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TOWARDS DETERMINATION OF THE THREONINE REQUIREMENT OF YEARLING HORSES FED VARYING DIETARY COMPOSITIONS USING THE INDICATOR AMINO ACID OXIDATION METHOD

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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2016

ABSTRACT OF THESIS

DETERMINATION OF THE THREONINE REQUIREMENT OF YEARLING HORSES FED VARYING DIETARY COMPOSITIONS USING THE INDICATOR AMINO ACID OXIDATION METHOD

The amino acid requirements of growing horses are currently unknown, and studies suggest that threonine is a limiting amino acid in common horse diets. Thus, the objective of this study was to determine the threonine requirement of growing horses fed two different forage to concentrate ratios using the indicator amino acid oxidation (IAAO) method. The study consisted of a high concentrate phase (HC; 60% concentrate and 40% forage) and a high forage phase (HF; 25% concentrate and 75% forage). Within each phase, 6 yearling horses were randomly assigned each of 6 dietary treatments in a 6 x 6 Latin square design. All 6 treatments were identical, apart from varying equimolar ratios of threonine to glutamate. After 6 days of adaptation, blood samples were collected for plasma urea nitrogen (PUN) and amino acid analysis. On day 7, horses underwent the IAAO protocol, during which regular breath and blood samples were collected. Phenylalanine flux, oxidation, non-oxidative disposal, and release from body protein, as well as total carbon dioxide production were calculated using plateau enrichment of samples. There was a significant linear effect of threonine intake on plasma threonine concentrations, and PUN had a significant linear response during the HC phase. There was no significant effect of treatment on phenylalanine oxidation during either phase ($P \geq 0.05$). It is unlikely that threonine was limiting in the experimental diets.

KEYWORDS: Equine, Nutrition, Indicator Amino Acid Oxidation, Dietary Protein, Amino Acid Requirements

Kelsey Michelle Smith
May 4th, 2016

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Chapter 1. Introduction

Optimal growth of young horses is dependent on adequate energy and protein intake (Ott and Asquith, 1986). The crude protein requirement of growing horses has been previously determined (NRC, 2007) and provides the basis for feeding practices in the United States. However, we understand that horses have a dietary requirement for each of the essential amino acids, which cannot be synthesized sufficiently by mammalian enzymes, as well as nitrogen for the synthesis of non-essential amino acids, rather than a requirement for crude protein. For convenience, the L- isomer of amino acids is implied in the present thesis unless otherwise noted. The requirements for individual amino acids have not been determined in horses. To ensure that individual amino acid requirements are met, horses are commonly fed crude protein far in excess of their requirement, with many consuming up to twice the recommended intake (Gallagher et al., 1992; Honoré and Uhlinger, 1994; Harper et al., 2009).

In previous studies, animals consuming less than the recommended crude protein, but balanced for the requirement for each individual amino acid, had reduced nitrogen excretion and equal gains and production compared with those consuming a standard diet (Le Bellego et al., 2001; Alagawany et al., 2014; Mantovani et al., 2014; Lee et al., 2015; Rachuonyo et al., 2015). Excess nitrogen excretion can result in harmful ammonia inhalation by the animal and its caretaker, as well as increased nitrogen runoff that could damage waterways (Harper et al., 2009). There is also evidence that consuming an excess of protein can lead to a negative calcium balance (Glade et al., 1985), metabolic acidosis (Graham-Thiers and Kronfeld, 2005b), and poor athletic performance in horses (Graham-Thiers et al., 1999). Due to the improvement of nitrogen excretion seen in other animals

fed diets balanced for amino acids, and the possible deleterious effects of excess crude protein intake, efforts are now underway to determine the requirements for each essential amino acid in horses.

An essential amino acid that is not adequately fed, and therefore limits protein synthesis, is known as the limiting amino acid. Lysine has been determined to be the first limiting amino acid in most horse diets, and the requirement has been determined by the National Research Council based on the lysine content of diets that result in maximal average daily gain and reduced plasma urea nitrogen in growing horses (NRC, 2007). Previous research suggests that threonine is the next limiting amino acid in horse diet (Graham et al., 1994b; Staniar et al., 2001; Graham-Thiers and Kronfeld, 2005a). Threonine is an essential amino acid found in most tissues, and is a major component of mucin, a protein responsible for lubricating the gastrointestinal tract. Mucin production increases in response to increased fiber content of the digesta in other species (Montagne et al., 2004) and, consequently, the threonine requirement for horses also may differ depending on the fiber content of the diet.

As animals consume increasing amounts of the limiting amino acid, protein synthesis increases until a different amino acid is limiting or until maximal rates of protein synthesis are achieved. Amino acids leave the free amino acid pool in the body via incorporation into protein, conversion to other metabolites, or oxidation. If the total flux of the free amino acid pool is known, and the rates of amino acid metabolism and oxidation are measured, then the estimated rate of protein synthesis can be derived. This principle is the basis for indicator amino acid oxidation method (IAAO), which has been used successfully to determine amino acid requirements of poultry, swine, and humans

(Tabiri et al., 2002; Myrie et al., 2014; Ishikawa-Takata, 2015). As intake of the limiting amino acid increases, the rate of oxidation of the indicator amino acid, typically phenylalanine, decreases until the requirement for that amino acid is met, at which point the rate will plateau. This breakpoint is the requirement for the amino acid.

The objectives of this study were to determine the threonine requirement for yearling horses, and to observe any differences of that requirement due to the fiber content of the diet.

Chapter 2. Literature Review

At birth, horses weigh an average of 9.7% of their adult weight. By 12 months of age, they weigh an average of 64.2% of their adult weight (NRC, 2007). For a horse with a mature body weight of 500 kg, that is a gain of 272.5 kg. The majority of that gain is protein, and it is essential that growing horses receive both sufficient dietary energy and sufficient dietary protein throughout their first year of life to achieve optimal rates of growth.

Dietary protein sources, digestion, and bioavailability

Fresh forages, grass and legume hays, and pelleted concentrates containing ingredients such as soybean meal are common sources of protein within horse diets. Alternative sources of nitrogen, such as urea, are not well-utilized by horses and can be toxic at very low levels (Godbee and Slade, 1981; Martin et al., 1991; Schubert et al., 1991).

Within non-ruminant mammalian species, protein digestion begins in the stomach, where it is denatured by hydrochloric acid and cleaved into polypeptides by pepsin. Upon entering the lumen of the small intestine, polypeptides are further cleaved to tri- and dipeptides through the action of pancreatic proteolytic enzymes (Silk et al., 1985). A portion of these small peptides are further digested to amino acids by brush border membrane enzymes. Peptides and amino acids are then absorbed by the small intestine. Following absorption into enterocytes, amino acids can be utilized by the cell for energy or protein synthesis, be converted to other metabolites, or exit through the basolateral membrane and enter the plasma. Plasma amino acids then travel to the liver,

which can also use them for energy, protein synthesis, or conversion to other metabolites. Amino acids exiting the liver are then available for use in peripheral tissues.

Much of the dietary and endogenous protein that reaches the large intestine is metabolized by the extensive microbial population. In contrast with ruminant species, microbial protein within the large intestine is minimally useful to horses (Reitnour et al., 1970). The majority of nitrogen absorbed at this site is in the form of ammonia (Reitnour and Salsbury, 1975), which is converted to urea or used to aminate the carbon moieties of amino acids. There is some evidence that amino acid transporters may be active within the large intestine (Woodward et al., 2010). However, the contribution of these transporters to the free amino acid pool is unknown (Hendriks et al., 2012).

The amount of protein reaching the large intestine is directly related to the form of the protein within the diet. Much of the protein found in forages is bound within cellulose, which is digested by microbes in the large intestine. This results in a lower pre-cecal digestibility of protein within forage based diets (Gibbs et al., 1996). Other factors that may affect protein digestibility include feed processing treatments (Raunio et al., 1978). Crystalline amino acids are commonly supplemented in swine diets to balance amino acid intakes, and have been shown to be 100% digestible (Chung and Baker, 1992).

Crude protein as a measure of dietary protein concentration

Crude protein is the standard measurement of the protein content of horse feeds within the U.S., and is calculated by dividing the nitrogen content of the diet by an assumed nitrogen concentration of protein, often 16% (Jordan and Myers, 1972). As the

protein content of feeds in the US is reported as crude protein, the protein requirements of horses are also expressed as crude protein.

The crude protein requirement of a growing horse can be estimated using the following equation, where E equals the efficiency of dietary protein use for gain, which gradually decreases from 0.5 in weanlings to 0.3 for yearling horses (NRC, 2007):

$$CP\ requirement = (BW\ \times 1.44\ g\ CP/kg\ BW) + \left(\frac{ADG \times 0.20}{E}\right)/0.79$$

Using this equation, a horse aged 12 mo and predicted to have a mature bodyweight of 500 kg is estimated to require 846 g of crude protein per day. Therefore, a yearling horse consuming 2.5% of his bodyweight in dry matter each day should consume a ration that is approximately 10.5% crude protein. This requirement was estimated by modeling the level of crude protein intake resulting in maximal nitrogen retention and average daily gain in horses fed various diets including coast-cross hay, corn, and soybean meal (de Almeida et al., 1998), soybean meal and alfalfa (Ott and Asquith, 1986), and soybean meal and Bermudagrass hay (Ott and Kivipelto, 2002).

The functional unit of protein is the amino acid, and amino acids are needed in varying amounts to support all protein synthesis needs within the body. Essential amino acids, by definition, cannot be synthesized in adequate quantities by mammalian enzymes and must be provided in the diet in the amounts required for both the synthesis of proteins and the synthesis of non-essential amino acids. Non-essential amino acids are also required by the body, but they can be synthesized by mammalian enzymes and do not need to be consumed. As most animals, including horses, have a requirement for amino acids rather than crude protein, the diets found to result in optimum nitrogen utilization in

these crude protein requirement studies actually contained amino acids in amounts most closely matching that needed for protein synthesis. Feeding for the requirement of each amino acid rather than total nitrogen is a concept known as “ideal protein”.

Although the National Research Council provides clear recommendations for crude protein intake, many horse owners believe that increasing crude protein intake will improve performance and health. Horse feed companies often market their feeds based on the concentration of protein, often listing the percentage without stating to what it refers. These factors, along with poor estimates of the protein content of fresh forage and total intake volumes, result in excess crude protein intake rates across the country. Racing thoroughbreds at a Detroit racetrack consumed an average of 126% of NRC recommendations for crude protein intake (Gallagher et al., 1992). In a survey of the owners of 50 adult riding horses in North Carolina, 72% consumed protein in excess of NRC recommendations (Honoré and Uhlinger, 1994). Eleven horse farms within the Chesapeake Bay Watershed, housing a total of 201 horses, fed an average of 157% of NRC recommendations for crude protein intake, with some as high as 260% (Harper et al., 2009). Crude protein is typically the most expensive portion of a diet, and most animal production systems work to minimize feed costs as much as possible. In contrast, many horse farms are recreational, or the perceived risk of decreasing performance by changing a feeding regimen outweighs the decrease in feed costs (Rotz, 2004). Consequently, the increased feed costs of high protein diets is not sufficient to deter horse owners from feeding excess nitrogen.

Consequences of a high level of protein intake

Free amino acids cannot be stored, and, therefore, any consumed in excess of requirements for protein synthesis, or use for synthesizing other metabolites, must be oxidized. Excess nitrogen released from catabolized amino acids is excreted in the urine. Thus, feeding excess amino acids results in increased nitrogen excretion (Williams et al., 2011).

Nitrogen loss to the environment

Urinary nitrogen is primarily in the form of urea, which is then converted to ammonia by microbes in the environment. Ammonia can then volatize into the atmosphere, or be converted to nitrate and remain dissolved in soil or ground water (De Vries et al., 2015). Volatilized ammonia can then dissolve into surface waters (Reed et al., 2015). Nitrates entering the soil in excess of plant uptake and soil binding capacity also leach and run off to ground and surface waters. Both of these processes result in increased nitrogen concentrations in bodies of water, which leads to eutrophication of the water and destruction of aquatic habitats (Bott et al., 2015).

Ammonia inhalation

In addition to damaging the environment, volatized ammonia can harm the respiratory health of horses and farmworkers. Ammonia concentrations have been shown to be high in horse stables, with some reports showing levels considered unsafe by the Center for Disease Control (Bott et al., 2015). It is well understood that high levels of ammonia inhalation can cause inflammation of the respiratory tract and loss of lung function in humans (Crook et al., 1991), as well as horses (Katayama et al., 1995). Ambient ammonia concentration has been shown to be significantly correlated with

exhaled ammonia concentration and the pH of exhaled breath condensate, both of which have been correlated with lower airway inflammation (Duz et al., 2009), when horses were stabled on a variety of bedding types in comparison to horses housed on pasture (Whittaker et al., 2009),.

Physiological effects

In addition to the harmful effects of ammonia inhalation, there is also evidence that the direct physiological effects of increased amino acid oxidation can affect the health and performance of the horse.

Heat production

Heat produced from the utilization of protein for energy is greater than for other substrates due to the inefficiency of catabolizing amino acids and the cost of synthesizing urea (Belko et al., 1986). This loss of energy as heat increases the energy intake requirements of the horse and may contribute to overheating during intense exercise in hot climates. The magnitude of these effects, however, has not been studied in horses and may be physiologically insignificant.

Alteration of acid-base balance

Excess dietary protein results in an acid load to the body, primarily from the release of sulfate from catabolized sulfur-containing amino acids (Mardon et al., 2008). Too large a load can overwhelm the kidney's capacity to maintain plasma pH homeostasis, resulting in decreased pH (Patience, 1990).

The prevalence and effects of this response in horses fed high protein diets is uncertain. Sedentary horses receiving a high protein diet had somewhat decreased plasma pH and plasma bicarbonate concentrations compared with those receiving a low protein diet (Graham-Thiers and Kronfeld, 2005b). This effect can be exacerbated by strenuous exercise, which increases the lactate concentration in the blood and further reduces pH. Studies with mature horses showed that protein restriction significantly reduced the magnitude of the acidogenic effect of exercise (Graham-Thiers et al., 1999; Graham-Thiers et al., 2000; Graham-Thiers et al., 2001; Connysson et al., 2006). These results conflict with other studies which did not observe a reduction in plasma pH (Spooner et al., 2013; Oliveira et al., 2014). However, Spooner et al. did observe a decrease in fecal pH, suggesting that the additional protein was primarily fermented in the hind-gut of the horses and excreted as microbial protein.

It has long been observed in human exercise science that a decrease in plasma pH reduces the rate of lactate efflux from skeletal muscle. This can lead to reduced force of contractility and time to fatigue (McCartney et al., 1983). While it is unlikely that high rates of protein intake affect the performance of horses in light to moderate work, it may be of some concern for horses in strenuous and prolonged work.

Another possible effect of low plasma pH is the mobilization of calcium from bone to buffer the plasma. High protein diets have been shown to increase urine calcium concentrations in horses (Glade et al., 1985), and a direct correlation has been observed between rates of sulfate excretion and rates of calcium excretion (Whiting and Draper, 1980). This increase in calcium excretion is typically coupled by an increase in calcium absorption from the intestines, resulting in zero net loss of calcium (Remer et al., 2014).

Additionally, there is evidence indicating that high dietary concentrations of amino acids other than methionine and cysteine may improve bone health (Gaffney-Stomberg et al., 2010). However, horses consuming calcium-deficient diets may not be able to mineralize bone at the rate that it is being resorbed, leading to bone loss.

Water loss

Urea must be dissolved in relatively large volumes of water to be excreted. Increasing urea production increases the volume of water excreted (Graham-Thiers et al., 2001; Connysson et al., 2006). A recent study found no effect of crude protein concentration of the diet on water intake but the volume of urine increased when crude protein concentrations increased from 7.5% to 13% (Oliveira et al., 2015). These increased rates of urination may negatively affect water balance, and are of particular concern for horses already at risk for dehydration, such as those doing intense work in hot climates.

Reducing crude protein intake

Other species

Due to the list of potentially negative effects of excess nitrogen excretion, it is clear that horse owners should strive to reduce their horses' crude protein intake while meeting their needs for protein synthesis. There is a large movement within the food animal industry to reduce nitrogen released from farms, and there have been countless successful attempts in swine (Girotto et al., 2013), poultry (Ospina-Rojas et al., 2012), and fish (Lima et al., 2015) to reduce crude protein intake without a negatively affecting feed efficiency, egg production, meat quality, or health. The majority of these studies

reduced crude protein intake to below the recommended intake, then supplemented with synthetic amino acids to meet the requirement for each amino acid. Nitrogen outputs from poultry and swine farms using this practice can be reduced by 40% or more without any loss in carcass yield (Nahm, 2002). Similar efforts are underway with ruminant species, but predicting the dietary amino acid requirements of foregut fermenters provides additional challenges due to microbial assimilation altering the composition of amino acids entering the small intestine (Arriola Apelo et al., 2014).

Reducing nitrogen excretion in horses

There has been some work to reduce crude protein intake in growing horses. A recent study using Italian heavy draft horses raised for slaughter found that reducing the dietary concentration of crude protein to 10 or 11% did not have any effect on growth or carcass traits (Mantovani et al., 2014). This is below the 13% requirement suggested by the French National Institute for Agricultural Research, which provides feeding recommendations that are widely used through Europe (Martin-Rosset et al., 2015). However, studies in horses have demonstrated that reduced intake of crude protein can slow protein synthesis and growth (Jordan and Myers, 1972; Ott et al., 1979a; Gibbs et al., 1989; Saastamoinen et al., 1994; Tanner et al., 2014). Therefore, it is essential that the amino acid requirements of horses are met with supplemented synthetic amino acids before crude protein intake is reduced to prevent deficiencies. This approach is limited, however, by the high forage content of horse diets, of which the crude protein content is not as easily manipulated.

Lysine requirements

It has been well-established that lysine is the first limiting amino acid in horses. Lysine supplementation in growing horses increased growth and feed to gain ratio (Hintz et al., 1971). Foals fed soybean meal experience greater weight, height, and girth gain when compared to those fed brewers dried grains, which has long been known to result in poor growth of horses. Those differences disappeared when foals fed brewers dried grains were supplemented with lysine so that its concentration was equivalent between treatments (Ott et al., 1979b), indicating that lysine was the primary nutrient limiting growth in diets consisting of brewers dried grains. Crude protein intake could also be reduced without an effect on growth when lysine was supplemented (Ott et al., 1981). Based on results of these studies and others that reported both lysine intakes and the resulting average daily gain, the NRC developed a requirement for lysine set at the point where average daily gain is maximal (NRC, 2007).

Threonine requirements

It was predicted that threonine was the next limiting amino acid for horses, based on the amino acid composition of common feedstuffs and muscle tissue (Bryden, 1991) and the results of amino acid requirement studies in swine (Wang and Fuller, 1989).

To approach the hypothesis that threonine is next limiting amino acid in horses, yearling horses were fed either a control diet, a diet supplemented with lysine, or a diet supplemented with both lysine and threonine, all of which contained slightly deficient concentrations of crude protein. Supplementing with lysine and threonine increased girth gain over just lysine (Graham et al., 1994a), but did not affect average daily gain, or increases in height or length. It should be noted that, due to incomplete intakes of offered

feed, the actual intake of lysine was increased to a mean of 45 g/d in the diet supplemented with threonine compared to 42 g/d in the diet without threonine. Therefore the effect of the increase in threonine intake may be confounded by the increase in lysine intake. However, from these findings, the authors concluded that threonine was the second limiting AA in growing horses.

Another study exploring this hypothesis fed yearling horses either a protein sufficient concentrate or a protein deficient concentrate which was supplemented with lysine and threonine, in addition to free access to pasture (Staniar et al., 2001). No overall differences in growth or plasma urea nitrogen were observed between groups, and an improved average daily gain was observed in the supplemented group during months of nutritional stress. Due to unexpectedly high crude protein concentrations in the pasture, the average total dietary crude protein concentrations were 16.8% and 15.5% for the control and supplemented diets respectively, which puts both diets within the recommended range of crude protein intakes. However, the improved average daily gain during colder months, when the energy and protein concentrations of forage were likely lower, indicate that there may have been a beneficial effect of amino acid supplementation which was masked during times of nutrient-dense forage.

In a recent study, weanling horses were fed a diet which met crude protein recommendations and was top-dressed with either crystalline threonine or crystalline glutamate in equimolar concentrations (Mastellar et al., 2016b). The authors did not observe any effect of treatment on plasma urea nitrogen concentrations or the rate of whole body protein synthesis estimated using the indicator amino acid oxidation method,

and concluded that threonine was not limiting protein synthesis in horses fed the provided diets.

Threonine structure, metabolism, and use

Threonine is an α-amino acid with an alcohol containing side chain, classifying it as a polar and neutral amino acid. Mammals lack the enzymes necessary to synthesize sufficient threonine, and therefore they must obtain the amino acid from the diet.

There are two general routes for threonine metabolism, as shown in Figure 2.1. The glycine-independent pathway involves a deamination reaction via the action of threonine dehydratase, which produces α-ketobutyrate, a precursor for propionate. The glycine dependent pathway can occur via the action of threonine aldolase or threonine dehydrogenase, and the eventual end-products of both enzyme-mediated reactions are acetyl-CoA and glycine (House et al., 2001). Glycine can be completely catabolized to carbon dioxide and ammonia, or form serine. Serine in turn can be converted to pyruvate, or combine with homocysteine, a product of methionine metabolism, to form cystathionine, which in turn is cleaved to cysteine and α-ketobutyrate (Finkelstein, 1990). These products can form substrates for glucose or lipid, or enter the citric acid cycle.

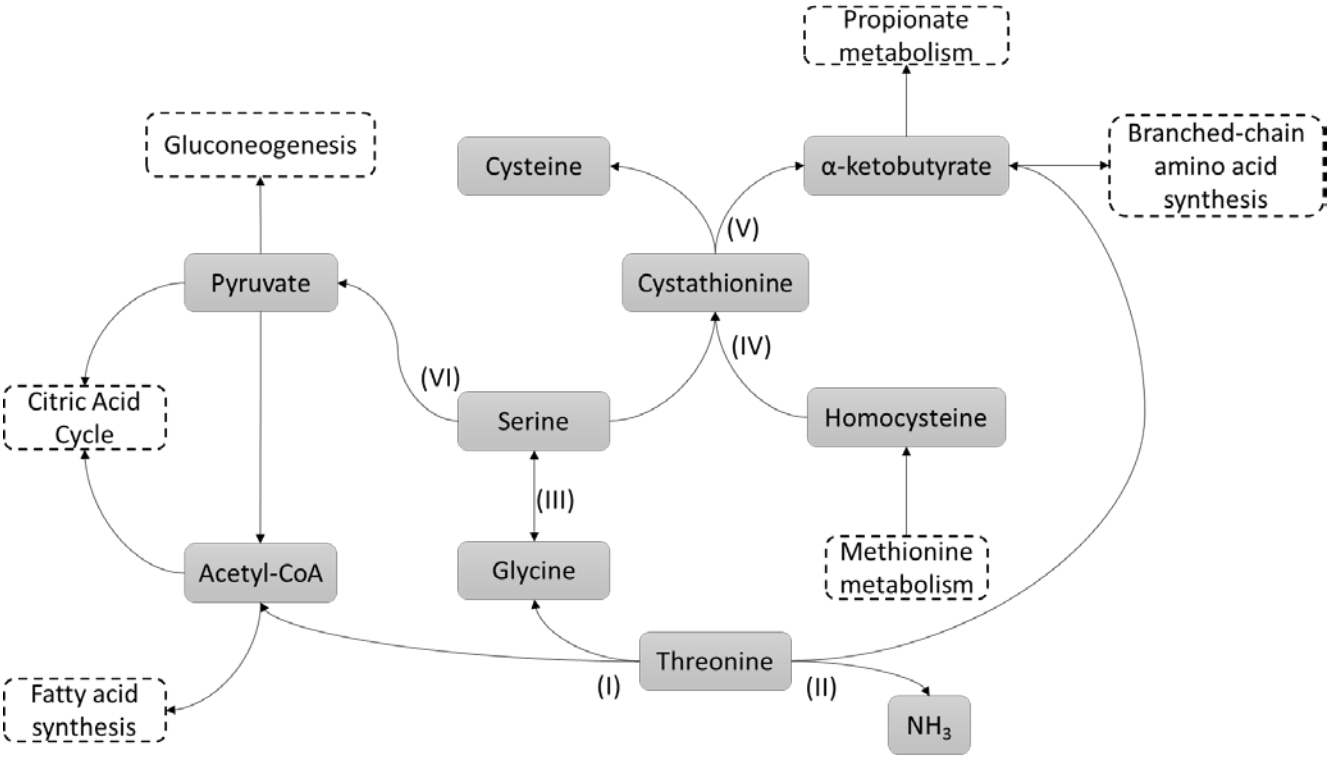

Figure 2.1. Metabolic pathways of threonine. Threonine can be deaminated (II) via threonine dehydratase, producing α-ketobutyrate and ammonia. Threonine also can be cleaved (I) via the actions of one of two enzymes. Threonine aldolase produces glycine and acetaldehyde, which can be metabolized to acetyl CoA. Threonine dehydrogenase produces 2-amino-acetoacetate, which is then cleaved to form glycine and acetyl-CoA. Other key enzymes: III, serine hydroxymethyltransferase; IV, cystathionine β-synthase; V, cystathionine γ-lyase; VI, serine dehydratase. Adapted from previous work (Kanehisa and Goto, 2000; House et al., 2001; Kanehisa et al., 2016).

Threonine, along with serine, composes up to 20 to 55% of mucin, a glycoprotein which is responsible for lubricating the gastrointestinal tract (Van Klinken et al., 1995). Mucin production and excretion into the intestinal lumen increases in response to increased fiber content of the digesta in swine (Montagne et al., 2004). Mucin secreted in the lumen of the intestine may flow to the large intestine, where the carbon moieties of indispensable amino acids such as threonine are irreversibly catabolized by microbes, resulting in endogenous losses of threonine. Therefore a large variation in forage intakes may alter an animal's need for threonine.

Methods of determining the amino acid requirement of horses

Tissue amino acid composition

Due to the conflicting results of these previous studies in horses, it is still unclear if threonine is next limiting after lysine in common horse diets, and what the requirement of this amino acid might be for growing horses. Many methods have been used to estimate or determine the amino acid requirements of horses and other species. The NRC suggests estimated indispensable amino acid requirements of horses based on the amino acid composition of muscle tissue (NRC, 2007), relative to lysine for which a requirement has been provided (Bryden, 1991), which is assumed to be representative of whole body protein composition in growing horses. Although this may provide an approximate picture of the requirement, there is likely a large amount of error in this assumption as the amino acid composition of different tissues varies and it does not consider differences in extraction by splanchnic tissues.

Plasma amino acid concentration

The amino acid requirements of swine, fish, and poultry have typically been determined by feeding increasing levels of an amino acid and determining the level that results in an optimal response. A variety of responses have been measured. Plasma amino acid concentrations have been proposed to be a reliable response (Broderick et al., 1974), based on the assumption that amino acids not used for protein synthesis will transiently build up in the blood, and therefore increasing the intake of an amino acid above its requirement will result in increased concentrations in the blood. This method does not consider, however, the rapid homeostatic mechanisms regulating plasma amino acid concentrations, and has not been widely successful (Patton et al., 2015). This method

was applied to mature thoroughbred horses to identify the requirement for lysine (Ohta et al., 2007), for which the current recommendation for horses of this class is 1.5 g/DE Mcal (NRC, 2007). The authors proposed 1.8 g/DE Mcal to be the requirement based on a breakpoint analysis of the dose-response curve, but the results of this study may not be accurate due to a small sample size and no visibly detectable breakpoint.

Plasma urea nitrogen concentration

Plasma urea nitrogen concentration is a more commonly used response to estimate requirements. When protein synthesis is limited by a single amino acid, the use of other dietary amino acids for protein synthesis is decreased and their oxidation increases. As limiting amino acids are increasingly supplied, protein synthesis increases and utilization of other amino acids increases, resulting in a decrease in oxidation until the point at which the test amino acid is no longer limiting. At this point, further increases in limiting amino acid intake do not result in increased oxidation. As amino acid oxidation increases, urea synthesis increases to rid the body of resulting ammonia (Eggum, 1970b). Therefore the plasma urea concentration should decrease until the requirement is met and then the concentration should level off. This method been regularly used and validated to measure the amino acid requirements of swine (Coma et al., 1995), and is often used in horses as supporting evidence for changes in protein or amino acid metabolism (Reitnour and Salsbury, 1976), but this method may not be sensitive enough in horses and is likely not sufficient evidence to make any claim as to a requirement (Geor et al., 2013).

Nitrogen balance

The original and most common method for determining amino acid requirements in other species is nitrogen retention. Nitrogen retained is the difference of nitrogen intake from the diet and nitrogen losses including fecal nitrogen and urine nitrogen. As discussed previously, a major goal within animal husbandry is to reduce nitrogen losses. Therefore the highest percentage of nitrogen retained is the most desirable. This method results in an opposite dose-response curve to that observed for plasma urea nitrogen. As oxidation decreases in response to increased limiting amino acid intake, urea synthesis and excretion decreases resulting in greater nitrogen retention. Further increasing supplementation results in excess total amino acids, and consequently nitrogen excretion again starts to increase and retention as a percentage of intake decreases. Although commonly applied to other species, this method may not practical or accurate in horses. Due to the size and exercise requirements of horses, total feces and urine collection for the required length of time is very labor intensive, and consequently studies using this method tend to have small sample sizes, typically 3 to 6 individuals. Crossover studies using this method are rare due to the necessary length of adaptation. Nitrogen losses in hair, sweat, and other uncollected excretions can be highly variable between horses, and this combined with small sample sizes may result in insufficient power (Antilley et al., 2007) .

Indicator amino acid oxidation method

A more recent method may provide a solution to the challenges and limitations of previous methods. The indicator amino acid oxidation method, as previously mentioned, estimates the rate of protein synthesis by directly measuring the rate of oxidation of a

labelled indicator amino acid. The stable isotope of a non-limiting amino acid is infused at a constant rate and the isotope enrichment of periodic blood and breath samples is used to calculate the rate of oxidation. Increasing the limiting test amino acid results in an increase in protein synthesis, and the oxidation of the indicator amino acid decreases until the requirement for the test amino acid is met. The World Health Organization has declared this method to be the gold standard for determining amino acid requirements in humans (Pencharz and Ball, 2003), and it has also been widely used in swine, poultry, and fish (Elango et al., 2008). The method is minimally invasive in comparison to nitrogen balance studies, and rapidly adapts to changes in diet, allowing for adaptation periods as short as two or three days (Elango et al., 2009).

Previous work has adapted and validated this method in horses (Urschel et al., 2010; Urschel et al., 2012; Mastellar et al., 2016a). The IAAO method is not only being used to determine the amino acid requirements of horses (Urschel et al., 2012; Mastellar et al., 2016b), but also to study the effects of other factors, such as age and disease, on protein synthesis in horses (Wagner et al., 2013; Mastro et al., 2014). Now that a clear protocol is in place for the use of this method in horses, it holds great promise to clarify the amino acid requirements of horses.

Chapter 3. Rationale and objectives of the thesis research

The objective of the following study was to add to the existing knowledge of amino acid requirements in growing horses, which is sparse. The requirement for lysine, the first limiting amino acid, in growing horses has been previously extrapolated from growth and nitrogen balance studies (NRC, 2007), and studies measuring various parameters of growth in response to threonine supplementation suggest that threonine is the second limiting amino acid for growing horses (Graham et al., 1994b; Staniar et al., 2001). Therefore threonine was selected as the next amino acid of interest.

Nutrient requirements are typically measured using a dose-response curve, in which a response to the nutrient is measured over increasing levels of intake of that nutrient. It has been suggested that at least 6 levels are necessary to observe a clear response (Pencharz and Ball, 2003). Thus, we formulated 6 levels of threonine intake. Dietary treatments were designed so that they were isocaloric and isonitrogenous and only varied in the concentrations of crystalline threonine and crystalline glutamate, allowing any observed effects of treatment to be the result of differences in the ratio of those two amino acids.

Previous efforts to define the amino acid requirements of horses using plasma amino acids, plasma urea nitrogen, or nitrogen balance as a response have been inconclusive and the methods may not be sensitive enough to clearly define requirements, although a requirement for lysine has been estimated using these methods (NRC, 2007). We chose the indicator amino acid oxidation method for determining amino acid requirements, which has recently been adapted for use in horses, due its sensitivity to amino acid intake and rapid adaptation time shown with other species including humans,

swine, poultry, and fish (Elango et al., 2008). The rapid adaptation time allowed for a relatively time-efficient 6 x 6 Latin square design to minimize the effect of individual variation.

Horses typically consume a diet consisting of 50 to 100% forage (NRC, 2007). The requirement of a nutrient is affected by the digestibility of that nutrient, which can differ dramatically between grains, protein meals, and fresh, dried, and ensiled forages (Takagi et al., 2003). Therefore, it is essential that the requirements of a nutrient be determined over a wide range of dietary compositions. This may be particularly true for threonine. Threonine, along with serine, composes up to 20 to 55% of mucin, a glycoprotein which is responsible for lubricating the gastrointestinal tract (Van Klinken et al., 1995). Mucin production and excretion into the intestinal lumen increases in response to increased fiber content of the digesta in other species (Montagne et al., 2004). Therefore, we predict that threonine requirements will increase in response to dietary fiber in horses. Consequently, we tested the lowest and highest levels of forage intake typically provided to growing horses.

Chapter 4 describes the high concentrate phase of our study, during which horses received 60% concentrate and 40% forage. Chapter 5 describes the high forage phase of our study, in which horses received 25% concentrate and 75% forage. Dietary rations were carefully formulated to be as similar as possible between phases so that comparisons could be made between the two levels of forage intake. However, there are many confounding variables between phases and phases could not be compared with inferential statistics.

The specific objective of this study was to determine the threonine requirements of growing horses receiving two different forage to concentrate ratios using the IAAO method.

Chapter 4. Threonine requirements of yearling horses fed a high concentrate diet

I. Introduction

Adequate protein intake is necessary for horses to attain optimal growth rates. Current dietary protein requirements are given in terms of crude protein, which is an estimate of dietary protein based on the amount of nitrogen in the feed (NRC, 2007). However, horses and other eukaryotes have a dietary requirement for each essential amino acid and the synthesis of other nitrogen containing metabolites, rather than total crude protein. Nitrogen is therefore typically fed in excess of its actual requirement for protein synthesis to ensure that amino acid requirements are met. This practice leads to increased nitrogen excretion, which can result in unsafe levels of ammonia inhalation by the animal and its caretaker (Rachuonyo et al., 2015), as well as increased nitrogen runoff that could damage waterways (Harper et al., 2009). There is also evidence that consuming an excess of protein can be harmful to the health and performance of the horse (Graham-Thiers et al., 1999; Graham-Thiers and Kronfeld, 2005b; Gaffney-Stomberg et al., 2014). These negative outcomes can be avoided by meeting the requirement for each individual amino acid and reducing excess nitrogen intake. However, research regarding amino acid requirements in horses is limited and primarily focuses on determining limiting amino acids rather than the actual requirement.

Previous efforts to determine amino acid requirements, using methods such as nitrogen balance studies or extrapolating from the amino acid composition of muscle, have resulted in a requirement for lysine and very tentative estimates for other amino acids that the National Research Council has chosen to exclude from their dietary recommendations for horses (NRC, 2007). A more recent approach, known as the

indicator amino acid oxidation (IAAO) method, estimates the rate of whole body protein synthesis by measuring the oxidation rate of a labelled amino acid. Amino acids cannot be stored, and therefore the rate that they enter the free amino acid pool in the body is the same as the rate they leave via incorporation into protein, conversion to other metabolites, or oxidation. If the rate of entry into the free amino acid pool is known, and the rates of amino acid oxidation are measured, then the rate of protein synthesis can be derived. This method has been extensively used in humans, swine, poultry, and fish to study the effects of various treatments on amino acid oxidation and the resulting estimation of protein synthesis (Elango et al., 2008). The IAAO method has recently been adapted for use in horses (Urschel et al., 2010; Mastellar et al., 2016a; Mastellar et al., 2016b), and efforts are now underway to determine the amino acid requirements of horses using this method.

Threonine has been suggested to be the second limiting amino acid after lysine in growing horses (Graham et al., 1994b; Staniar et al., 2001), based on observed improvements in parameters of growth when threonine was supplemented. The objective of this study was to determine the threonine requirement for growing horses using the IAAO method. It was expected that feeding increasing amounts of threonine would result in decreased rates of indicator amino acid oxidation until threonine was no longer limiting, at which point the rate of oxidation would plateau.

II. Materials and methods

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals, Housing, and Feeding

Six female Thoroughbred horses aged 9 to 11 months were obtained from the University of Kentucky Department of Animal and Food Sciences' research herd. Initial body weights ranged from 274.5 kg to 401.5 kg, and the average was 318.6 (± 42.9) kg. Horses were housed individually in 3.7 × 3.7 m stalls overnight, and turned out together with muzzles to prevent grazing between the hours of 0900 and 1500. Horses were fed their experimental diets at 0600 and 1500, and refusals were collected before the morning meal and weighed each day to calculate total daily intake.

Experimental Design

Horses were randomly assigned to 1 of 6 dietary treatments in a 6 × 6 randomized Latin square design. On day 0 of each period, horses began consuming their experimental diet at the afternoon meal and continued to consume that diet for 7 days. As will be later described in detail, pre- and post- meal blood samples for urea and amino acid analysis were taken on day 6, and the isotope infusion protocol took place on day 7. Horses were then switched to the next dietary treatment on the afternoon of day 7 following infusion procedures.

Dietary Treatments

The experimental diets were formulated to meet or exceed the estimated requirements for all nutrients (NRC, 2007). Daily feed offered was 2.50% of bodyweight on an as-fed basis, and the composition of the diet was 60% pelleted concentrate and 40% timothy hay cubes. Two concentrate pellets were formulated and manufactured (Buckeye Feeds, Dayton, OH): a high threonine pellet containing additional crystalline threonine and a low threonine pellet containing additional crystalline glutamate in equimolar

concentrations to the threonine in the high threonine pellet, to ensure all treatments were isonitrogenous. All other ingredients remained the same, as shown in Table 4.1. The 6 dietary treatments were produced by combining the high threonine (High Thr) and low threonine (Low Thr) concentrates in ratios of 0:1, 1:4, 2:3, 3:2, 4:1, and 1:0, resulting in expected threonine intakes of 98, 110, 122, 134, 146, and 158 mg/(kg BW·d). Scented apple oil was added the high thr concentrate to prevent technician error in mixing feeds. Each meal was top dressed with 100 g of canola oil to meet energy requirements. The analyzed nutrient compositions of the dietary components are shown in Table 4.2.

Table 4.1. Feed ingredient composition of high and low threonine concentrate[1], DM basis, %

Ingredient	High Thr	Low Thr
Ground corn	30.4	30.5
Corn distiller dried grains	27.0	27.0
Soy hulls	17.7	17.7
Oatmill byproduct	11.7	11.7
Soybean oil	3.7	3.7
Apple flavor oil	0.11	0.00
Amino acids		
L-Glutamate	0.00	0.69
L-Threonine	0.57	0.00
L-Lysine HCl	0.50	0.50
L-Histidine monohydrate HCl	0.18	0.18
Minerals and vitamins		
Calcium carbonate	2.5	2.5
Monocalcium phosphate, 21%	2.0	2.0
Salt	0.73	0.73
Dynamate[2]	0.66	0.66
Vitamin E Acetate, 20%	0.42	0.42
Gro N Win pre-mix[3]	0.04	0.04
Equine trace mineral mix[4]	0.04	0.04
Magnesium oxide, 56%	0.22	0.22
Zinc Sulfate, 36%	0.02	0.02
Selenium premix[5]	0.02	0.02

[1]Concentrates formulated for study by Buckeye Nutrition, Dalton, OH

[2] Contains: 180 g of potassium, 110 g of magnesium, and 220 g of sulfur /kg, Mosaic Feed Ingredients, South Riverview, FL

[3]Contains: magnesium oxide, sodium bentonite, zinc sulfate, iron sulfate, Copper sulfate, manganese sulfate, Optimin Zinc, DL-Methionine, Optimin Copper , Selenium PX, Optimin Manganese, L-Lysine HCL, Optimin Iron, mineral oil, Optimin Selenium Yeast 3000, Organic Iodine EDDI, Vitamin C Monophosphate Stay-C, Buckeye Nutrition, Dalton, OH

[4]Contains: ferrous sulfate, ferrous carbonate, iron oxide, manganese sulfate, manganous oxide, chromium tripicolinate, copper sulfate, copper carbonate, tribasic copper chloride, copper oxide, zinc oxide, zinc sulfate, zinc methionine complex, calcium iodate, potassium iodide, eddi, pentacalcium orthoperiodate, cobalt carbonate, cobalt sulfate, sodium selenite, sodium molybdate, sodium sulfate, sodium bicarbonate, sodium chloride, sulfur, potassium sulfate, potassium chloride, potassium magnesium sulfate, magnesium sulfate, magnesium oxide, calcium sulfate, calcium carbonate, tricalcium phosphate, dicalcium phosphate, monoammonium phosphate, monosodium phosphate, ammonium chloride, ammonium sulfate, clay, bentonite, calcium stearate, zeolex (sodium aluminosilicate), zeofree (precipitate amorphous hydrated silicon dioxide), mineral oil, natural flavors and artificial flavors, Buckeye Nutrition, Dalton, OH

[5]Contains calcium carbonate, sodium selenite, crystalline quartz silica, Prince Agri Products, Inc., Quincy, IL

Table 4.2. Nutrient composition of each component of treatment diet, as-fed basis

	High threonine concentrate	Low threonine concentrate	Timothy hay cubes
Overall nutrient composition			
Dry matter, %	88.9 ± 0.5	89.2 ± 0.3	90.1 ± 0.1
DE, Mcal/kg	2.75 ± 0.05	2.74 ± 0.04	1.83 ± 0.02
Crude protein, %	13.4 ± 0.6	13.3 ± 0.2	9.5 ± 0.0
Acid detergent fiber, %	15.5 ± 1.1	15.8 ± 1.3	35.2 ± 1.1
Neutral detergent fiber, %	27.9 ± 0.4	28.2 ± 1.9	52.0 ± 0.5
Calcium, %	1.29 ± 0.06	1.27 ± 0.03	0.50 ± 0.03
Phosphorus, %	0.70 ± 0.02	0.73 ± 0.01	0.23 ± 0.02
Iron, mg/kg	295 ± 16	319 ± 9	514 ± 66
Zinc, mg/kg	183 ± 12	182 ± 28	105 ± 8
Amino acid composition, %			
Alanine	0.67 ± 0.02	0.67 ± 0.02	0.47 ± 0.05
Arginine	0.54 ± 0.03	0.56 ± 0.03	0.42 ± 0.05
Aspartate + Asparagine	0.62 ± 0.02	0.66 ± 0.03	0.73 ± 0.06
Glutamate + Glutamine	1.82 ± 0.07	2.36 ± 0.07	0.86 ± 0.09
Glycine	0.45 ± 0.01	0.46 ± 0.02	0.37 ± 0.04
Histidine	0.37 ± 0.03	0.37 ± 0.03	0.15 ± 0.02
Isoleucine	0.35 ± 0.05	0.37 ± 0.05	0.34 ± 0.06
Leucine	1.14 ± 0.05	1.15 ± 0.05	0.62 ± 0.07
Lysine	0.63 ± 0.03	0.65 ± 0.04	0.36 ± 0.05
Methionine	0.14 ± 0.02	0.15 ± 0.04	0.11 ± 0.01
Phenylalanine	0.50 ± 0.05	0.52 ± 0.02	0.39 ± 0.05
Proline	0.84 ± 0.03	0.84 ± 0.02	0.55 ± 0.03
Serine	0.56 ± 0.02	0.55 ± 0.01	0.36 ± 0.02
Threonine	0.81 ± 0.03	0.41 ± 0.02	0.36 ± 0.03
Tyrosine	0.40 ± 0.02	0.39 ± 0.02	0.21 ± 0.02
Valine	0.46 ± 0.06	0.48 ± 0.06	0.43 ± 0.07

Sampling and isotope infusion

Animals were weighed on day 6 of each experimental period at 1100 h. Blood samples were collected on day 6 of each study period prior to the morning meal and 90 and 180 minutes following the meal to measure the effects of threonine intake level on plasma amino acid and plasma urea concentrations. Blood was collected via venipuncture from the jugular vein into heparinized vacutainers (BD, Franklin Lakes, NJ) and immediately centrifuged at 1,500 × g for 10 min at 4 ºC. The supernatant was collected and stored at -20 ºC until the time of analysis.

On day 7 of each study period, whole-body phenylalanine kinetics were determined using a 2 h primed, constant intravenous infusion of [^{13}C] sodium bicarbonate at a prime rate of 5.30 μmol/(kg BW·h) and a constant rate of 4.41 μmol/(kg BW·h) followed by a 4 h primed, constant oral administration of [1-^{13}C] phenylalanine at a prime rate of 10.23 and a constant rate of 7.22 μmol/(kg BW·h) (Cambridge Isotope Laboratories, Andover, MA). The prime to constant ratio was previously validated in horses (Urschel et al., 2012), and the dose of phenylalanine has been shown to result in stable, measurable plateaus of isotope within the breath (Mastellar et al., 2016a). To maintain a steady metabolic state, horses received 1/48th of their daily ration every half hour throughout sampling, beginning 90 minutes before the start of the bicarbonate infusion. Any feed remaining by the time of next feeding was weighed and removed. Baseline blood and breath samples were collected immediately prior to isotope infusion. Blood was collected from a catheter placed in the jugular vein and processed as described for day 6 sampling. Breath was collected into gas impermeable bags using a modified equine Aeromask (BreathEazy Ltd, Malvern, Worcestershire) and immediately analyzed (Urschel et al., 2012).

Following baseline sample collection, [^{13}C] sodium bicarbonate was infused into the intravenous catheter using a cordless infusion pump (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc.) attached to a surcingle worn by the horse. During the infusion, breath samples were collected every 30 min for the first hour, then every 15 min during the second hour. After 2 h, infusion was stopped and horses received their [1-^{13}C] phenylalanine prime and first constant dose top-dressed on their meal. Horses continued to receive constant doses every half hour. Throughout administration, blood and breath samples were collected every half hour. After 4 h, catheters were removed and horses received their day 0 meal for the next treatment period.

Sample Analysis

Pre- and post-feeding blood samples from day 6 were analyzed for plasma amino acid and urea nitrogen concentrations. Plasma urea nitrogen concentrations were measured using a colorimetric spectrophotometric assay. A 10-μL aliquot of each plasma sample and was pipetted in duplicate into microcentrifuge tubes containing 125 μL of urease buffer (Sigma-Aldrich Co., St. Louis, MO). After 20 min of incubation at room temperature, 250 μL of phenol nitroprusside solution, 250 μL of alkaline hypochlorite solution, and 1000 μL of distilled water were added to each sample. After 25 min of incubation at room temperature, 200 μL of each sample were transferred in duplicate to 96 well plates, placed in a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA,) and read at 570 nm of wavelength. The intra-assay variation was < 10% for the urea nitrogen analysis.

Plasma amino acid concentrations were determined using high performance liquid chromatography (HPLC) with a previously described method (Bidlingmeyer et al., 1984).

Briefly, samples were deproteinated using 10-kd cutoff centrifugal filters, and filtrate was freeze-dried. Norleucine was added as an internal standard. Ethanol, water, and trimethylamine (TEA) (Fisher Scientific, Fair Lawn, NJ) were then added in a 2:2:1 ratio, and samples were re-dried. Amino acids were then derivatized by adding ethanol, TEA, water, and phenylisothiocyanate (Acro Organics, Geel, Belgium) in a 7:1:1:1 ratio and incubating in a sealed container at room temperature for 20 min. Samples were then freeze-dried and reconstituted with HPLC eluent and injected onto a 3.9x300mm PICO-TAG reverse phase column (Waters Corporation, Milford, MA).

Samples of timothy hay cubes, chopped timothy hay, and the high and low threonine pelleted concentrates were collected each week and sent for proximate analysis via wet chemistry (Dairy One Cooperative Inc., Ithaca, NY). Feed amino acid concentrations were determined using acid hydrolysis (AOAC International, 2005). Samples were ground, and 0.2 g was weighed into ashed vials. To these vials 12 mL of 6N hydrochloric acid (Fisher Scientific, Fair Lawn, NJ) was added and samples were capped tightly and incubated at 110 °C for 24 h. After incubation, samples were filtered using a 0.45 μm syringe filter (Sarstedt, Numbrecht, Germany) into microcentrifuge tubes. Samples were then derivatized and analyzed by HPLC as described for plasma samples. To measure methionine concentrations, a separate assay was performed in which 2 mL of performic acid (J.T. Baker Chemicals, Center Valley, PA) and 0.42 g of metabisulfite (Fisher Scientific, Fair Lawn, NJ) were added to samples and incubated overnight at 4 °C prior to the addition of hydrochloric acid (AOAC International, 2005).

The isotope enrichment of plasma samples collected during isotope infusion was determined by negative chemical ionization GC-MS analysis of a heptafluorobutyric, n-

propyl derivative (Metabolic Solutions Inc., Nashua, NH), as previously described (Matthews et al., 1990; Urschel et al., 2011). A Phenomenex ZB-1MS capillary column was used to separate the derivative of phenylalanine. Selected ion chromatograms were obtained by monitoring ions at a m/z 383 and 384 for phenylalanine and [1-13C]phenylalanine, respectively. The isotope enrichment of breath samples was determined by measuring the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the breath using an infrared isotope analyzer (IRIS-3; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany) (Urschel et al., 2012).

Calculations

The average enrichment at isotopic stable state was determined for both plasma and breath samples. The plateau was defined as at least 3 points with a coefficient of variation less than 10%. If the plateau was not obtained, the data was discarded. Total CO_2 production rate was determined using the average enrichment of breath samples during [^{13}C] sodium bicarbonate infusion with the following formula:

$$CO_2 \text{ production} = i \times [(Ei/Eb) - 1] \times [0.0224 \text{ mL}/\mu\text{mol } CO_2]$$

where i is rate of the isotope infusion (μmol//(kg BW·h)), Ei is the enrichment of isotope solution, and Eb is the plateau breath enrichment (Hoerr et al., 1989).

Whole-body phenylalanine flux was calculated using the following equation where Q is flux (μmol//(kg BW·h)), i is rate of the isotope infusion (μmol//(kg BW·h)), E_i is isotope solution enrichment, and Ep is the plateau plasma enrichment (Hsu et al., 2006):

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

The different processes affecting flux include amino acids entering the blood 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) (Picou and Taylor-Roberts, 1969):

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

Phenylalanine entering the plasma amino acid pool from dietary intake (I) was corrected for pre-cecal digestibility by multiplying phenylalanine intake from forage by factor of 0.4 (Gibbs et al., 1988) and multiplying the phenylalanine intake from concentrate by a factor of 0.7 (Farley et al., 1995), and further corrected by assuming that 26.5% of the digestible phenylalanine was extracted by the splanchnic tissues (Mastellar et al., 2016a). Mammals cannot synthesize phenylalanine de novo, so the phenylalanine entering the blood amino acid pool from protein breakdown (B) can be determined from the following equation:

$$B = Q - I$$

[$^{1\text{-}13}$C] phenylalanine oxidation was calculated using the following equation (Hsu et al., 2006):

$$E = F^{13}CO_2(1/Ep - 1/Ei) \times 100$$

where E represents phenylalanine oxidation (μmol /(kg BW·h)) and $F^{13}CO_2$ is the product of isotope enrichment of the breath and the rate of carbon dioxide production (μmol/(kg BW·h)). Phenylalanine conversion to tyrosine was assumed to be minimal and any that did occur should be the same for all treatments because both phenylalanine and tyrosine intakes were not different between treatments. Therefore, protein synthesis can be calculated using the following equation:

$$Z = Q - E$$

Statistical Analysis

All experimental data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Repeated measures analysis was used for analyzing plasma amino acids and plasma urea nitrogen from pre and post feeding blood samples. Fixed effects were treatment, time, period, time × period, treatment × period, and time × treatment interactions, and horse was included as a random effect. A one-way ANOVA was conducted to estimate treatment effects on intake and whole-body phenylalanine kinetics. Orthogonal polynomial contrasts were used to test linear, quadratic, and cubic effects of treatment and time, with the IML procedure of SAS used to determine contrast coefficients. The orrelation of threonine intake and plasma threonine concentrations were determined using simple linear regression for descriptive purposes. The break point in [$^{1\text{-}13}$C] phenylalanine oxidation rate was determined using a biphasic linear regression model with the slope of the 2nd phase restricted to 0. Significance was determined at $P < 0.05$. Measurements are shown as least squares means, and the standard error of the mean is denoted as SEM.

III. Results

Growth

The mean average daily gain for the length of the study was 0.43 (± 0.09) kg/day, which is within the normal range for thoroughbred horses of 0.4 to 0.7 kg (NRC, 2007).

Intake

As shown in Table 4.3, there was no significant effect of threonine intake level on daily total ration intake ($P \geq 0.05$) or daily forage intake ($P \geq 0.05$). There was a significant linear effect of treatment on concentrate intake, with decreasing levels of threonine inclusion in the diet associated with lower amounts of concentrate intake ($P = 0.009$).

The mean intake rates shown in Table 4.3 and the composition of the feed components resulted in the daily nutrient intakes shown in Table 4.4. The diets exceeded all nutrient intake recommendations.

Table 4.3. Effect of threonine intake level on average daily intake in growing horses fed a high concentrate diet, as-fed basis, g/(kg BW·d)

	Level of threonine intake, mg/(kg BW·d)							*P*-values			
Intake	98	110	122	134	146	158	SEM	Treatment	Linear	Quadratic	Cubic
Total	22.9	22.2	22.8	23.1	22.7	22.8	0.5	0.791	0.719	0.856	0.376
Forage	8.3	7.8	7.8	8.0	7.7	7.7	0.5	0.846	0.331	0.816	0.524
Concentrate	14.8	14.8	15.0	15.1	15.0	15.1	0.1	0.208	0.009	0.531	0.758
High Thr[1]	0.0	3.0	6.0	9.1	12.0	15.1					
Low Thr[1]	14.8	11.8	9.0	6.0	3.0	0.0					

[1]Calculated from mean concentrate intake and the ratio of high thr to low thr of the dietary treatment

Table 4.4. Calculated average daily nutrient intakes of growing horses fed a high concentrate diet containing increasing levels of threonine

	Level of threonine intake, mg/(kg BW·d)						NRC (2007) Recommendation [1]
Nutrient	98	110	122	134	146	158	
Digestible energy, kcal/kg BW[2]	61.5	60.6	61.3	61.9	61.2	61.4	58.6
Crude protein, g/kg BW	2.76	2.71	2.75	2.78	2.74	2.75	2.64
NDF, g/kg BW	8.46	8.20	8.28	8.40	8.21	8.21	
ADF, g/kg BW	5.24	5.07	5.11	5.18	5.05	5.05	
Calcium, mg/kg BW	229.6	227.6	230.9	233.8	231.7	233.4	117.4
Phosphorus, mg/kg BW	127.3	125.2	125.8	126.0	123.7	123.3	65.1
Iron, mg/kg BW	8.97	8.65	8.67	8.73	8.46	8.41	1.25
Zinc, mg/kg BW	3.57	3.52	3.56	3.60	3.55	3.57	1.00
Amino acids, mg/kg BW							
Alanine	139.1	136.7	138.3	139.9	137.8	138.3	
Arginine	117.7	115.1	115.9	116.8	114.4	114.4	
Aspartate + Asparagine	159.2	154.5	155.2	156.1	152.2	151.5	
Glutamate + Glutamine	422.7	402.3	391.1	378.8	358.0	343.5	
Glycine	100.2	98.1	98.9	99.8	97.9	97.9	
Histidine	66.5	65.8	66.6	67.3	66.6	66.9	
Isoleucine	82.5	80.5	81.0	81.6	79.8	79.7	
Leucine	222.7	219.5	222.0	224.3	221.1	222.0	
Lysine	126.1	123.7	124.5	125.2	122.8	122.6	113.4
Methionine	29.8	29.6	30.2	30.9	30.7	31.1	
Phenylalanine	110.2	107.8	108.6	109.4	107.2	107.1	
Proline	171.5	168.8	170.8	172.7	170.2	170.9	
Serine	112.4	110.7	112.2	113.7	112.3	112.9	
Threonine	90.8	100.9	114.2	127.7	138.1	151.0	
Tyrosine	75.8	74.9	76.0	77.0	76.1	76.6	
Valine	106.8	104.2	104.9	105.7	103.4	103.3	

[1]Calculated from the average body weight of 12 month old horses (NRC, 2007)

[2]Includes 200 g canola oil top dressed on meal

Plasma Urea and Amino Acid Concentrations

There was a significant cubic response of plasma urea nitrogen (PUN) to threonine inclusion ($P = 0.010$), with an initial decrease, followed by an increase, and ending with a decrease of PUN with increasing threonine intake. There was a significant positive linear association of threonine intake level and the plasma concentrations of methionine, threonine, and asparagine ($P < 0.05$). Glutamate and serine had a significant cubic responses to threonine level ($P < 0.05$). Aspartate had a significant linear decrease in response to increasing threonine ($P = 0.029$). These results are summarized in Table 4.5. There was a significant time by treatment interaction only for aspartate ($P = 0.0471$), however this amino acid has very low plasma concentrations relative to other amino acids and small significant differences are unlikely to be physiologically significant or relevant to the objectives of the study.

Table 4.5. Main effect of threonine intake level on plasma concentrations of urea nitrogen and amino acids in growing horses fed a high concentrate diet

	Level of threonine intake, mg/(kg BW·d)							*P*-values			
Metabolite	98	110	122	134	146	158	SEM[1]	Treatment	Linear	Quadratic	Cubic
Plasma urea nitrogen, mmol/L	4.0	3.7	3.5	3.8	3.8	3.5	0.19	0.0278	0.039	0.501	0.010
Indispensable amino acids, μmol/L											
Histidine	83	99	90	89	91	88	6	0.150	0.984	0.178	0.116
Isoleucine	38	37	39	34	35	37	3	0.623	0.373	0.617	0.463
Leucine	104	112	104	103	104	105	7	0.755	0.656	0.955	0.328
Lysine	137	160	147	142	139	136	8	0.104	0.186	0.132	0.063
Methionine	28	31	35	34	36	38	3	0.001	<0.0001	0.317	0.355
Phenylalanine	60	66	61	61	62	61	3	0.349	0.671	0.384	0.261
Threonine	72	142	232	255	352	462	39	<0.0001	<0.0001	0.523	0.468
Tryptophan	61	64	64	61	62	62	3	0.908	0.811	0.731	0.369
Valine	136	134	134	125	124	127	8	0.578	0.104	0.718	0.515
Dispensable amino acids, μmol/L											
Alanine	276	323	270	284	248	285	22	0.146	0.639	0.899	0.176
Arginine	84	95	91	89	83	81	5	0.161	0.143	0.062	0.154
Asparagine	37	44	44	42	47	47	3	0.047	0.006	0.487	0.223
Aspartate	9	9	9	8	7	8	1	0.204	0.029	0.773	0.145
Glutamate	54	56	53	49	49	53	6	0.046	0.043	0.214	0.016
Glutamine	484	526	500	493	505	493	62	0.676	0.872	0.500	0.391
Glycine	590	541	556	514	592	585	58	0.804	0.833	0.291	0.734
Proline	121	138	128	129	133	131	9	0.246	0.334	0.404	0.200
Serine	257	306	323	294	317	343	22	0.016	0.003	0.496	0.032
Tyrosine	68	79	75	75	75	74	6	0.324	0.524	0.133	0.226

The correlation of threonine intake level with plasma threonine is shown in Figure 4.1. The correlation is strong ($R^2 = 0.66$; $P < 0.0001$) and the regression function is y = 5.97x – 514.19. There was no significant effect of time relative to feeding ($P = 0.23$) on plasma threonine concentration.

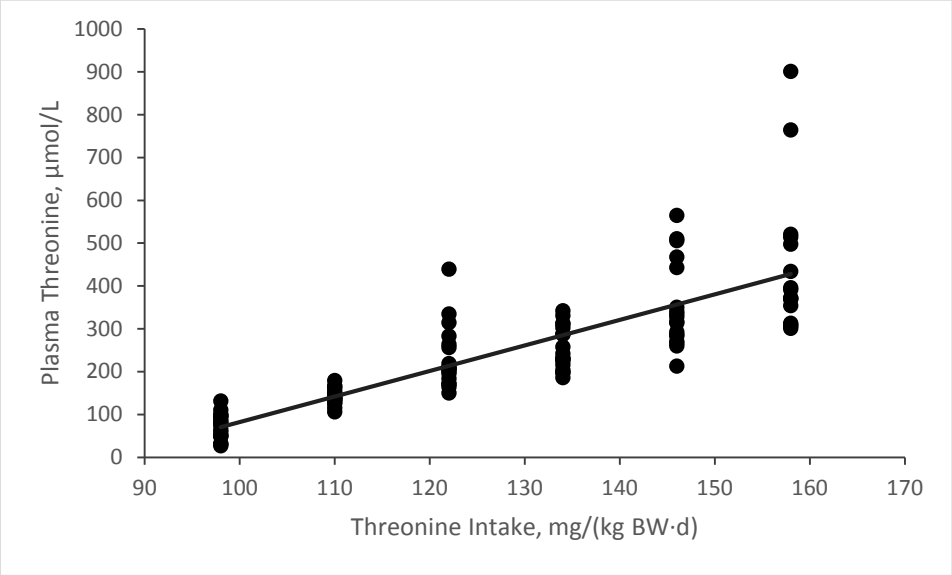

Figure 4.1. Correlation of threonine intake with plasma threonine concentrations in growing horses fed a high concentrate diet.

Plasma urea nitrogen, histidine, lysine, and arginine plasma concentrations increased linearly over time after feeding ($P < 0.05$). Leucine and alanine showed a significant quadratic response to time, with an initial decrease 90 min after feeding, followed by an increase at 180 min ($P < 0.005$). Plasma glutamate and tryptophan concentrations decreased linearly ($P < 0.05$) in response feeding. These results are summarized in Table 4.6.

Table 4.6. Main effect of time relative to feeding on the plasma concentrations of urea nitrogen and amino acids in growing horses fed a high concentrate diet

	Time post-feeding, min				*P*-values		
Item	0	90	180	SEM	Time	Linear	Quadratic
Plasma urea nitrogen, mmol/L	3.6	3.7	3.8	0.2	0.052	0.021	0.418
Indispensable amino acids, μmol/L							
Histidine	80	89	101	5	<0.0001	<0.0001	0.644
Isoleucine	38	35	37	2	0.456	0.795	0.226
Leucine	108	98	110	6	0.008	0.742	0.003
Lysine	118	147	165	7	<0.0001	<0.0001	0.344
Methionine	34	33	34	3	0.881	0.959	0.618
Phenylalanine	63	60	63	3	0.171	0.969	0.064
Threonine	243	260	255	17	0.232	0.399	0.142
Tryptophan	66	62	59	3	0.074	0.023	0.717
Valine	130	124	136	7	0.156	0.403	0.083
Dispensable amino acids, μmol/L							
Alanine	307	266	288	15	0.002	0.129	0.002
Arginine	83	85	93	5	0.041	0.015	0.410
Asparagine	42	42	46	3	0.083	0.059	0.206
Aspartate	9	8	8	1	0.176	0.091	0.427
Glutamate	57	51	49	6	<0.0001	<0.0001	0.100
Glutamine	500	486	514	60	0.096	0.347	0.051
Glycine	547	598	544	46	0.419	0.961	0.190
Proline	130	124	136	8	0.116	0.262	0.079
Serine	310	301	308	18	0.302	0.761	0.134
Tyrosine	77	73	72	5	0.192	0.091	0.497

Whole Body Phenylalanine Kinetics

Phenylalanine flux was calculated from the infusion rate of [$^{1\text{-}13}$C] phenylalanine and the isotope enrichment of plasma, and represents the rate of entry and exit of phenylalanine into the free amino acid pool. Two horses were removed from treatments 1, 3, and 6, and one horse was removed from treatments 2 and 4 due to high coefficients of variation of enrichment plateaus. There was a significant quadratic response of flux to level of threonine intake (P =0.016). Oxidation was calculated from the infusion rate of [^{13}C] sodium bicarbonate, and the average plateau enrichment of $^{13}CO_2$ of breath during both [^{13}C] sodium bicarbonate and [1-^{13}C] phenylalanine infusion. Oxidation was not significantly correlated with threonine intake level ($P \geq 0.05$). Phenylalanine used for protein synthesis was calculated as the difference between flux and oxidation. There was a significant quadratic response of protein synthesis to threonine intake level (P = 0.042). Phenylalanine influx from protein breakdown was calculated as the difference of flux and phenylalanine intake. There was a significant quadratic response to threonine intake level (P = 0.017). There was no significant effect of treatment on carbon dioxide production ($P \geq 0.05$). These results are summarized in Table 4.7.

Table 4.7. Effect of threonine intake level on parameters of phenylalanine flux in growing horses fed a high concentrate diet, μmol/(kg·h)

	Graded threonine intakes, mg/kg BW/d							*P*-values			
	98	110	122	134	146	158	SEM	Treatment	Linear	Quadratic	Cubic
Phe flux[1]	114.2	108.1	90.7	93.0	83.3	103.2	11.5	0.018	0.025	0.016	0.161
Phe oxidation[2]	23.8	22.3	25.5	20.6	19.4	24.9	3.3	0.457	0.726	0.522	0.199
Phe for protein synthesis[3]	89.6	90.1	65.1	72.5	63.9	77.7	11.2	0.024	0.022	0.042	0.223
Dietary Phe intake[4]	10.3	10.3	10.2	10.1	10.1	10.0	0.1	0.578	0.063	1.000	1.000
Phe from protein breakdown[5]	104.0	97.8	80.5	82.8	73.2	93.2	11.5	0.019	0.027	0.017	0.160
CO_2 production[6]	17982	19933	20514	18359	20015	18506	829	0.129	0.912	0.073	0.317

[1]Flux = isotope infusion rate × [(isotope solution enrichment/ plateau plasma enrichment)-1]

[2]Oxidation = 100 × [(1/plateau plasma enrichment – 1/isotope solution enrichment) × rate of $^{13}CO_2$ release during phenylalanine infusion]

[3]Non-oxidative Phe disposal = Flux – Oxidation

[4]Assumes a pre-cecal digestibility of 42% (Gibbs et al., 1988; Farley et al., 1995) and a splanchnic extraction of 26.5% (Mastellar et al., 2016a)

[5]Phenylalaine entering pool from protein degradation = Flux – Phe from diet

[6]Total carbon dioxide production = isotope infusion rate × [(isotope solution enrichment/ plateau breath enrichment)-1] × [0.0224 mL/μmol CO_2]

The two-phase linear regression of phenylalanine oxidation with threonine intake level did not reach significance (P = 0.53), and a breakpoint could not be determined. Figure 4.2 shows the spread of rates of phenylalanine oxidation at each level of threonine intake.

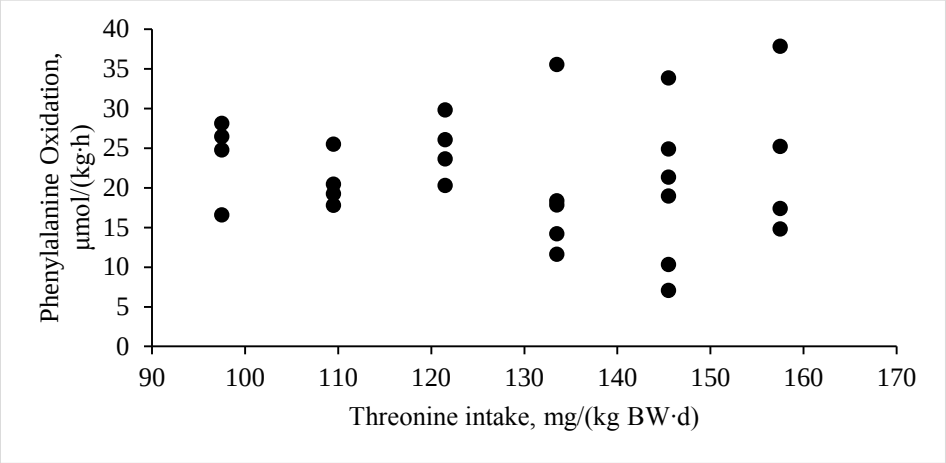

Figure 4.2. Effect of threonine intake level on the rate of phenylalanine oxidation.

IV. Discussion

Efforts are currently underway to determine the amino acid requirements of horses. The requirement for the first limiting amino acid, lysine, has already been reported (NRC, 2007), and previous studies in growing horses suggest that threonine may be the next limiting amino acid (Graham et al., 1994b; Staniar et al., 2001). However, the results of the present study suggest that threonine is not a limiting amino acid for growing horses fed a 12% crude protein ration consisting of 60% concentrate and 40% timothy hay cubes.

It was expected that the yearlings would completely consume their daily rations, as they were provided with 2.2% of bodyweight per day in feed which is near the lower

end of the expected range for yearling horses of 2.0% to 3.0% (NRC, 2007). However, the horses in our study consumed approximately 2.0% of bodyweight per day on an as-fed basis. One possible explanation for the lower intake rates is gastric ulceration. The presence of ulcers was not confirmed, but the horses were experiencing many of the risk factors associated with ulceration as well as presenting clinical signs including decreased appetite, slowed growth, and a few cases of mild colic immediately following consumption of concentrate (Videla and Andrews, 2009).

Despite the decreased intake, horses were still consuming above the amounts of digestible energy and crude protein required for optimum rates of growth (NRC, 2007). The mean average daily gain of the study animals was at the lower end of the expected range for thoroughbred horses (NRC, 2007). This study took place in February and March of 2015, and it is likely that the particularly cold weather during that time played a role in the depressed growth, with eleven days of high temperatures below 0° Celsius and average high temperature of 2° Celsius during the month of February. A decrease in average daily gain during late winter has been observed in other studies (Pagan et al., 1996; Staniar et al., 2004).

It was expected that by feeding increasing levels of threonine, protein synthesis would increase and amino acid oxidation would decrease, resulting in decreased urea synthesis. This response has previously been shown in horses and other species receiving amino acid supplementation (Eggum, 1970a). The results of the present study show a cubic response of plasma urea nitrogen concentration, with an initial decrease, followed by an increase, and decreasing again as threonine intake increases. This pattern of response has previously been observed in weanling horses receiving increasing levels of

methionine (Winsco et al., 2011a). Winsco et al. suspected that this may be due to decreased mean intake of lysine and threonine at the peak of plasma urea. This was not the case in our study, as none of the average amino acid intakes were elevated at the 4th and 5th levels of threonine intake relative to other levels, and a biological explanation for the peak of PUN cannot be determined.

Plasma concentrations of methionine, threonine, and asparagine increased linearly and serine increased in a cubic pattern with increasing threonine intakes. The increases in plasma concentration of these amino acids despite no difference in intake has been previously observed in mature and weanling horses (Mok, 2015; Mastellar et al., 2016b).

The rates of synthesis and catabolism of an amino acid is determined by the concentration of its substrates and products, and the activity of the enzymes associated with its metabolism (Cynober, 2002). Threonine can be metabolized through multiple pathways. The first is a deamination reaction via the action of threonine dehydratase, which produces in α-ketobutyrate, a precursor for propionate. The other two are via the action of threonine aldolase or threonine dehydrogenase, which both produce acetyl-CoA and glycine (House et al., 2001). Glycine can be completely catabolized to carbon dioxide and ammonia, or form serine. Serine can then be converted to pyruvate, or combine with homocysteine, a product of methionine metabolism, to form cystathionine, which in turn is cleaved to cysteine and α-ketobutyrate (Finkelstein, 1990).

The synthesis and cleavage of cystathionine are irreversible processes mediated by the enzymes cystathionine β-synthase and cystathionine γ-lyase respectively (Stipanuk, 2004). As mediators of the only catabolic pathway for methionine, these enzymes are directly responsible for plasma methionine homeostasis. An increase in the

production of α-ketobutyrate as a result of increased threonine intake and catabolism may have some capacity to inhibit these enzymes, although the primary regulator is the concentration of S-adenosyl methionine (Stipanuk, 2004) and regulation of these enzymes by dietary threonine or α-ketobutyrate has not been explored. It does stand as a possibility that threonine inhibits the catabolic pathway of methionine, explaining the increased plasma methionine concentration observed in response to an increase in dietary threonine intake.

It was expected that the plasma concentrations of most amino acids would exhibit a typical postprandial increase (Millward et al., 1996). An increase of plasma amino acid concentration 90 minutes following a meal was previously observed in a very similar study with male yearling horses receiving a 55% concentrate and 45% forage diet containing nearly identical ingredients (Tanner, 2014). In contrast, most amino acids, including threonine did not experience a change in plasma concentrations in response to the meal. Horses were not fasted prior to sample collection, and it was expected that horses would consume most of the previous evening's meal within a few hours of feeding. However, horses had feed remaining at the time of the pre-feeding sample collection, and it is possible that horses fed gradually throughout the night and within a few hours of sampling. It may be advisable for sampling to occur immediately after horses are brought in from turnout when feeding has been prohibited with muzzles for at least six hours.

There was a significant effect of treatment on some phenylalanine kinetics parameters as a result of a significant change in phenylalanine flux, from which they were calculated. A significant effect of treatment on flux violates the principles of the

IAAO method, as it indicates that the level of threonine intake affects the size of the free phenylalanine pool, and which, as an indicator amino acid, should be independent of the treatment (Elango et al., 2009). Flux is calculated from the rate of phenylalanine infusion and plateau isotope enrichment of the plasma. Oral [$^{1\text{-}13}$C] phenylalanine dosing has been previously shown to result in stable plasma enrichment values that are not affected by test amino acid intake level in adult horses (Mastellar et al., 2016a). However, this method relies on complete intake of each dose of phenylalanine isotope, which was not always achieved in the present study. The young horses were often too distracted, agitated, or tired to finish their meals. Oral dosing was chosen to avoid the trauma of inserting two catheters into each horse, but it may be necessary in future studies with finicky horses to obtain stable isotope enrichment.

It was expected that threonine would be limiting in horses consuming the lower levels of threonine intake, and that phenylalanine oxidation would decrease with increasing levels of intake until threonine is no longer limiting. The results of our study show that there was no significant effect of threonine intake level on phenylalanine oxidation, suggesting that threonine was not limiting within our range of intakes. The low threonine pelleted concentrate was formulated to contain 0.25% threonine, but the results of our analysis indicate that it contained approximately 0.41% threonine, which may have resulted a range of threonine intakes above the actual threonine requirement. The estimated threonine requirement of horses based on the ratio of lysine to threonine in the muscle is 69 mg/(kg BW·d) (Bryden, 1991), well below our lowest level of 91 mg/(kg BW·d), although estimating the requirement based on muscle composition may not be

accurate due to the proportionally large amount of threonine used for proteins other than muscle within the body, including intestinal mucins (Montagne et al., 2004).

A previous study did show a significant increase in girth gain in yearling horses when threonine intake increased from 93 to 110 mg/kg BW/d and lysine intake increased from 116 to 127 mg/kg BW/d (Graham et al., 1994b). These intakes of threonine are within the range of the present study, however these horses were group-fed Bermudagrass hay and provided concentrate ad-libitum for 1.5 h each day. These differences in feed intake, form, and composition may account for the conflicting results of threonine supplementation. Additionally, there were no other significantly affected growth parameters in the previous study and the effect of increased threonine intake cannot be separated from increased lysine intake (Graham et al., 1994b).

Based on the results of the present study, we conclude that threonine was not limiting in the provided dietary conditions and reported rates of growth. Due to the negative effects of nitrogen excretion on the environment and the health of horses and farmworkers, it is imperative that research on the amino acid requirements of growing horses continues.

Chapter 5. Threonine requirements of yearling horses fed a high forage diet

I. Introduction

The individual amino acid requirements of horses have not yet been determined. Efforts are currently underway to determine the requirement for threonine, which has been suggested to be the second limiting amino acid in common horse diets (Graham et al., 1994b; Staniar et al., 2001). As with other dietary nutrients, the digestibility and endogenous losses of threonine has been shown to vary with increasing fiber content in swine (Myrie et al., 2008). Horses typically consume a diet ranging from 50 to 100% forage (NRC, 2007), resulting in a relatively high daily intake of fiber, particularly in comparison to other non-ruminant species. Consequently, it is important that nutrient requirements are determined over a wide range of forage intakes.

The dietary requirement for threonine previously has been explored in growing horses receiving a diet consisting of 40% forage and 60% concentrate (see Chapter 4), which reflects the minimum intake of forage (1% of BW) observed in standard equine productions systems (see Ch. 4). No effect of threonine intake levels ranging from 90.8 to 151.0 mg/(kg BW·d) was observed, leading the authors to conclude that threonine was not limiting at any of the provided levels of threonine intake when horses were fed a high concentrate diet. It is predicted, however, that an effect will be observed as the percentage of forage in the diet increases. Threonine, along with serine, composes up to 20 to 55% of mucin, a glycoprotein which is responsible for lubricating the gastrointestinal tract (Van Klinken et al., 1995). Mucin production and secretion into the intestinal lumen increases in response to increased fiber content of the digesta in other species (Montagne et al., 2004). The amino acids within mucin cannot be reabsorbed

within the large intestine, and are therefore excreted, resulting in large endogenous losses of threonine. It is expected that increasing the forage content in the diet of horses will increase endogenous losses of threonine and increase the dietary requirement for this amino acid.

The objective of this study was to determine the threonine requirements of growing horses receiving a high forage diet consisting of 75% forage and 25% concentrate, using the indicator amino acid oxidation method.

II. 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. Six female Thoroughbred horses aged 10 to 12 months were obtained from the University of Kentucky research herd. Initial body weights ranged from 293.5 kg to 423.0 kg, and the average was 336.6 (± 44.4) kg. Horses were housed individually in 3.7 × 3.7 m stalls overnight, and turned out together with muzzles to prevent grazing between the hours of 0900 and 1500. Horses were transitioned from a previous high concentrate study diet to a high forage diet over the course of one week, then were given one week to adapt to treatment diets before sampling. Horses were fed their experimental diets at 0600 and 1500, and refusals were collected before the morning meal and weighed each day to calculate total daily intake.

Experimental Design

Horses were randomly assigned to 1 of 6 dietary treatments in a 6 × 6 randomized Latin square design. On day 0 of each period, horses began consuming their experimental

diet at the afternoon meal and continued to consume that diet for 7 days. As will be later described in detail, pre- and post- meal blood samples for urea and amino acid analysis were taken on day 6, and the isotope infusion protocol took place on day 7. Horses were then switched to the next dietary treatment on the afternoon of day 7 following infusion procedures.

Dietary Treatments

The experimental diets were formulated to meet or exceed the estimated requirements for all nutrients (NRC, 2007). Daily feed offered was 2.75% of bodyweight on an as-fed basis. The composition of the diet was 25% pelleted concentrate and 75% roughage. Roughage composition varied from 100% timothy cubes to 50% timothy cubes and 50% chopped timothy hay to maximize forage intake, but was consistent among all horses within a period. Two concentrate pellets were formulated and manufactured specifically for the high forage phase of the study (Buckeye Feeds, Dayton, OH): a high threonine pellet containing additional crystalline threonine and a low threonine pellet containing additional crystalline glutamate in equimolar concentrations to the threonine in the high threonine diet to ensure all treatments were isonitrogenous. All other ingredients remained the same, as shown in Table 5.1. The dietary treatments were produced by combining the high threonine (High Thr) and low threonine (Low Thr) concentrates in ratios of 0:1, 1:4, 2:3, 3:2, 4:1, and 1:0, resulting in expected threonine intakes of 109, 119, 128, 137, 146, and 155 mg/(kg BW·d). Scented apple oil was added the high thr concentrate to prevent technician error in mixing feeds. analyzed nutrient compositions of the dietary components are shown in Table 5.2.

Table 5.1. Feed ingredient composition of high and low threonine concentrate[1], DM basis, %

Ingredient	High Thr	Low Thr
Corn distiller dried grains	43.5	43.1
Soy hulls	17.6	17.4
Ground corn	15.3	15.8
Oatmill byproduct	6.5	6.4
Soybean oil	6.1	6.1
Apple flavor oil	0.11	0.00
Amino Acids		
L-Glutamate	0.00	0.95
L-Threonine	0.80	0.00
L-Lysine HCl	0.73	0.74
L-Histidine monohydrate HCl	0.46	0.46
Minerals and Vitamins		
Calcium carbonate	2.4	2.5
Magnesium oxide, 56%	2.0	2.1
Monocalcium phosphate, 21%	1.9	1.9
Salt	0.69	0.69
Vitamin E Acetate, 20%	0.41	0.41
Gro N Win pre-mix[2]	0.06	0.06
Equine trace mineral mix[3]	0.04	0.04
Selenium premix[4]	0.03	0.03
Zinc Sulfate, 36%	0.02	0.02

[1]Concentrates formulated for study by Buckeye Nutrition, Dalton, OH

[2] Contains: magnesium oxide, sodium bentonite, zinc sulfate, iron sulfate, Copper sulfate, manganese sulfate, Optimin Zinc, DL-Methionine, Optimin Copper , Selenium PX, Optimin Manganese, L-Lysine HCL, Optimin Iron, mineral oil, Optimin Selenium Yeast 3000, Organic Iodine EDDI, Vitamin C Monophosphate Stay-C, Buckeye Nutrition, Dalton, OH

[3]Contains: ferrous sulfate, ferrous carbonate, iron oxide, manganese sulfate, manganous oxide, chromium tripicolinate, copper sulfate, copper carbonate, tribasic copper chloride, copper oxide, zinc oxide, zinc sulfate, zinc methionine complex, calcium iodate, potassium iodide, eddi, pentacalcium orthoperiodate, cobalt carbonate, cobalt sulfate, sodium selenite, sodium molybdate, sodium sulfate, sodium bicarbonate, sodium chloride, sulfur, potassium sulfate, potassium chloride, potassium magnesium sulfate, magnesium sulfate, magnesium oxide, calcium sulfate, calcium carbonate, tricalcium phosphate, dicalcium phosphate, monoammonium phosphate, monosodium phosphate, ammonium chloride, ammonium sulfate, clay, bentonite, calcium stearate, zeolex (sodium aluminosilicate), zeofree (precipitate amorphous hydrated silicon dioxide), mineral oil, natural flavors and artificial flavors, Buckeye Nutrition, Dalton, OH

[4]Contains calcium carbonate, sodium Selenite, crystalline quartz silica, Prince Agri Products, Inc., Quincy, IL

Table 5.2. Nutrient composition of each component of treatment diet, as-fed basis

	High threonine concentrate	Low threonine concentrate	Timothy hay cubes	Chopped timothy hay
Overall nutrient composition				
Dry matter, %	88.5 ± 0.3	90.5 ± 1.6	91.1 ± 0.3	93.3 ± 0.0
DE, Mcal/kg	2.75 ± 0.05	2.80 ± 0.11	1.84 ± 0.10	1.98 ± 0.02
Crude protein, %	16.9 ± 0.2	17.6 ± 0.3	10.7 ± 0.3	7.1 ± 0.4
Acid detergent fiber, %	15.1 ± 0.1	16.0 ± 0.7	34.8 ± 2.5	38.3 ± 1.0
Neutral detergent fiber, %	28.9 ± 2.4	29.7 ± 1.3	53.4 ± 1.0	57.7 ± 1.1
Calcium, %	1.18 ± 0.02	1.29 ± 0.07	0.60 ± 0.03	0.28 ± 0.03
Phosphorus, %	0.73 ± 0.02	0.78 ± 0.02	0.22 ± 0.04	0.26 ± 0.05
Iron, mg/kg	560 ± 15	563 ± 20	451 ± 340	NA
Zinc, mg/kg	200 ± 10	225 ± 32	83 ± 51	NA
Amino acid composition, %				
Alanine	0.86 ± 0.05	0.92 ± 0.03	0.47 ± 0.03	0.43 ± 0.07
Arginine	0.65 ± 0.05	0.72 ± 0.02	0.39 ± 0.02	0.39 ± 0.08
Aspartate + Asparagine	0.57 ± 0.05	0.62 ± 0.06	0.76 ± 0.09	0.87 ± 0.16
Glutamate + Glutamine	2.34 ± 0.06	3.08 ± 0.25	0.83 ± 0.07	0.84 ± 0.2
Glycine	0.57 ± 0.03	0.61 ± 0.03	0.37 ± 0.03	0.35 ± 0.07
Histidine	0.60 ± 0.05	0.66 ± 0.03	0.15 ± 0.01	0.18 ± 0.04
Isoleucine	0.46 ± 0.09	0.51 ± 0.06	0.28 ± 0.05	0.28 ± 0.04
Leucine	1.47 ± 0.11	1.56 ± 0.04	0.59 ± 0.04	0.55 ± 0.08
Lysine	0.90 ± 0.08	0.94 ± 0.10	0.32 ± 0.03	0.30 ± 0.05
Methionine	0.19 ± 0.01	0.19 ± 0.01	0.10 ± 0.01	0.10 ± 0.02
Phenylalanine	0.63 ± 0.06	0.67 ± 0.03	0.36 ± 0.03	0.33 ± 0.04
Proline	1.09 ± 0.05	1.16 ± 0.03	0.58 ± 0.05	0.53 ± 0.05
Serine	0.68 ± 0.02	0.73 ± 0.06	0.4 ± 0.04	0.41 ± 0.06
Threonine	0.94 ± 0.14	0.57 ± 0.06	0.35 ± 0.02	0.33 ± 0.06
Tyrosine	0.51 ± 0.03	0.54 ± 0.02	0.19 ± 0.01	0.19 ± 0.03
Valine	0.59 ± 0.1	0.66 ± 0.05	0.37 ± 0.05	0.36 ± 0.05

Sampling and isotope infusion

Animals were weighed on day 6 of each experimental period at 1100 h. To analyze plasma urea and amino acid concentrations, blood samples were collected on day 6 of each study period prior to the morning meal and 90 and 180 minutes following the meal to measure the effects of threonine intake level on plasma amino acid and plasma urea concentrations. Blood was collected via venipuncture from the jugular vein into heparinized vacutainers (BD, Franklin Lakes, NJ) and immediately centrifuged at 1,500 × g for 10 min at 4 ºC. The supernatant was collected and stored at -20 ºC until the time of analysis.

On day 7 of each study period, whole-body phenylalanine kinetics were determined using a 2 h primed, constant intravenous infusion of [^{13}C] sodium bicarbonate at a prime rate of 5.30 μmol/(kg BW·h) and a constant rate of 4.41 μmol/(kg BW·h) followed by a 4 h primed, constant oral administration of [1-^{13}C] phenylalanine at a prime rate of 10.23 and a constant rate of 7.22 μmol/(kg BW·h) (Cambridge Isotope Laboratories, Andover, MA). The prime to constant ratio was previously validated in horses (Urschel et al., 2012), and the dose of phenylalanine has been shown to result in stable, measurable plateaus of isotope within the breath (Mastellar et al., 2016a). to maintain a steady metabolic state, horses received 1/48th of their daily ration every half hour throughout sampling, beginning 90 minutes before the start of the bicarbonate infusion. Any feed remaining by the time of next feeding was weighed and removed. Baseline blood and breath samples were collected immediately prior to isotope infusion. Blood was collected from a catheter placed in the jugular vein and processed as described for day 6 sampling. Breath was collected into gas impermeable bags using a modified

equine Aeromask (BreathEazy Ltd, Malvern, Worcestershire) and immediately analyzed (Urschel et al., 2012).

Following baseline sample collection, [^{13}C] sodium bicarbonate was infused into the intravenous catheter using a cordless infusion pump (J-1097 VetPro Infusion Pump, Jorgensen Laboratories Inc.) attached to a surcingle worn by the horse. During the infusion, breath samples were collected every 30 min for the first hour, then every 15 min during the second hour. After 2 h, infusion was stopped and horses received their [1-^{13}C] phenylalanine prime and first constant dose top-dressed on their meal. Horses continued to receive constant doses every half hour. Throughout administration, blood and breath samples were collected every half hour. After 4 h, catheters were removed and horses received their day 0 meal for the next treatment period.

Sample Analysis

Pre- and post-feeding blood samples from day 6 were analyzed for plasma amino acid and urea nitrogen concentrations. Plasma urea nitrogen concentrations were measured using a colorimetric spectrophotometric assay. A 10-μL aliquot of each plasma sample was pipetted in duplicate into microcentrifuge tubes containing 125 μL of urease buffer (Sigma-Aldrich Co., St. Louis, MO). After 20 min of incubation at room temperature, 250 μL of phenol nitroprusside solution, 250 μL of alkaline hypochlorite solution, and 1000 μL of distilled water were added to each sample. After 25 min of incubation at room temperature, 200 μL of each sample were transferred in duplicate to 96 well plates, placed in a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA,) and read at 570 nm of wavelength. The intra-assay variation was < 10% for the urea nitrogen analysis.

Plasma amino acid concentrations were determined using high performance liquid chromatography (HPLC) as previously described (Bidlingmeyer et al., 1984; Urschel et al., 2010). Briefly, samples were deproteinated using 10-kd cutoff centrifugal filters, and filtrate was freeze-dried. Norleucine was added as an internal standard. Ethanol, water, and trimethylamine (TEA) (Fisher Scientific, Fair Lawn, NJ) were then added in a 2:2:1 ratio, and samples were re-dried. Amino acids were then derivatized by adding ethanol, TEA, water, and phenylisothiocyanate (Acro Organics, Geel, Belgium) in a 7:1:1:1 ratio and incubating in a sealed container at room temperature for 20 min. Samples were then freeze-dried and reconstituted with HPLC eluent and injected onto a 3.9x300mm PICO-TAG reverse phase column (Waters Corporation, Milford, MA).

Sub-samples of timothy hay cubes, chopped timothy hay, and the high and low threonine pelleted concentrates were collected each week and sent for proximate analysis via wet chemistry (Dairy One Cooperative Inc., Ithaca, NY). Feed amino acid concentrations were determined using acid hydrolysis (AOAC International, 2005). Samples were ground, and 0.2 g was weighed into ashed vials. To these vials 12 mL of 6N hydrochloric acid (Fisher Scientific, Fair Lawn, NJ) was added and samples were capped tightly and incubated at 110 °C for 24 h. After incubation, samples were filtered using a 0.45 μm syringe filter (Sarstedt, Numbrecht, Germany) into microcentrifuge tubes. Samples were then derivatized and analyzed by HPLC as described for plasma samples. To measure methionine concentrations, a separate assay was performed in which 2 mL of performic acid (J.T. Baker Chemicals, Center Valley, PA) and 0.42 g of metabisulfite (Fisher Scientific, Fair Lawn, NJ) were added to samples and incubated overnight at 4 °C prior to the addition of hydrochloric acid (AOAC International, 2005).

The isotope enrichment of plasma samples collected during isotope infusion was determined by negative chemical ionization GC-MS analysis of a heptafluorobutyric, n-propyl derivative (Metabolic Solutions Inc., Nashua, NH), as previously described (Matthews et al., 1990; Urschel et al., 2011). A Phenomenex ZB-1MS capillary column was used to separate the derivative of phenylalanine. Selected ion chromatograms were obtained by monitoring ions at a m/z 383 and 384 for phenylalanine and [1-13C]phenylalanine, respectively. The isotope enrichment of breath samples was determined by measuring the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the breath using an infrared isotope analyzer (IRIS-3; Wagner Analysen Technik Vetriebs GmbH, Bremen, Germany) (Urschel et al., 2012).

Calculations

The average enrichment at isotopic stable state was determined for both plasma and breath samples. The plateau was defined as at least 3 points with a coefficient of variation less than 10%. If the plateau was not obtained, the data was discarded. Total CO_2 production rate was determined using the average enrichment of breath samples during [^{13}C] sodium bicarbonate infusion with the following formula:

$$CO_2 \text{ production} = i \times [(E_i/E_b) - 1] \times [0.0224 \text{ mL}/\mu\text{mol } CO_2]$$

where i is rate of the isotope infusion (μmol//(kg BW·h)), E_i is the enrichment of isotope solution, and E_b is the plateau breath enrichment (Hoerr et al., 1989).

Whole-body phenylalanine flux was calculated using the following equation where Q is flux (μmol//(kg BW·h)), i is rate of the isotope infusion (μmol//(kg BW·h)), E_i

is isotope solution enrichment, and Ep is the plateau plasma enrichment (Hsu et al., 2006):

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

The different processes affecting flux include amino acids entering the blood 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) (Picou and Taylor-Roberts, 1969):

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

Phenylalanine entering the plasma amino acid pool from dietary intake (I) was corrected for pre-cecal digestibility by multiplying phenylalanine intake from forage by factor of 0.4 (Gibbs et al., 1988) and multiplying the phenylalanine intake from concentrate by a factor of 0.7 (Farley et al., 1995), and further corrected by assuming that 26.5% of the digestible phenylalanine was extracted by the splanchnic tissues (Mastellar et al., 2016a). Mammals cannot synthesize phenylalanine de novo, so the phenylalanine entering the blood amino acid pool from protein breakdown (B) can be determined from the following equation:

$$B = Q - I$$

[$^{1\text{-}13}$C] phenylalanine oxidation was calculated using the following equation (Hsu et al., 2006):

$$E = F^{13}CO_2 \times (1/E_p - 1/E_i) \times 100$$

where E represents phenylalanine oxidation (μmol/(kg BW·h)) and $F^{13}CO_2$ is the product of isotope enrichment of the breath and the rate of carbon dioxide production (μmol/(kg BW·h)). Phenylalanine conversion to tyrosine was assumed to be minimal and any that did occur should be the same for all treatments because both phenylalanine and tyrosine intakes were not different between treatments. Therefore, the rate of protein synthesis can be estimated using the following equation:

$$Z = Q - E$$

Statistical Analysis

All experimental data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Repeated measures analysis was used for analyzing plasma amino acids and plasma urea nitrogen from pre- and post-feeding blood samples. Fixed effects were treatment, time, period, time × period, treatment × period, and time × treatment interactions, and horse was included as a random effect. A one-way ANOVA was conducted to estimate treatment effects on intake and whole-body phenylalanine kinetics. Orthogonal polynomial contrasts were used to test linear, quadratic, and cubic effects of treatment and time, with the IML procedure of SAS used to determine contrast coefficients. The correlation of threonine intake and plasma threonine concentrations were determined using simple linear regression for descriptive purposes. The break point in [$^{1\text{-}13}$C] phenylalanine oxidation rate was determined using a biphasic linear regression model with the slope of the 2nd phase restricted to 0. Significance was determined at $P < 0.05$. Measurements are shown as least squares means, and the standard error of the mean is denoted as SEM.

III. Results

Growth

The mean average daily gain for the length of the study was 0.91 (± 0.14) kg/day, which is above the normal range for thoroughbred yearling horses of 0.4 to 0.7 kg (NRC, 2007).

Intake

As shown in Table 5.3, there was no significant effect of threonine intake level on daily total ration intake, daily forage intake, or daily concentrate intake ($P \geq 0.05$). The mean intake rates and the composition of the feed components resulted in daily nutrient intakes shown in Table 5.4. The average intake of digestible energy was 95.2% of recommended intake. The amount consumed of all other nutrients exceeded recommendations.

Table 5.3. Effect of threonine intake level on average daily intake in growing horses fed a high forage diet, as-fed basis, g/(kg BW·d)

	Level of threonine intake, mg/(kg BW·d)							*P*-values			
Intake	109	119	128	137	146	155	SEM[1]	Treatment	Linear	Quadratic	Cubic
Total	26.2	26.2	26.3	27.2	26.6	25.5	0.8	0.472	0.824	0.134	0.197
Forage	19.3	19.3	19.6	20.3	19.8	18.6	0.8	0.396	0.788	0.089	0.189
Hay cubes	12.7	13.0	13.4	13.5	13.0	12.5	0.7	0.809	0.858	0.170	0.810
Chopped hay	6.6	6.3	6.2	6.9	6.8	6.1	0.4	0.489	0.890	0.615	0.079
Concentrate	6.9	6.9	6.7	6.9	6.9	6.9	0.1	0.408	0.771	0.276	0.523
High Thr[2]	0.0	1.4	2.7	4.1	5.5	6.9					
Low Thr[2]	6.9	5.5	4.0	2.8	1.4	0.0					

[1]SEM, pooled standard error of the mean

[2]Calculated from mean concentrate intake and the ratio of high thr to low thr of the dietary treatment

Table 5.4. Calculated average daily nutrient intakes of growing horses fed a high forage diet containing increasing levels of threonine

Nutrient	Level of threonine intake, mg/(kg BW·d)						NRC (2007) Recommendation[1]
	109	119	128	137	146	155	
Digestible energy, kcal/kg BW	55.7	55.6	55.6	57.5	56.4	54.0	58.6
Crude protein, g/kg BW	3.04	3.04	3.04	3.12	3.05	2.93	2.64
NDF, g/kg BW	12.63	12.61	12.71	13.20	12.86	12.18	
ADF, g/kg BW	8.05	8.02	8.09	8.40	8.18	7.72	
Calcium, mg/kg BW	183.4	182.8	181.6	184.5	179.7	173.2	117.4
Phosphorus, mg/kg BW	98.7	97.9	96.7	99.2	97.2	93.6	65.1
Iron, mg/kg BW	9.60	9.73	9.82	9.95	9.72	9.49	1.25
Zinc, mg/kg BW	2.60	2.59	2.56	2.56	2.49	2.41	1.00
Amino acids, mg/kg BW							
Alanine	152.8	151.7	150.6	154.4	150.5	143.9	
Arginine	126.4	125.3	124.3	127.4	123.9	118.1	
Aspartate + Asparagine	196.2	194.9	195.1	201.8	196.1	185.4	
Glutamate + Glutamine	381.5	369.5	355.6	354.6	337.6	315.6	
Glycine	112.6	111.8	111.2	114.2	111.2	106.1	
Histidine	76.4	75.5	74.1	75.6	73.8	71.0	
Isoleucine	88.7	88.1	87.6	90.0	87.7	83.7	
Leucine	219.7	218.5	216.7	222.3	217.5	209.4	
Lysine	125.2	124.7	123.7	126.9	124.5	120.2	113.4
Methionine	22.9	22.9	22.8	23.4	23.0	22.2	
Phenylalanine	114.0	113.5	113.2	116.4	113.8	109.1	
Proline	190.2	189.2	188.0	192.8	188.2	180.4	
Serine	129.0	127.8	126.7	129.8	126.2	120.3	
Threonine	105.5	110.7	115.8	124.7	127.7	128.8	
Tyrosine	73.5	73.2	72.7	74.7	73.2	70.6	
Valine	116.2	115.5	114.9	118.0	115.0	109.8	

[1]Calculated based on the average body weight of 12 month old horses as determined by the NRC

Plasma Urea and Amino Acid Concentrations

As shown in Table 5.5, there was a significant positive linear association of threonine intake level and the plasma concentrations of methionine and threonine ($P < 0.05$). There was a significant negative linear association of arginine and glutamine with

level of threonine intake ($P < 0.05$). Serine had a significant cubic response to dietary threonine level ($P = 0.006$). The dispensable amino acids glutamine, arginine, and asparagine also had significant responses to treatment. There were no time by treatment interactions.

Table 5.5. Main effect of threonine intake level on plasma concentrations of urea nitrogen and amino acids in growing horses fed a high forage diet

	Level of threonine intake, mg/(kg BW·d)							*P*-values			
Metabolite	109	119	128	137	146	155	SEM	Treatment	Linear	Quadratic	Cubic
Plasma urea nitrogen, mmol/L	4.3	4.0	4.3	4.0	4.3	4.4	0.3	0.549	0.581	0.205	0.785
Indispensable amino acids, μmol/L											
Histidine	96	107	110	105	100	106	5	0.387	0.565	0.196	0.091
Isoleucine	57	50	54	52	50	54	3	0.226	0.264	0.189	0.784
Leucine	128	127	129	122	130	128	9	0.946	0.908	0.722	0.956
Lysine	140	149	158	156	152	151	10	0.822	0.459	0.259	0.760
Methionine	32	34	36	36	35	37	2	0.058	0.005	0.277	0.519
Phenylalanine	66	67	71	70	67	71	3	0.117	0.093	0.323	0.279
Threonine	99	143	165	188	188	252	23	<0.0001	<0.0001	0.913	0.134
Tryptophan	71	68	75	77	65	73	4	0.059	0.950	0.383	0.583
Valine	204	190	195	188	190	189	11	0.457	0.120	0.382	0.544
Dispensable amino acids, μmol/L											
Alanine	360	383	416	375	378	385	19	0.175	0.536	0.132	0.115
Arginine	112	108	112	108	99	107	4	0.031	0.036	0.723	0.155
Asparagine	54	56	59	57	55	65	4	0.085	0.048	0.434	0.055
Aspartate	10	10	10	8	10	7	1	0.109	0.070	0.521	0.436
Glutamate	51	46	49	47	50	43	6	0.531	0.274	0.878	0.188
Glutamine	477	495	525	526	499	529	61	0.131	0.046	0.273	0.344
Glycine	446	506	549	494	462	526	49	0.408	0.491	0.416	0.092
Proline	179	182	188	180	174	198	12	0.386	0.251	0.653	0.094
Serine	291	303	320	315	295	356	25	0.002	0.002	0.322	0.007
Tyrosine	81	79	84	79	79	76	7	0.640	0.297	0.316	0.674

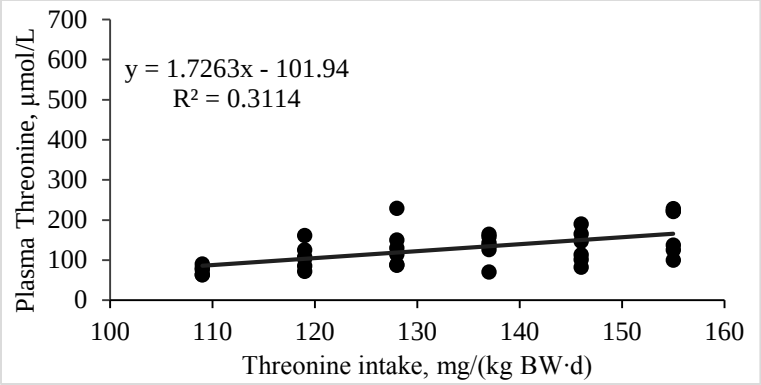

Figure 5.1. Correlation of level of threonine intake with plasma threonine concentration prior to the morning meal in growing horses fed a high forage diet.

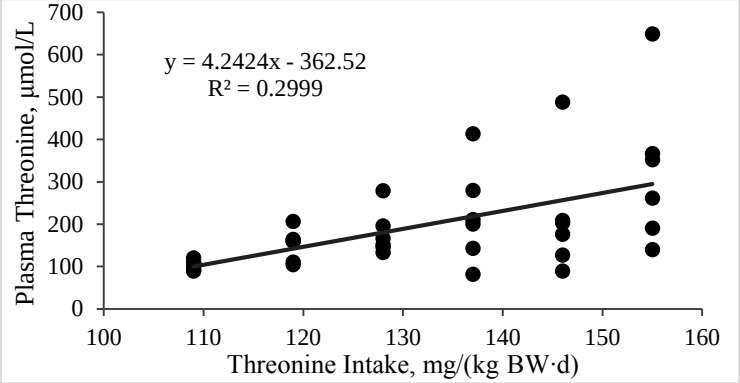

Figure 5.2. Correlation of threonine intake with plasma threonine concentration 90 minutes after the morning meal in growing horses fed a high forage diet.

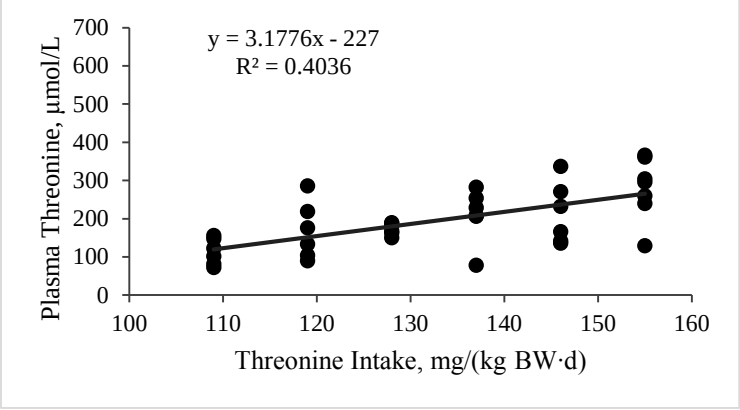

Figure 5.3. Correlation of threonine intake with plasma threonine concentration 180 minutes in growing horses fed a high forage diet.

There was a weak but significant correlation of threonine intake level with plasma threonine ($R^2 = 0.25$; $y = 1.6808x - 78.219$). There was also a significant effect of time relative to feeding ($P < 0.0001$) on plasma threonine concentration, and the interaction of time and treatment was not significant.

As shown in Table 5.6, the concentration of plasma urea nitrogen (PUN) and 14 of the amino acids had a significant response to the time of sampling relative to feeding. The majority of dispensable amino acids, including asparagine, glutamine, alanine, proline, tyrosine, arginine, as well as phenylalanine had plasma concentrations increase linearly in response to the time of feeding ($P < 0.05$). The majority of indispensable amino acids, specifically leucine, isoleucine, histidine, threonine, and lysine as well as serine showed a significant quadratic response to time, with an initial increase 90 min after feeding, followed by an decrease at 180 min ($P < 0.05$).

Table 5.6. Main effect of time relative to feeding on the plasma concentrations of urea nitrogen and amino acids in growing horses fed a high forage diet

	Time post-feeding, min				*P*-values		
Item	0	90	180	SEM	Time	Linear	Quadratic
Plasma urea nitrogen, mmol/L	4.1	4.3	4.3	0.2	0.041	0.068	0.075
Indispensable amino acids, μmol/L							
Histidine	77	117	118	5	<0.0001	<0.0001	0.009
Isoleucine	50	57	51	3	0.051	0.693	0.018
Leucine	109	140	133	8	<0.0001	0.001	0.005
Lysine	106	182	165	8	<0.0001	<0.0001	<0.0001
Methionine	31	37	37	2	0.001	<0.0001	0.047
Phenylalanine	64	71	71	3	0.001	0.001	0.064
Threonine	127	199	191	19	<0.0001	<0.0001	0.003
Tryptophan	74	72	68	3	0.016	0.005	0.462
Valine	184	197	197	10	0.090	0.062	0.231
Dispensable amino acids, μmol/L							
Alanine	354	393	402	15	0.001	<0.0001	0.138
Arginine	96	113	113	4	<0.0001	<0.0001	0.070
Asparagine	47	61	65	4	<0.0001	<0.0001	0.106
Aspartate	9	9	9	1	0.176	0.091	0.427
Glutamate	51	47	45	6	0.185	0.076	0.598
Glutamine	461	519	545	60	<0.0001	<0.0001	0.293
Glycine	485	495	511	49	0.655	0.366	0.866
Proline	159	186	204	11	<0.0001	<0.0001	0.464
Serine	290	329	321	24	0.002	0.002	0.004
Tyrosine	74	83	81	6	0.020	0.028	0.068

Whole Body Phenylalanine Kinetics

As shown in Table 5.6, plasma phenylalanine flux, CO_2 production, and phenylalanine balance were not significantly affected by the level of threonine intake ($P \geq 0.05$). Data values of one horse were removed from treatments 1, 2, and 4, and three horses were removed from treatment 5 due unstable isotope enrichment values. This resulted in each horse being tested at 5 of the 6 treatment levels. Phenylalanine entering the free phenylalanine pool from protein breakdown, and phenylalanine leaving the free

amino acid pool by oxidation and non-oxidative disposal were also unaffected by threonine intake ($P \geq 0.05$). As demonstrated in Fig. 5.4, a breakpoint in phenylalanine oxidation rate was not detected by broken line analysis ($P = 0.77$).

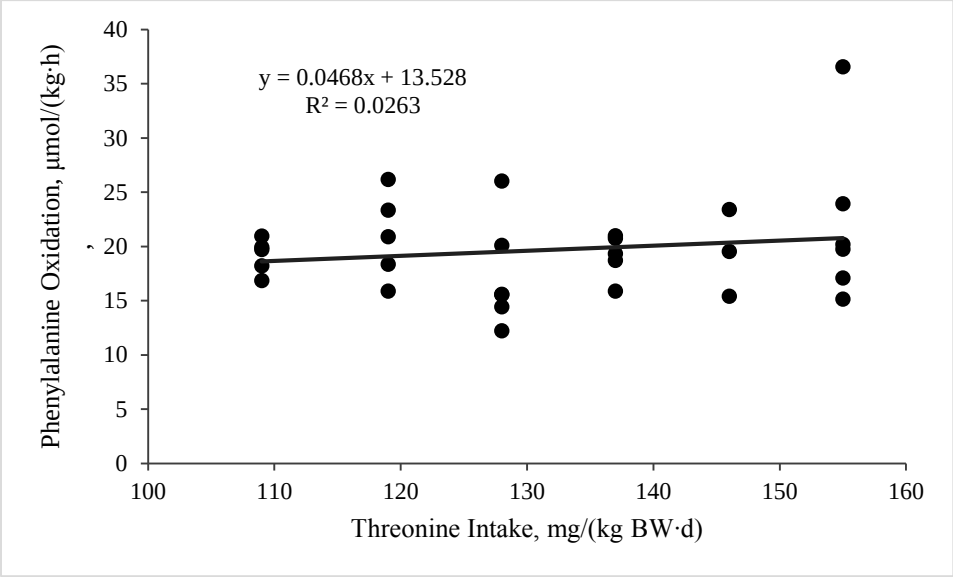

Figure 5.4. Effect of threonine intake level on the rate of phenylalanine oxidation in growing horses fed a high forage diet.

Table 5.7. Effect of level of threonine intake on parameters of phenylalanine flux in growing horses fed a high forage diet, μmol/(kg·h)

	Level of threonine intake, mg/(kg BW·d)							*P*-values			
	109	119	128	137	146	155	SEM	Treatment	Linear	Quadratic	Cubic
Phe flux[1]	81.1	85.5	79.7	84.6	85.9	82.2	3.4	0.381	0.553	0.579	0.619
Phe oxidation[2]	18.2	20.1	17.3	17.9	17.2	22.1	3.0	0.550	0.549	0.266	0.251
Phe for protein synthesis[3]	64.6	66.8	62.3	66.4	67.8	60.1	4.1	0.391	0.539	0.288	0.282
Dietary Phe intake[4]	10.1	10.2	10.2	10.2	10.2	10.2	0.03	0.141	0.471	0.021	0.563
Phe from protein breakdown[5]	71.0	75.2	69.5	74.4	75.7	72.1	3.4	0.385	0.555	0.596	0.619
CO_2 production[6]	19338	18175	17730	19213	18853	18643	706	0.525	0.985	0.386	0.136

[1]Flux = isotope infusion rate × [(isotope solution enrichment/ plateau plasma enrichment)-1]

[2]Oxidation = 100 × [(1/plateau plasma enrichment – 1/isotope solution enrichment) × rate of $^{13}CO_2$ release during phenylalanine infusion]

[3]Non-oxidative Phe disposal = Flux – Oxidation

[4]Assumes a pre-cecal digestibility of 42% (Gibbs et al., 1988; Farley et al., 1995) and a splanchnic extraction of 26.5% (Mastellar et al., 2016a)

[5]Phenylalaine entering pool from protein degradation = Flux – Phe from diet

[6]Total carbon dioxide production = isotope infusion rate × [(isotope solution enrichment/ plateau breath enrichment)-1] × [0.0224 mL/μmol CO_2]

IV. Discussion

Due to the importance of reducing nitrogen excretion and crude protein intake in horses, efforts are currently underway to determine the requirement of each individual essential amino acid. Previous work has shown that threonine is likely to be the next limiting amino acid after lysine for horses fed common feedstuffs (Graham et al., 1994b; Staniar et al., 2001). In a previous phase of the present study, it was found that threonine was not limiting for horses fed a diet consisting of 60% concentrate and 40% forage with a crude protein content of 12%. It was predicted that increasing the forage content of the diet would increase the requirement for threonine due to its use for mucin synthesis, which aids in lubricating the digestive tract (Van Klinken et al., 1995). Calculated neutral detergent fiber and acid detergent fiber intake was increased by 53% and 58% respectively, relative to the high concentrate phase of the study. However, the results of the present study suggest that threonine is not a limiting amino acid for growing horses fed an 11.5% crude protein ration consisting of 25% concentrate and 75% timothy hay cubes and chopped timothy hay.

Horses were provided with 2.5% of bodyweight per day in dry matter, and consumed approximately 2.4% which is within the expected range for yearling horses of 2.0% to 3.0% (NRC, 2007). This rate of intake was improved over that observed in the high concentrate phase of the study. During the high concentrate phase of the study, animals were exhibiting symptoms of gastric ulcers, which has been shown to reduce dry matter intake (Videla and Andrews, 2009). The combination of antacid treatment and

changing to a high forage diet likely allowed potential ulcers to heal and appetite to improve.

The horses were consuming less digestible energy than recommended, however they were experiencing high rates of growth. The mean average daily gain of the study animals was 0.91 kg/day, which is well above the typical range of 0.4 to 0.7 kg/day. The horses were experiencing depressed rates of growth in the months before this study, and were presenting growth patterns typical of compensatory gain often observed in yearlings in cold climates (Pagan et al., 1996; Staniar et al., 2004).

It was expected that by feeding increasing levels of threonine, protein synthesis would increase and amino acid oxidation would decrease, resulting in decreased urea synthesis. This response has previously been shown in horses and other species receiving amino acid supplementation (Eggum, 1970a; Mantovani et al., 2014). In contrast, the results of the present study did not show a significant response of plasma urea nitrogen (PUN) concentration to the level of threonine intake. This suggests that the rate of amino acid oxidation did not change throughout treatment levels. It is interesting to note that the average concentrations of PUN in the high forage phase of this study were greater than those observed in the high concentrate study. Protein within forages has been shown to be less digestible than that found within grains (Gibbs et al., 1988; Farley et al., 1995; Takagi et al., 2003), resulting in greater amounts bypassing digestion within the small intestine and entering the hindgut, where it is metabolized by microbes. Microbial protein metabolism results in the production of ammonia, which is absorbed by the epithelial cells of the large intestine and converted to urea (Schmitz et al., 1991). In this way, an increase in microbial protein metabolism increases plasma urea.

Plasma concentrations of threonine and arginine increased linearly and serine increased in a cubic pattern in response to increasing threonine intakes. The catabolism of threonine produces acetyl-CoA and glycine. Glycine can be completely catabolized to carbon dioxide and ammonia, or form serine (Wang et al., 2013). This pathway is likely the cause for the increase in serine concentrations in response to increased threonine intake. Arginine did have a statistically significant response to threonine, however, the difference between the highest and lowest mean concentration was 13 μmol/L, which is unlikely to be physiologically significant. As described in detail previously, methionine and threonine can both form α-ketobutyrate through their catabolic pathways, and it is conceivable that a buildup of that metabolite from increased threonine metabolism slows the catabolic enzymes of methionine, but this possibility has not been explored.

As was expected, most amino acids exhibited an increase in plasma concentration following the meal (Mastellar et al., 2016b). This is in contrast to the high concentrate phase of the study, which did not display this increase. The smaller amounts of concentrate provided in the high forage phase and improved appetites may have allowed the horses to consume the majority of their dietary amino acids the afternoon before sampling, resulting in conditions more reflective of fasting prior to the morning meal.

It was expected that threonine would be limiting in horses consuming the lower levels of threonine intake, and that the rate of phenylalanine oxidation would decrease with increasing levels of intake until threonine was no longer limiting. This effect was not observed, as the level of threonine intake did not significantly affect the rate of phenylalanine oxidation. This suggests that threonine is not the second limiting amino acid, or the requirement for dietary threonine is below the lowest level of intake. The low

threonine pelleted concentrate was formulated to contain 0.25% threonine, but the results of our analysis indicate that it contained approximately 0.57% threonine, which may have caused us to miss the requirement. The lowest level of intake in the present study was 109 mg/(kg BW·d). A previous study in yearlings observed an increase in girth gain when threonine intake increased from 93 to 110 mg/(kg BW·d) (Graham et al., 1994b), suggesting that the requirement is within or above that range. In contrast, the results of our study suggest that the threonine requirement for horses fed a high forage diet may be even lower than that. The high concentrate phase of our study included threonine intakes as low as 91 mg/(kg BW·d) and still we did not observe an effect of treatment on the rate of phenylalanine oxidation.

As previously described, it was expected that horses fed a high forage diet would have even greater requirements for threonine. The elevated rate of gain and consequently protein synthesis during the present phase was expected to further increase the threonine requirement. The estimated threonine requirement of horses based on the ratio of lysine to threonine in the muscle is 69 mg/(kg BW·d) (Bryden, 1991). It was expected that the actual requirement would be much greater than that, due to the disproportionately higher amounts of threonine in proteins such as mucin when compared with muscle tissue. However, based on our analysis, the estimate based on the composition of muscle may be closer to the actual requirement than we predicted. Another possibility is that 7 days of adaptation is not sufficient to change the synthesis of mucin production.

There were no other significant effects of threonine intake on phenylalanine kinetics parameters. We conclude that threonine is not a limiting amino acid for growing horses fed a high forage diet consisting of 11.5% protein. It is imperative for the health

of the environment, horses, and farmworkers that research into the amino acid requirements of horses continues.

Chapter 6. Overall summary and direction for future studies

The hypotheses of our studies were that the threonine requirement of growing horses would be observed within the range of threonine intakes we provided, and that that requirement would be greater for horses fed the high forage diet compared with those fed the high concentrate diet. Our range of intakes was based on those previously observed to have resulted in increased rates of growth in yearling horses (Graham et al., 1994b). The estimated increase of the requirement in response to increased forage intake was based on the observation that mucin, of which threonine is a major component, has increased production rates in response to dietary fiber in other species (Montagne et al., 2004).

The results of the high concentrate phase presented in Chapter 4 and the high forage phase presented in Chapter 5 suggest that threonine was not limiting even at the lowest levels of intake, regardless of diet composition. Using the indicator amino acid oxidation protocol, phenylalanine oxidation was measured at each level of threonine intake. According to the principle that amino acids are not stored, all amino acids which are not used for protein synthesis or converted to other metabolites must be oxidized. As intake of the amino acid limiting protein synthesis increases, the use of other amino acids for synthesis also increases and less amino acid oxidation occurs. Therefore, if threonine were limiting protein synthesis at any level of intake in our study, we would have seen decreased rates of oxidation of phenylalanine as threonine intake increased above that level. In contrast, phenylalanine oxidation was not significantly affected by the level of threonine intake and appeared to remain constant throughout all levels. This observation, combined with a lack of evidence for a threonine deficiency based on plasma urea

nitrogen and amino acid concentrations, leads us to conclude that threonine was not limiting protein synthesis at any level of threonine intake.

It could be that all amino acids were provided in sufficient amounts to achieve optimal rates of protein synthesis. A second explanation is that another amino acid was limiting protein synthesis. Previous work suggests that histidine may be a limiting amino acid in common horse diets (Tanner, 2014). Histidine was supplemented so that it was provided in levels above that fed in the previous study, and is unlikely to be limiting. A possible alternate limiting amino acid is methionine, which is commonly limiting in swine diets (Wang and Fuller, 1989). A recent study explored this possibility by feeding weanling quarter horses four different levels of methionine for 56 d and measuring growth, nitrogen retention, and plasma urea nitrogen concentrations (Winsco et al., 2011b). There was no effect of methionine level on growth or nitrogen balance. There was a significant quadratic effect of treatment on plasma urea nitrogen concentration, and the authors suggest that the initial decrease may indicate that the requirement is within their range of intakes, however the data is inconclusive.

to compare the methionine intake of the differently aged horses in the present study and the study by Winsco et al., values can be shown as a percentage of crude protein requirements (NRC, 2007). Corrected accordingly, the methionine intakes of the weanling study ranged from 1.3 to 1.7 % of their CP requirement, whereas the methionine intakes of the present study averaged 1.00 and 0.75 % during the high concentrate and high forage phases respectively. If Winsco et al. did identify a requirement within their range of intakes, then our diets may have been deficient in methionine. This is in contrast, however, with the observation that there was an increase

of plasma methionine as threonine intake increased. If methionine was limiting protein synthesis, then it should be diverted for use in protein synthesis rather than buildup in the plasma.

An interesting difference between the high concentrate and high forage phases of the study is the response of plasma threonine concentration to level of threonine intake. As shown in Figure 6.1, the high concentrate phase of the study (Chapter 4) resulted in higher plasma threonine concentrations compared with the high forage phase at similar levels of threonine intake (Chapter 5). This agrees with the differences in plasma urea nitrogen and suggests that amino acids were less bioavailable in the high forage phase of the study. A second explanation is that more threonine was utilized in the intestine for mucin synthesis. Despite possible decreased absorption or increased utilization of threonine during the high forage phase, threonine still did not appear to be limiting in either phase of the study.

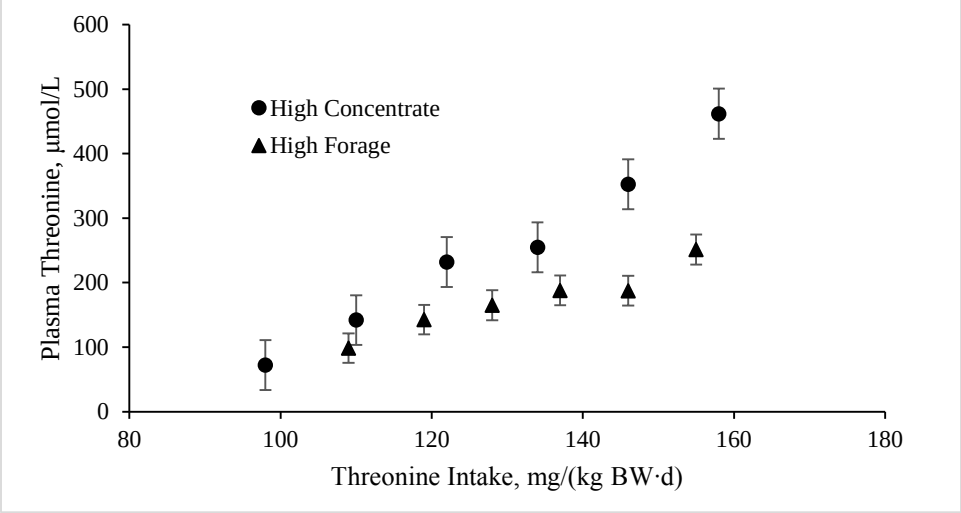

Figure 6.1. Effect of level of threonine intake on plasma threonine concentrations, comparing the high concentrate phase with the high forage phase.

While we were not able to determine a requirement for threonine from the present study, we did narrow down the range of possibilities and/or provide evidence that threonine may not be the second limiting amino acid. We were also able to find weaknesses within our current protocol to be improved upon in future studies. These include the inability to achieve stable isotope enrichment when horses did not completely consume their meals, which may necessitate insertion of a second catheter to infuse phenylalanine isotope instead of providing oral doses. Additionally, the slow-eating habits of young horses may mean that a fasting state must be forced through muzzling or feed withholding before pre- and post-feeding blood samples can be taken.

Based on the observations of the present and previous studies, I believe the next step we need to take is to determine the order of limiting amino acids. I propose a method similar to that previously described in swine (Wang and Fuller, 1989), in which the crude protein content of the feed is reduced to well below the recommended intake and basal diets are supplemented with equimolar amounts of the most likely limiting amino acids, for example a basal + Glu diet is compared with basal + Met, basal + Thr, basal + Val, etc. The IAAO method then can be used to measure any decreases in amino acid oxidation in response the supplemented amino acid. The amino acid resulting in the greatest decrease would be indicative of the most limiting amino acid, as described by Wang and Fuller. After a limiting amino acid has been determined, studies such as the present one can proceed to narrow the possible range containing the requirement and eventually determine the requirement. Once a requirement has been determined for a specific diet and physiological state, studies can be performed to measure how variation in these factors affects the requirement. This procedure should then be performed for all

amino acids, eventually resulting in knowledge equal to the extensive information and numerous ideal amino acid ratios known within the swine industry.

The research presented in this thesis is the first to use the IAAO method to take on the challenge of determining the threonine requirement of growing horses. Although a requirement could not be determined, we were able narrow the possible range of threonine intakes containing the requirement, as well as further perfect the IAAO protocol for use in growing horses. There is a great amount of research left to do regarding the amino acid requirements of horses, but the importance of animal health and environmental sustainability requires that this investigation continue.

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