move about their stalls, to infuse the [$^{13}$C]sodium bicarbonate and intravenous [1-$^{13}$C]phenylalanine isotopes. Oral administration of [1-$^{13}$C]phenylalanine, including administration of the prime dose, was accomplished by topdressing feed with a saline solution containing the isotope every half hour. Beginning 90 minutes prior to the start of isotope infusion procedures 1/24 of the morning meal was given every half hour during infusion in order to reduce fluctuations in phenylalanine oxidation (Moehn et al., 2004a; Mohn et al., 2003). Meals fed prior to the [1-$^{13}$C]phenylalanine infusion were top-dressed with non-isotopic phenylalanine to ensure that phenylalanine entering the system during the measurement of $CO_2$ production and [1-$^{13}$C]phenylalanine oxidation to $CO_2$ were as equivalent as possible. Breath samples

were collected every 30 minutes beginning 30 minutes prior to the start of the [$^{13}$C]sodium bicarbonate infusion until the end of the [1-$^{13}$C]phenylalanine infusion into gas impermeable bags using a modified equine Aeromask (Urschel et al., 2009). Blood samples were collected every 30 minutes beginning 30 minutes prior to the start of the [1-$^{13}$C]phenylalanine infusion until the end of the infusion procedures. Sampling schedules were the same whether the [1-$^{13}$C]phenylalanine was administered intravenously or orally.

At the end of the isotope infusion procedures, catheters were removed and the horses were allocated to the next treatment at their afternoon concentrate meal. All horses were returned to University of Kentucky's Maine Chance Farm research herd at the completion of the study.

**Sample analyses**

Blood sampling and isotope enrichment analyses were conducted as previously described (Chapter 4 and Chapter 5).

**Calculations**

Blood and breath phenylalanine enrichment were used to calculate phenylalanine flux, phenylalanine oxidation and non-oxidative phenylalanine disposal. Splanchnic extraction was calculated using the following formula (Chapman et al., 2013; Kriengsinyos et al., 2002; Stoll et al., 1997), where $Q_{IV}$ is phenylalanine flux when the [1-$^{13}$C]phenylalanine isotope was administered intravenously and $Q_O$ is the phenylalanine flux when the [1-$^{13}$C]phenylalanine isotope was administered orally:

$$\text{Splanchnic extraction} = 1 - Q_{IV}/Q_O$$

**Statistical analysis**

Data were analyzed using SAS (version 9.3, SAS Institute Inc., Cary, NC), using the MIXED procedure. In all cases, differences were considered significant at $P < 0.05$ and statistical trends were considered where $0.05 < P < 0.10$. The phenylalanine kinetics parameters were determined using a one-way analysis of variance with treatment as the fixed effect and horse as the random effect. In all cases, when differences were significant, means were separated using the Tukey-Kramer adjusted pdiff option.

**Results**

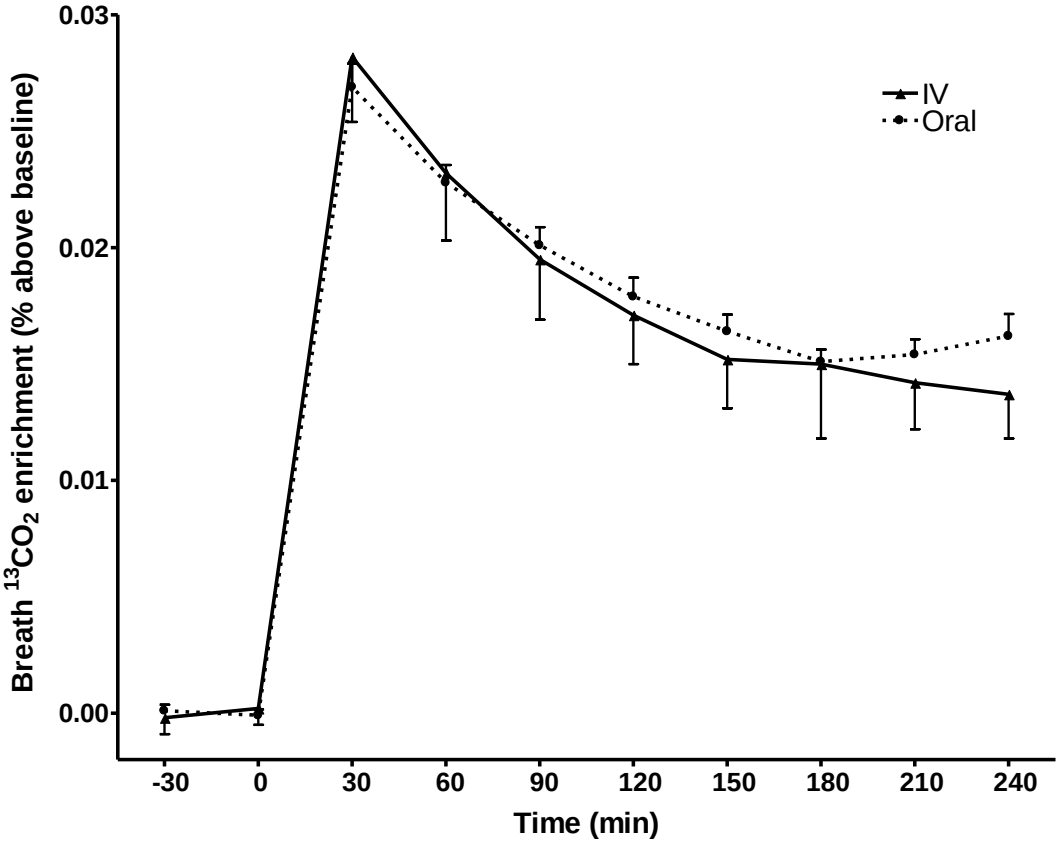
All horses completed all treatments, with the exception of one horse that was unable to complete the intravenous infusion of [1-$^{13}$C]phenylalanine when fed the +Thr diet due to a catheter problem. Composition of the diet before the amino acid supplements were added is listed in Table 7.3. Total dietary threonine intake was 119 mg/kg/d on the +Thr treatment and 58 mg/kg/d on the +Glu treatment, which is well above the NRC dietary requirement estimate of 33 mg/kg/d for adult horses at maintenance.

**Table 7.3: As-fed nutrient composition of the feeds used in both diets fed to the adult mares.**

| | Timothy | | | Low Thr Concentrate | | |
|---|---|---|---|---|---|---|
| ***Overall nutrient composition*** | | | | | | |
| Dry matter (%) | 90.0 | ± | 0.8 | 91.3 | ± | 0.1 |
| DE (Mcal/kg) | 1.97 | ± | 0.04 | 2.25 | ± | 0.19 |
| Crude protein (%) | 7.3 | ± | 0.2 | 15.1 | ± | 0.1 |
| Lignin (%) | 5.4 | ± | 0.3 | 3.0 | ± | 2.1 |
| ADF (%) | 36.3 | ± | 0.4 | 26.6 | ± | 7.3 |
| NDF (%) | 53.1 | ± | 1.0 | 43.0 | ± | 5.8 |
| Water soluble carbohydrates (%) | 19.6 | ± | 0.9 | 3.7 | ± | 0.4 |
| Ethanol soluble carbohydrates (%) | 9.2 | ± | 1.1 | 5.0 | ± | 2.5 |
| Starch (%) | 1.2 | ± | 0.4 | 10.5 | ± | 1.2 |
| Crude fat (%) | 1.9 | ± | 0.0 | 4.5 | ± | 0.2 |
| Calcium (%) | 0.44 | ± | 0.03 | 1.20 | ± | 0.19 |
| Phosphorus (%) | 0.23 | ± | 0.01 | 0.78 | ± | 0.07 |
| Iron (mg/kg) | 144 | ± | 6 | 367 | ± | 17 |
| Zinc (mg/kg) | 17 | ± | 1 | 139 | ± | 19 |
| ***Amino acid composition (g/100g feed)*** | | | | | | |
| Alanine (%) | 0.30 | ± | 0.01 | 0.77 | ± | 0.03 |
| Arginine (%) | 0.25 | ± | 0.02 | 0.67 | ± | 0.02 |
| Aspartate + Asparagine (%) | 0.63 | ± | 0.01 | 0.84 | ± | 0.03 |
| Glutamate + Glutamine (%) | 0.58 | ± | 0.04 | 2.08 | ± | 0.02 |
| Glycine (%) | 0.23 | ± | 0.02 | 0.59 | ± | 0.03 |
| Histidine (%) | 0.12 | ± | 0.01 | 0.34 | ± | 0.02 |
| Isoleucine (%) | 0.21 | ± | 0.28 | 0.46 | ± | 0.33 |
| Leucine (%) | 0.37 | ± | 0.04 | 1.32 | ± | 0.04 |
| Lysine (%) | 0.20 | ± | 0.01 | 0.54 | ± | 0.01 |
| Methionine (%) | 0.32 | ± | 0.04 | 0.80 | ± | 0.06 |
| Phenylalanine (%) | 0.40 | ± | 0.01 | 0.75 | ± | 0.01 |
| Proline (%) | 0.36 | ± | 0.02 | 0.91 | ± | 0.03 |
| Serine (%) | 0.24 | ± | 0.01 | 0.67 | ± | 0.01 |
| Threonine (%) | 0.19 | ± | 0.02 | 0.47 | ± | 0.04 |
| Tyrosine (%) | 0.13 | ± | 0.01 | 0.48 | ± | 0.02 |
| Valine (%) | 0.27 | ± | 0.02 | 0.56 | ± | 0.03 |

The horses readily consumed the [1-$^{13}$C]phenylalanine as delivered in saline on top of their feed. The oral infusion resulted in stable plateaus, confirming the doses and length of oral infusion (Figure 7.2 and Figure 7.3). Phenylalanine intake and carbon dioxide production (Table 7.4) did not differ between any treatments ($P>0.10$). Phenylalanine kinetics (Table 7.4) were not affected by diet ($P>0.10$), although phenylalanine flux, oxidation and non-oxidative disposal values were greater when the isotope was administered orally ($P<0.05$). The differences in flux between the oral and intravenous infusions allowed for the calculation of phenylalanine splanchnic extraction of 26±5% and 27±4% for the +Thr and +Glu treatments, respectively.

**Figure 7.2: Breath $^{13}CO_2$ enrichment of six adult Thoroughbred mares when [1-$^{13}$C]phenylalanine was administered either intravenously[1] (IV) or orally[2]**

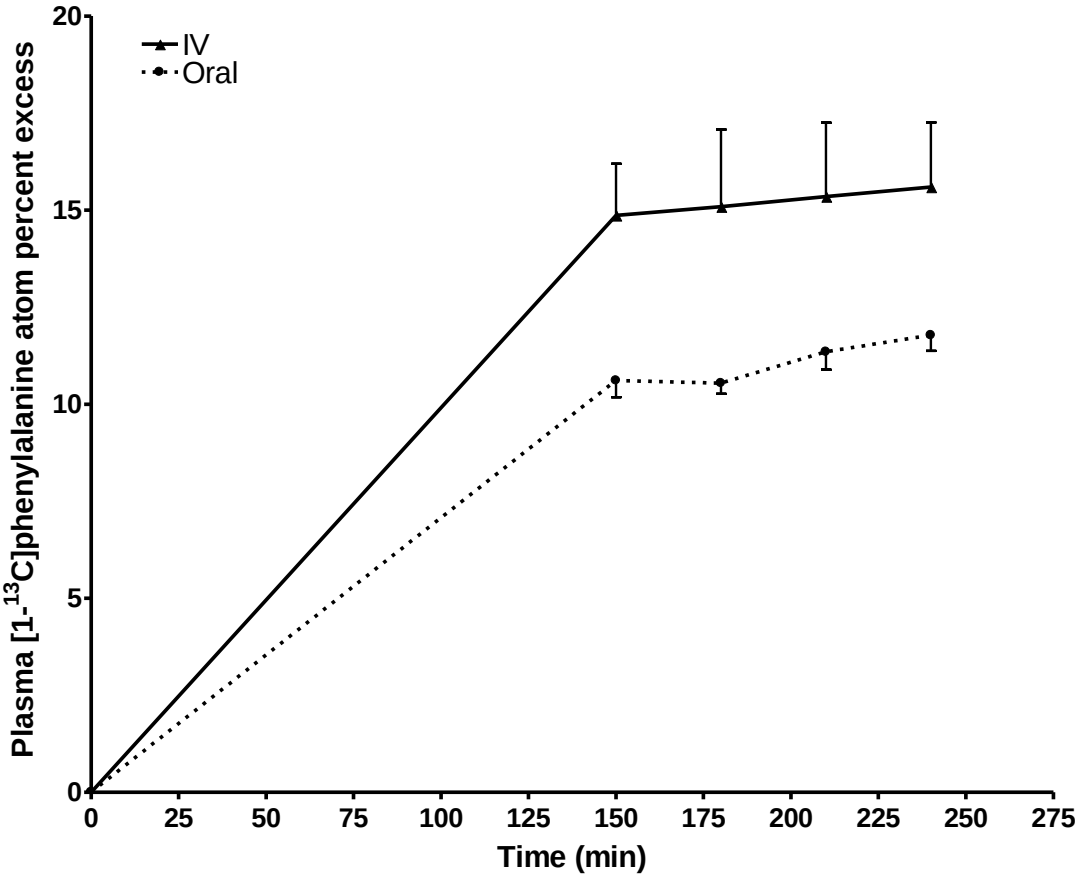



Values are mean ± standard deviation from the data as pooled by infusion route meaning all but one horse was studied twice for each infusion route

[1] n=11

[2] n=12

**Figure 7.3: Plasma [1-$^{13}$C]phenylalanine enrichment of adult Thoroughbred mares when [1-$^{13}$C]phenylalanine was administered either intravenously[1] (IV) or orally[2]**




Values are mean ± standard deviation from the data as pooled by infusion route meaning all but one horse was studied twice for each infusion route

[1] n=11

[2] n=12

**Table 7.4: Whole-body phenylalanine kinetics in mature Thoroughbred mares that were receiving either 72 mg/kg/d of threonine supplement (+Thr) or an isonitrogenous amount of glutamate (+Glu; 89 mg/kg/d) when [1-$^{13}$C]phenylalanine was administered either intravenously (IV) or orally (O) [1]**

| | +Thr | | +Glu | | Pooled SE | p-value |
|---|---|---|---|---|---|---|
| | IV | O | IV | O | | |
| *Phenylalanine flux [μmol/(kg·h)]* | | | | | | |
| | 50[a] | 71[b] | 52[a] | 73[b] | 3 | <0.001 |
| *Carbon dioxide production [μmol/(kg·h)]* | | | | | | |
| | 14864 | 14905 | 14588 | 14377 | 491 | 0.8272 |
| *Phenylalanine leaving the free phenylalanine pool [μmol/(kg·h)][2]* | | | | | | |
| Phenylalanine oxidation | 11.0[a] | 17.6[b] | 11.3[a] | 18.6[b] | 1.4 | <0.001 |
| Non-oxidative phenylalanine disposal | 39.2[a] | 53.6[b] | 41.0[a] | 54.0[b] | 2.6 | 0.0003 |

[1]Values are least squares means ± SE, as determined using a one-way analysis of variance
[2]The following stochastic model of phenylalanine kinetics was used: flux = rate of phenylalanine entry = rate of phenylalanine leaving; rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown; rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal; a 50% rate of splanchnic phenylalanine extraction was assumed when calculating the amount of dietary phenylalanine reaching general circulation
[a,b] Different superscripts within a row indicate statistically significant differences ($P<0.05$)

## Discussion

Splanchnic extraction of phenylalanine in other species (Table 7.1) ranges from 19% (Kriengsinyos et al., 2002) to 47% (Volpi et al., 1999), with most values between 20% and 30% (Cvitkovic et al., 2004; Gooding et al., 2013; Matthews et al., 1993; Volpi et al., 1999). Splanchnic extraction of lysine and leucine in piglets is also approximately 30% (Stoll et al., 1998). The values that we calculated for phenylalanine splanchnic extraction in horses fall neatly within that range. For future studies in horses using

phenylalanine kinetics, splanchnic extraction of phenylalanine will no longer have to be estimated using values from other species.

The estimate used previously in estimating the amount of dietary phenylalanine that entered the free amino acid pool was 50% (Chapter 4). In the only study to measure ileal amino acid digestibility in horses, phenylalanine was found to have a prececal apparent digestibility of ~84% and a true prececal digestibility of ~100% (Almeida et al., 1999). Almeida et al. (1999) used different diet ingredients, specifically *Cynodon dactylon* hay and a corn/soybean meal based concentrate, than has been used in the studies presented here. Differences in dietary ingredients could result in differences in the prececal digestibility of phenylalanine. True digestibility of all amino acids together ranged from 40-85% in horses depending on feedstuff, with the highest values for concentrates and the lowest for grass hay (Martin-Rosset, 2000). In swine true phenylalanine digestibility has been measured to be 90% for soybean meal (Smiricky et al., 2002). In broilers true phenylalanine digestibility has been measured at 73% for blood and bone meal and 91% for soybean meal (Angkanaporn et al., 1996). Given that the diet in this study was 60% grass forage the true digestibility of the phenylalanine in this diet is likely to be lower than the previous report by Almeida et al. (1999). Given the splanchnic extraction value of 26% found in this study, a true phenylalanine digestibility of 76% would be needed to account for our previous estimate of 50% of the dietary phenylalanine makes it into general circulation (Urschel et al., 2012).

The phenylalanine that was extracted by the splanchnic tissues was both oxidized and incorporated into protein as evidenced by the greater oxidation and non-oxidative disposal of phenylalanine when the isotope was administered orally (Table 7.4). It is

unlikely that the non-oxidative disposal of phenylalanine was to tyrosine as a final product, as the diets contained the same amount of tyrosine and that amount of tyrosine was adequate according to estimates from the NRC (2007). The diets also contained equal amounts of phenylalanine and infusion rates were the same for orally infused and intravenously infused horses, meaning the differences in flux were not due to different levels of phenylalanine intake.

Oral infusion of [1-$^{13}$C]phenylalanine successfully allowed for the appearance of the label to be detected in both blood and breath samples in horses, allowing future studies using these techniques to be less invasive compared to an intravenous infusion. Oral infusions have been used with the IAAO method in swine (Samuel et al., 2012) and humans (Elango et al., 2007; Kriengsinyos et al., 2002). Use of the oral method of isotope infusion in future studies will decrease the number of catheters required and make for less invasive studies. As phenylalanine splanchnic extraction was not influenced by dietary levels of threonine in our study, it is likely that it will not be affected by differing levels of a test amino acid in future studies.

Examples of future studies would include determination of amino acid requirements, such as lysine, threonine, and methionine, via the IAAO method. Threonine is suspected to be a limiting amino acid in some equine diets (Graham et al., 1994). The current estimate for equine threonine requirements based on equine muscle tissue amino acid composition (NRC, 2007) may be inadequate given threonine's use in the gut for mucin synthesis in other species (Wu, 2009). Given the lack of knowledge regarding threonine requirements in the horse and the potential for current estimates to be underestimating the requirement, threonine was chosen as the test amino acid for this

study. However, flux, oxidation, and non-oxidative disposal were not affected by threonine intake (Table 7.4), indicating that the threonine level provided was adequate at both levels of intake.

## Chapter 8. Overall discussion and directions for future research

The studies presented in this dissertation are the first to investigate amino acid adequacy and requirements using isotopic methods in growing horses. As these methods are novel to the horse, the efficacy of their use may be questioned. Although three out of four studies evaluating amino acid supplemented diets presented did not find differences in phenylalanine kinetics, differences were detected in Chapter 4. As discussed previously in Chapter 5 and Chapter 6, it is likely that the lack of differences between treatments was due to the diets and does not reflect on the effectiveness of the method. Furthermore, isotopic methods have been used very successfully in humans as reviewed by Elango et al. (2012a) and swine as reviewed by NRC (2012).

### Diet formulation for studies of amino acid requirements in horses

Diet formulation for the determination of amino acid requirements in horses presents several challenges. In order to make the test amino acid limiting, diets are generally made low in protein. Diets low in protein have the potential for more than one amino acid to be limiting. Additionally, the lack of empirically determined amino acid requirements for horses make it difficult to know which indispensable amino acids are limiting and need to be added to an experimental diet for only the test amino acid to be limiting. Future researchers may wish to supplement experimental diets with all indispensable amino acids, with the exception of the test amino acid. If the diets utilized in Chapter 5 had been supplemented with additional indispensable amino acids, we may have been able to determine a lysine requirement for yearling horses.

Another challenge in precision formulating of equine diets is variability of feed ingredients (Table 8.1), particularly forage from year to year. The forage used in Chapter 5 was ~3% lower in crude protein and the threonine concentration was also less than the forage used in the subsequent weanling study (Chapter 6). As forage makes up a major component of equine diets, forage quality can play a large role in the amino acid adequacy of equine diets. Amino acid content of legumes and grasses has been known to change as the plants mature (NRC, 2001). Drought and nitrogen fertilization can also cause changes in amino acid content (Belesky et al., 1984). Seasonal changes in amino acid content of pasture have been shown to be reflected in equine plasma concentrations (Graham-Thiers and Bowen, 2009). Variation in forage amino acid content will likely continue to be problematic for equine nutritionists attempting to precisely formulate for amino acids in equine diets.

**Table 8.1: Threonine and lysine concentrations of packaged forages for horses**

| **Forage** | **Alfalfa cubes** | **Timothy cubes** | **Timothy cubes** | **Chopped timothy** |
|---|---|---|---|---|
| Chapter | 4 | 5 | 6 | 6 and 7 |
| Year | 2010 | 2011 | 2012 | 2013 |
| *AA Composition (%)* | | | | |
| Threonine | 0.40 | 0.18 | 0.24 | 0.19 |
| Lysine | 0.62 | 0.25 | 0.41 | 0.20 |
| *Proximate Analysis "As Fed"* | | | | |
| DE Mcal/kg | 2.06 | 1.69 | 1.78 | 1.97 |
| CP % | 17.9 | 7.1 | 10.5 | 7.3 |

**Amino acid supplementation of equine diets**

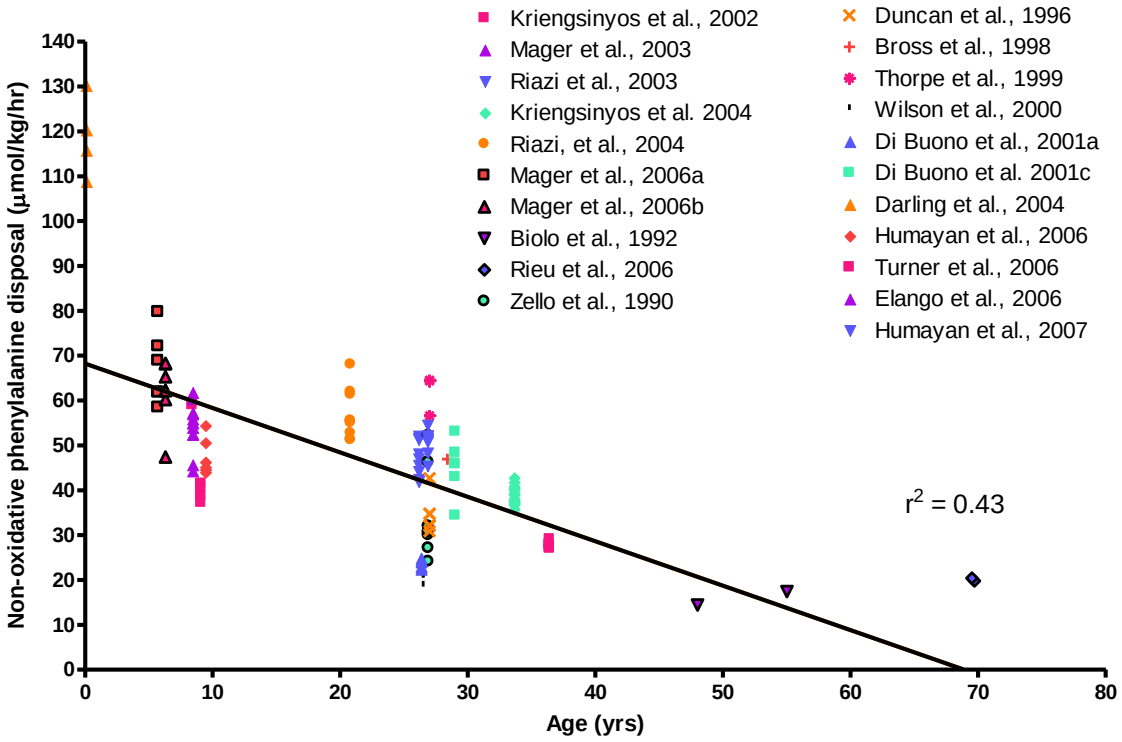
Amino acid supplementation of equine diets is likely to continue. Owners/caretakers top dress added supplements and the commercial companies often add amino acids to account for the aforementioned variability in forages. As demonstrated by the plasma amino acid concentration data in Chapter 5 and Chapter 6, changing the

intakes of two amino acids in the diet affects the metabolism of many more amino acids. Additional studies aimed at understanding the implications of long term supplementation on metabolism could be helpful in determining guidelines for supplementation. So far no negative side effects have been reported in horses consuming diets supplemented with crystalline amino acids (Fisher et al., 1989; Graham-Thiers and Kronfeld, 2005a; Graham-Thiers et al., 2000; Graham et al., 1994; Staniar et al., 2001). However, negative effects due to imbalances (Harper et al., 1970) and over supplementation (Garlick, 2004) of amino acids have been reviewed in other species.

**Phenylalanine non-oxidative disposal and balance across the lifespan**

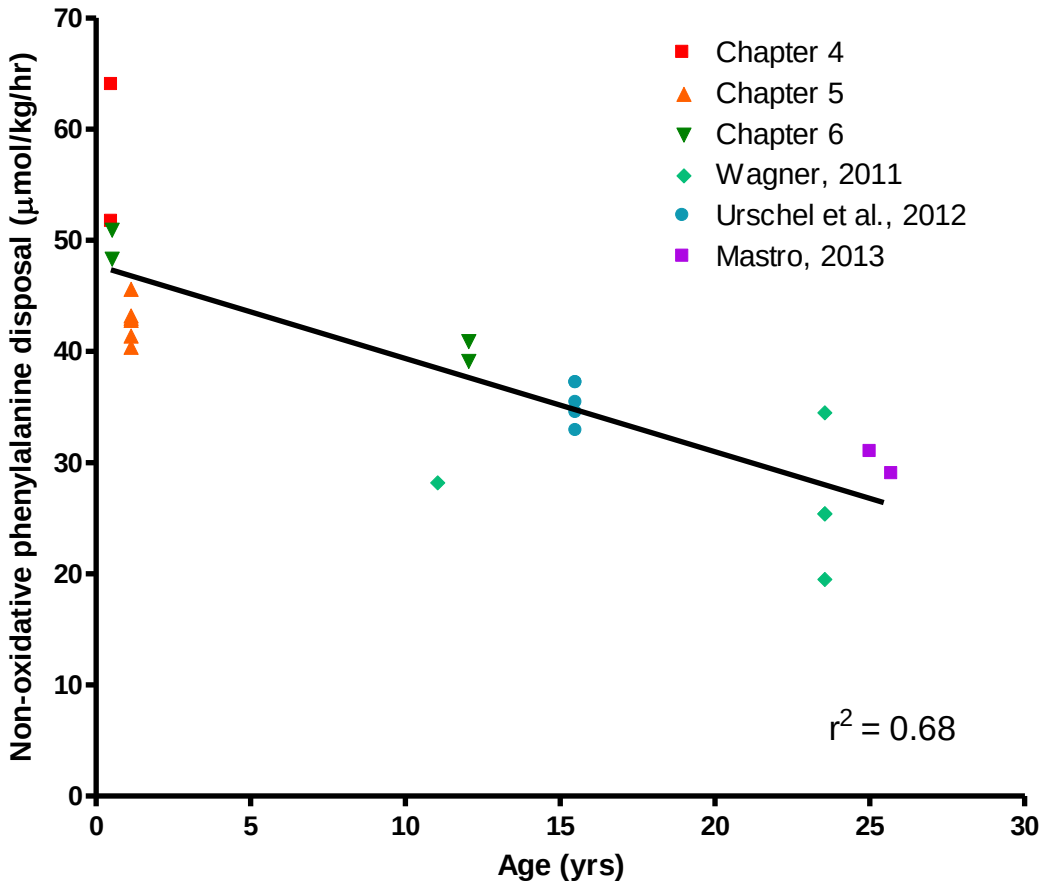
Unfortunately, studies using phenylalanine kinetics are often short term and differences in oxidation or non-oxidative disposal have not been correlated with measures of animal production goals, such as growth rate. However, phenylalanine kinetics are dependent on age in humans (Figure 8.1 and Figure 8.3) and horses (Figure 8.2 and Figure 8.4). In both species, non-oxidative phenylalanine disposal decreases with age. Interestingly, this relationship holds true, despite the different studies having different diets, genders, treatments and subject criteria. Similar figures for swine data were not created as much of the swine data was reported in graph form or non-oxidative disposal and/or flux were not reported.

**Figure 8.1: Relationship between non-oxidative phenylalanine disposal and age in humans[1]**



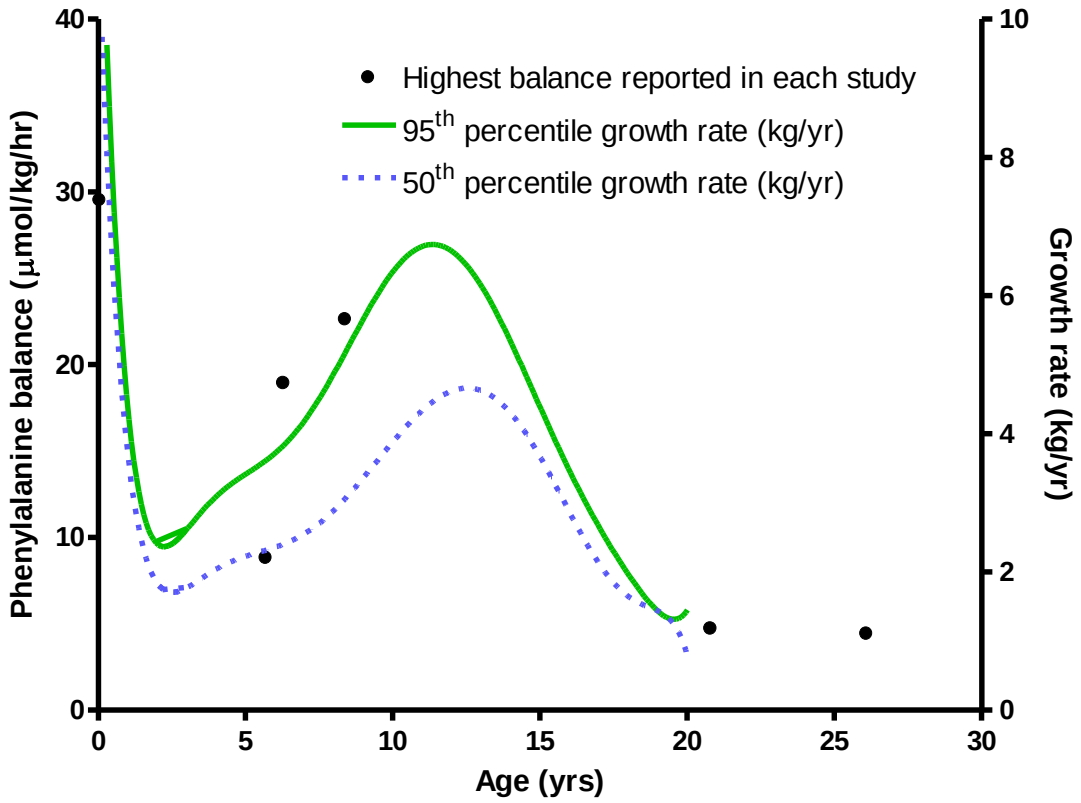

[1] Data for this figure was taken from literature using [1-$^{13}$C]phenylalanine on humans reporting age, and either non-oxidative phenylalanine disposal and/or flux and oxidation (flux - oxidation = non-oxidative disposal) (Biolo et al., 1992; Bross et al., 1998; Darling et al., 2004; Di Buono et al., 2001a, c; Duncan et al., 1996; Elango et al., 2007; Humayun et al., 2007a; Humayun et al., 2006; Kriengsinyos et al., 2002; Kriengsinyos et al., 2004; Mager et al., 2003; Mager et al., 2006a, b; Riazi et al., 2004; Riazi et al., 2003; Rieu et al., 2006; Thorpe et al., 1999; Turner et al., 2006; Wilson et al., 2000; Zello et al., 1990). All treatments were included.

**Figure 8.2: Relationship between non-oxidative phenylalanine disposal and age in horses**[1]



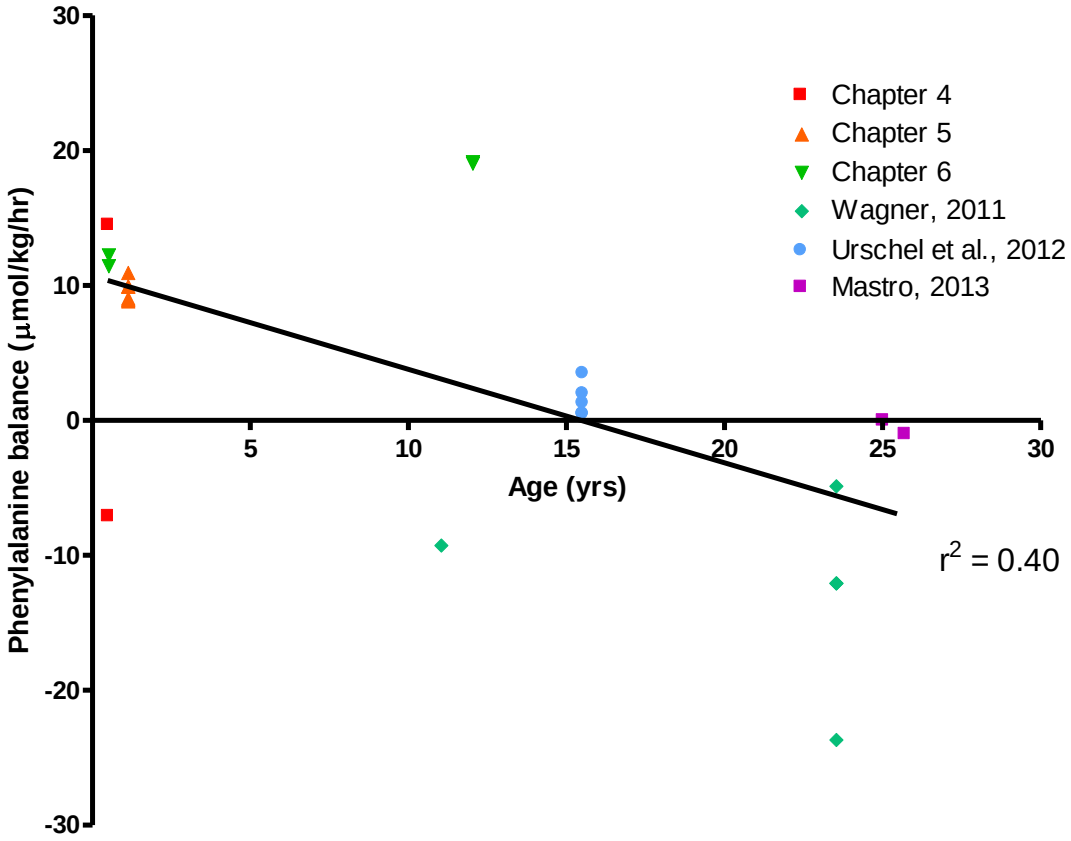

[1] Data for this figure was taken from literature, including the present studies (Chapter 4, Chapter 5, and Chapter 6), using [1-$^{13}$C]phenylalanine infusion in horses reporting age and non-oxidative phenylalanine disposal (Mastro, 2013; Urschel et al., 2012; Wagner, 2011). All treatments were included.

**Figure 8.3: Relationship between phenylalanine balance, age, and growth rate in humans**[1]




[1] Phenylalanine balance data for this figure was taken from articles reporting age, phenylalanine intake and oxidation in humans for [1-$^{13}$C]phenylalanine infusion studies (Darling et al., 2004; Mager et al., 2003; Mager et al., 2006a, b; Riazi et al., 2004; Riazi et al., 2003). Only the highest balance was used from each study in an attempt to minimize variation imposed by the various treatments of the studies. The growth curves are calculated from CDC growth reference charts using projected growth in the 95$^{th}$ and 50$^{th}$ percentiles (CDC, 2001).

**Figure 8.4: Relationship between phenylalanine balance and age in horses[1]**




[1] Data for this figure was taken from literature, including the present studies (Chapter 4, Chapter 5, and Chapter 6), using [1-$^{13}$C]phenylalanine infusion in horses reporting age, phenylalanine intake, and phenylalanine flux (Mastro, 2013; Urschel et al., 2012; Wagner, 2011). All treatments were included.

Phenylalanine balance can be taken as a measure of protein accretion. In horses, phenylalanine balance decreases with age (Figure 8.4), making the growth obtained in the young horse important for the life of the horse. Protein accretion would be expected to correlate with fat free gain and fat free gain has been correlated with growth rate in humans (Xiong et al., 2012). Phenylalanine balance appears to follow a similar pattern as growth rate in humans, but there is a distinct lack of phenylalanine kinetics data on humans between the ages of 10 and 20 during the adolescent growth spurt (Figure 8.3).

More data is needed in the horse to determine a correlation between phenylalanine balance and growth rate. As horses are athletic animals, muscle growth is particularly important. Maintaining muscle is equally important, as horses are often kept and used well into old age. Future studies on different ages of horse will add to our understanding of protein metabolism over the life-span of the horse.

**Determining requirements for different growth rates**

By definition, the indicator amino acid oxidation method of determining amino acid requirements determines the lowest intake of the test amino acid where whole-body protein synthesis is maximized. If whole-body protein synthesis is maximized, growth rate is also likely maximized. However, maximal growth rate is not necessarily optimal in horses. High rates of growth have been associated with osteochondrosis in horses as reviewed by Jeffcott (1991) and Stromberg (1979). Maximal growth rate can be influenced by a limiting nutrient, such as an amino acid or energy, as well as genetic potential. Theoretically, the indicator amino acid oxidation method should be able to determine amino acid requirements at submaximal growth rates if another nutrient, such as energy is used to "cap" growth to the desired rate by being made deliberately limiting equally across treatments. If optimal growth for horses is submaximal, the question of how to best control growth rate becomes an interesting one. There may be advantages/disadvantages for regulating growth rate via dietary amino acids, as opposed to dietary energy or selective breeding.

**Opportunities for future research involving fiber and the splanchnic extraction of threonine**

Knowing the splanchnic extraction of phenylalanine is useful for phenylalanine kinetics calculations, however, future research on the splanchnic extraction of other amino acids may be able to increase our understanding of amino acid metabolism in the horse. Splanchnic extraction of lysine (Chapman et al., 2013; Kriengsinyos et al., 2002), threonine (Chapman et al., 2013), and glutamine (Boza et al., 2001) have been determined in humans. Measuring splanchnic extraction of threonine in horses on diets with varying amounts and types of fiber would be of interest as threonine is used for mucin production, which is influenced by dietary fiber in other species, such as rats (Ito et al., 2009; Satchithanandam et al., 1990). If splanchnic extraction of threonine in horses is lower than other species, it would help explain why supplementation of threonine did not increase whole body protein synthesis in Chapter 6.

**Opportunities for future research in amino acid requirements and bioavailability**

None of the studies presented here determined an amino acid requirement. Amino acid requirements are needed in order to move past using crude protein in defining protein requirements for horses. Lysine (Tome and Bos, 2007), threonine (Laurichesse et al., 1998), and methionine (Bornstein and Lipstein, 1975) will likely be the first amino acids investigated given that they are commonly limiting in diets of other species. Although the possibility of histidine as a limiting amino acid in horse diets is discussed in Chapter 4 and Chapter 5, it has not been investigated directly. Histidine has been found to be first limiting in at least one cattle diet (Vanhatalo et al., 1999), which may be related to the fact that histidine is first limiting in rumen-isolated proteins fed to rats (Bergen et al.,

1968). Future research will need to be conducted to determine amino acid requirements in a variety of ages and physiological states. Physiological states of particular interest are the exercising horse and the aged horse, as these have been largely uninvestigated in other livestock species. The indicator amino acid oxidation method could be used for the determination of these amino acid requirements, especially now that an oral infusion of isotope can now be used for phenylalanine kinetics studies Chapter 7.

In addition to amino acid requirements, research on amino acid bioavailability in horses is greatly lacking. Only one study investigates ileal amino acid digestibility (Almeida et al., 1999). Studies using phenylalanine kinetics have been used to determine amino acid bioavailability in feedstuffs for swine (Moehn et al., 2005). Use of the indicator amino acid oxidation method may be preferred to ileal cannulation because of expertise needed to perform cannulation surgery (Simmons, 1988) and the difficulty and expense of maintaining cannulated horses (Horney et al., 1973).

**Conclusion**

Although no amino acid requirements were determined in the studies presented in this dissertation, phenylalanine kinetics can be a valuable tool to fill in the research gaps compared to other species described in Chapter 2. Diet formulation will be key to the success of future studies. There are many opportunities for additional research in this vein, as amino acid requirements, bioavailability, and metabolism remain largely unstudied in the horse. These questions are particularly pertinent for growing and athletic horses.

## Appendices

## Appendix A - Extended abstract: Amino acid concentrations in late lactation mares' milk

### Citation:

Tanner, S. L., A. L. Wagner, R. B. Ennis, and K. L. Urschel. 2011. Amino acid concentrations in late lactation mares' milk. Journal of Equine Veterinary Science 31: 267-268.

**Introduction:** The domestic horse is weaned typically between 4 and 6 months of age [1]. The weaning period is often stressful and can be accompanied with an associated decrease in growth rate, especially if weaned at 4 months of age [2]. Elevated stress due to weaning increases the importance of good nutrition prior to this life stage [1]. The National Research Council (NRC) [3] provides nutrient requirement guidelines for 4-6 mo old foals; however, research on protein and amino acid (AA) requirements for this age remains scarce. The free AA profiles of mares' milk over the first 45 d of lactation have been detailed finding that concentrations were higher in colostrum and early lactation (2-5 d) than during middle lactation (8-45 d) [5]. The total AA profile of post-colostrum mares' milk has also been compared to that of other mammalian species with interspecies similarities and differences seen [6]. However, there is also little known about the AA composition of late lactation mares' milk, the main source of nutrition for the foal pre-weaning. The purpose of this study was to determine the free and total AA profiles of late lactation (75-150 d) mares' milk and to determine if milk AA concentrations change during this period.

**Materials and Methods:** *Animals and experimental design.* This was a longitudinal study where milk was collected from 6 Thoroughbred (TB) mares in late lactation (75-

150 d), on a weekly basis over a period of 8 weeks. Approximately 5 mL of milk was obtained from each mare between 0900 and 1000 on each sampling day.

*Sample preparation.* Samples were frozen immediately after collection and stored at -20_C for further processing. The process for determining milk free AA concentrations was slightly modified from a previously described methodology [6]. Briefly, norleucine was added to thawed milk aliquots of 100 mL. Fat and protein were removed using 10,000 kDa spin filter centrifuge tubes at 14,000 x g for 30 minutes for the determination of free AAs via HPLC. Milk total AA concentrations were determined by hydrolyzing 200 mL of milk with 4 mL 6 N HCl at 110°C for 24 hrs prior to removing fat with spin filter centrifuge tubes. Total AAs were also determined via HPLC adapted from Davis et al. [6].

*Calculations and statistics.* Samples were divided into lactation class for statistical analysis (75 - 100 d, n = 12; 101 - 125 d, n = 20; or 126 - 150 d, n = 12). The effect of lactation class on individual total and free AA content was analyzed using repeated measures in the mixed models procedure of SAS (SAS Institute, Cary, NC). The amount of protein per kg of milk was determined by summing AA amounts per kg.

**Results and Discussion:** The average protein content of the TB milk collected for this study was 21 ± 2.8 g/kg and this is consistent with the amount of protein found in QH milk (21 g/kg) [7] and Portuguese Lusitano milk (18 g/kg) [4]. In general, milk composition was relatively unchanged during this period (Table 1). Free Met (29 ± 3 mmol/L 75-100 d; 23 ± 3 mmol/L 101-125 d; 20 ± 3 mmol/L 126-150 d) decreased ($P < .05$) as lactation progressed. Lactation period also affected total concentrations of Thr

(5220 ± 288 mmol/L 75-100 d; 4197 ± 196 mmol/L 101-125 d; 3860 ± 287 mmol/L 126-150 d) ($P < .01$) and Asp+Asn (10225 ± 938 mmol/L 75 - 100 d; 9304 ± 834 mmol/L 101 - 125 d; 8149 ± 960 mmol/L 126 - 150 d) ($P < .1$), which decreased as lactation continued, although there was no effect of lactation day on the free concentrations of these AA (Thr: 113 ± 14; Asn+Asp: 96 ± 3). The results of this study show that the majority of all AAs exist as a part of protein in milk (Table 1). Subsequent research should be more thorough and include direct measurements of foal intake and growth, so that amino acid intakes in the context of actual growth rates can be quantified.

**Table 1:** Total and Free AA Content of Late Lactation Mares' Milk

| AA | Total† | SD | Free† | SD |
|---|---|---|---|---|
| Glu+Gln | 23103 | 1726 | 1295 | 22 |
| Ser | 7177 | 1214 | 155 | 6.6 |
| Gly | 5358 | 262 | 101 | 12.6 |
| His | 2938 | 196 | 18 | 1.2 |
| Arg | 6741 | 348 | not available | - |
| Ala | 7005 | 423 | 86 | 6.6 |
| Pro | 12927 | 574 | 7 | 1.4 |
| Tyr | 3941 | 371 | 27 | 0.5 |
| Val | 9470 | 462 | 57 | 8 |
| Met | 2593 | 218 | in text | in text |
| Ile | 7408 | 484 | 9 | 0.9 |
| Leu | 13186 | 761 | 14 | 0.4 |
| Phe | 5213 | 272 | 4 | 0.1 |
| Lys | 9928 | 567 | 12 | 0.3 |

† Values are least squares means and standard deviations as determined by the SAS mixed procedure expressed as mmol/L milk. Asp+Asn and Thr are shown in text.

**Conclusion:** The purpose of this study was to gain information about the AA composition of late lactation mares' milk and we found that AA content did not change significantly during the 75-150 d phase of lactation and that most of the AAs present

were bound in protein. Knowledge of milk amino acid concentrations in combination with a greater knowledge of AA requirements during the critical pre-weaning period could lead to the formulation of more nutritionally effective diets for foals pre-weaning.

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**Appendix B - 1° antibody sources, dilutions, and incubation times used in the Western blotting procedures**[†]

| **Protein** | **Antibody source company** | **Dilution factor**[‡] | **Incubation length and conditions** |
|---|---|---|---|
| p-4E-BP1 | Cell Signaling | 1 : 1000 | Overnight rocking @ 4°C |
| 4E-BP1 total | Cell Signaling | 1 : 1000 | Overnight rocking @ 4°C |
| rpS6-P ($Ser^{235/236}$ $Ser^{240/244}$) | Cell Signaling | 1 : 2000* | Overnight rocking @ 4°C |
| rpS6 total | Cell Signaling | 1 : 10,000 | Overnight rocking @ 4°C |
| p-p70 ($Thr^{389}$) | Santa Cruz | 1 : 25,000 | 1 hr rocking at room temperature |
| p70 total | Cell Signaling | 1 : 1000 | Overnight rocking @ 4°C |
| P-Akt ($Ser^{473}$) | Cell Signaling | 1 : 2000 | Overnight rocking @ 4°C |
| Akt total | Cell Signaling | 1 : 2000 | Overnight rocking @ 4°C |

[†]The Western blotting procedure is detailed in Chapter 5
*Each of the 2 antibodies in a half and half mix
[‡]1° antibody in 1X TBST + 5% fat-free milk powder

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**Vita**

Sara L. Tanner

**Education:**

**PhD, Animal and Food Sciences,** in progress
*University of Kentucky, Lexington, KY*
Additional certificate: Graduate Certificate in College Teaching and Learning

**Bachelor of Professional Studies in Management,** May 2009
*Cazenovia College, Cazenovia, NY*
Specialization: Equine Business Management
Minors/Certificates: Biology, Pre-Law, Certificate in Management & Supervision

**Experience:**

**South Dakota State University,** Brookings, SD, August 2014 – present
*Instructor, Department of Animal Sciences*

**University of Kentucky,** Lexington, KY, August 2010 – August 2014
*Graduate Research Fellow, Department of Animal and Food Sciences*
*Teaching Assistant for ASC 378, Animal Nutrition,* August 2011-December 2011

**Midway College,** Midway, KY, August 2013-May 2014
*Adjunct for EQS 106: Applied Equine Studies and EQS 215: Equine Science Lab*

**Corning Community College,** Corning, NY, March 2010-July 2010
*Health and Sciences - Bio/Chem Clerk*

**Smith-Photography,** Sayre, PA, August 2009-July 2010
*Photo Studio Assistant*

**Cazenovia College,** Cazenovia, NY
*Layout Coordinator/Editorial Assistant,* August 2007-July 2011
*Academic Learning Center Peer Tutor for Equine Courses,* August 2008-May 2009
*Equine Anatomy Master Student,* August 2008-December 2008
*Assistant Coordinator Cargill Animal Nutrition Feeding Trial,* January 2007-May 2007

**Ledgewood Equine Veterinary Clinic,** Ontario, NY, December 2007-January 2008
*Business Management Intern*

**Foxmoor Farm,** Horseheads, NY, June 2006-August 2008
*Employee*

**Tanner Farms LLC,** Elmira, NY
*Family farm*

## Honors:

**University of Kentucky**, Lexington, KY

- 1st place poster 2014 Animal & Food Sciences Graduate Association annual poster symposium, 2nd place in 2013
- North American Regional Winner and Global Finalist for the Alltech Young Scientist Competition 2013
- 3rd place poster 2013 Experimental Animal RIS of the American Society of Nutrition in conjunction with Experimental Biology, Boston, MA
- $400 travel award to attend the 2012 American Academy of Veterinary Nutrition Clinical Nutrition and Research Symposium by the American Academy of Veterinary Nutrition
- Selected as a pre-doctoral scholar in the Research Training in Muscle Biology of Cardiopulmonary Disease NIH training program November 2011-November 2013
- Graduate School Academic Year non-service fellowship for 2010-2011 and 2011-2012

**Cazenovia College**, Cazenovia, NY

- Valedictorian Cazenovia College Class of 2009
- Most Outstanding Senior Capstone in Equine Business Management 2009
- Excellence in Equine Business Management Program Award 2009
- Dressage Team MVP 2009
- Tied for Best Argument Overall in the Debate Society's 2009 Great Debate
- Who's Who Among Students in American Universities and Colleges 2009
- Emerson Scholarship 2006-2009
- Don Roy Scholarship 2008

## Affiliations and additional activities:

**Professional memberships:**

- American Society of Animal Science (ASAS) Member 2012-present
- Equine Science Society (ESS) Member 2011-present
- American Academy of Veterinary Science (AAVN) Member 2012-present

**University of Kentucky,** Lexington, KY

- 4H volunteer
- Kentucky Equine Survey volunteer, May 2012 – October 2012
- Animal & Food Sciences Graduate Association (AFSGA) Member 2010-present
- University of Kentucky's Equine Programs 2010-present

**Cazenovia College,** Cazenovia, NY

- Alpha Chi 2009
- Student Athlete Advisory Committee 2008-2009
- Equine Ambassador Club 2006-2009
- Intercollegiate Dressage Association (IDA) Team 2005-2009

**Publications and presentations:**

*Journal article:*

**Tanner, S.L.**, A.L. Wagner, Digianantonio, R.N., P.A. Harris, J. Sylvester, & K.L. Urschel. 2014. Dietary protein intake influences rates of whole-body protein synthesis in weanling horses. The Veterinary Journal. http://dx.doi.org/10.1016/j.tvjl.2014.06.002

*Grant submitted:*

Validation of oral [1-13C]phenylalanine administration procedures to study amino acid requirements in horses using the indicator amino acid oxidation procedure. University of Kentucky Internal Grant 2012. Status: Not funded – PI already well funded.

*Abstracts:*

**Tanner**, **S. L.**, T. Barnes, K. Cybulak, and K. L. Urschel. 2014. Splanchnic Extraction of Phenylalanine in Adult Thoroughbred Mares Fed Two Different Levels of Threonine. 2014 ADSA ASAS Joint Annual Meeting, Kansas City, MO. 4336 (Poster)

**Tanner**, **S.L.**, L.R. Good, E. A. DeLuca, R. J. Coleman, & K. L. Urschel. 2013. Muscle free amino acids are affected by varying levels of lysine intake in yearling Thoroughbred colts. 2013 Animal & Food Sciences Graduate Association Poster Symposium, Lexington, KY. (Poster)

Mastro, L.M., N.A. Holownia, **S.L. Tanner**, T. Barnes, M.G. Sanz, A.A. Adams & K.L. Urschel. 2013. Pituitary pars intermedia dysfunction does not affect various measures of insulin sensitivity in old horses. 2013 ESS Meeting, Mescalero, NM. (#PG1)

**Tanner, S.L.**, A. Moffet, P.A. Harris & K.L. Urschel. 2013. Threonine supplementation does not increase protein synthesis in weanlings receiving a grass forage and commercial concentrate. 2013 ESS Meeting, Mescalero, NM. (#NG15)

**Tanner, S.L.**, L.R. Good, E. A. DeLuca, R. J. Coleman, & K. L. Urschel. Free Amino Acid Content of Equine Muscle Changes in Response to Graded Levels of Lysine Intake. 2013 Experimental Biology. Boston, MA. 4316. (Poster)

**Tanner**, **S.L.**, L.R. Good, E. A. DeLuca, R. J. Coleman, & K. L. Urschel. 2012. Increased lysine intake in yearling Thoroughbred colts results in increased plasma lysine after a meal, but not activation of the mTOR signaling pathway. 2012 University Center for Muscle Biology Retreat, Lexington, KY. (Poster)

**Tanner**, **S.L.**, R.N. Digianantonio, A.L. Wagner, P.A. Harris, J. Sylvester, and K.L. Urschel. 2012. Reducing dietary crude protein intake limits whole body protein synthesis in growing horses even when NRC requirements are met. 2012

American Academy of Veterinary Nutrition Clinical Nutrition and Research Symposium, New Orleans, LA. (Presentation)

**Tanner**, **S. L.**, R. J. Coleman, K. L. Urschel. 2012. Feeding graded amounts of lysine to yearling thoroughbred colts does not activate the mTOR signaling pathway. 2012 ADSA ASAS Joint Annual Meeting, Phoenix, AZ. 51326 (Presentation)

**Tanner**, **S. L.**, A. L. Wagner, R. N. Digianantonio, P. A. Harris, J. T. Sylvester & K. L. Urschel. 2012. Reducing dietary crude protein intake limits whole body protein synthesis in growing horses, even when NRC requirements are met. 2012 Animal & Food Sciences Graduate Association Poster Symposium, Lexington, KY. (Poster)

L.M. Mastro, **S.L. Tanner**, T. Barnes, K.L. Urschel. 2012. 24 hours of acid hydrolysis is sufficient to detect maximum concentrations of most amino acids in pelleted feeds and forages fed to horses. 2012 Animal & Food Sciences Graduate Association Poster Symposium, Lexington, KY. (Poster)

**Tanner, S.L.** and K.L. Urschel. 2011. Determining lysine requirements in yearling horses using indicator amino acid oxidation. 2011 Animal & Food Sciences Graduate Association Poster Symposium, Lexington, KY. (Poster)

Wagner, A.L., R.N. Digianantonio, **S.L. Tanner**, R.B. Ennis, P.A. Harris, J. Sylvester, and K.L. Urschel. 2011. Glycemic and insulinemic responses of weanling horses to high and low protein diets. 2011 ADSA ASAS Joint Annual Meeting, New Orleans, LA. 45513. (Poster)

**Tanner, S.L.**, A.L. Wagner, R.B. Ennis, and K.L. Urschel. 2011. Amino Acid Concentrations in Late Lactation Mare's Milk. 2011 ESS Meeting, Murfreesboro, TN. 44224. (Presentation)

Digianantonio, R.N., A.L. Wagner, **S.L. Tanner**, R.B. Ennis, P.A. Harris, J. Sylvester, and K.L. Urschel. 2011. Postprandial changes in plasma free amino acids in weanling horses. 2011 ESS Meeting, Murfreesboro, TN. 44243. (Presentation)

**Tanner, S.L.**, A.L. Wagner, and K.L. Urschel. 2010. Biopsy depth had no effect on amino acid concentrations or translation initiation factor activation in equine gluteus medius muscle. 2010 University Center for Muscle Biology Retreat, Lexington, KY. (Poster)