INFLUENCE OF METHIONINE ON GROWTH AND NITROGEN BALANCE IN WEANLING QUARTER HORSES

A Thesis

by

KELLY NICOLE WINSCO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2009

Major Subject: Animal Science

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Approved by:

Chair of Committee,	Josie Coverdale
Committee Members,	Tryon Wickersham
	John Carey
Head of Department,	Gary Acuff

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ABSTRACT

Influence of Methionine on Growth and Nitrogen Balance in Weanling Quarter Horses.

(December 2009)

Kelly Nicole Winsco, B.S., University of Georgia

Chair of Advisory Committee: Dr. Josie Coverdale

Twenty-four Quarter horse weanlings (120 d ± 10 d) were blocked by age into 4 groups (n = 6) for a 56 d trial to evaluate the influence of methionine on growth and nitrogen retention. Weanlings were housed by block and individually fed 1 of 4 concentrate diets twice daily at 1.5% BW (as fed). Weanlings were randomly assigned to 1 of 4 treatments: basal (0.20 MET), basal + 0.03% methionine (0.23 MET), basal + 0.07% methionine (0.27 MET), and basal + 0.11% methionine (0.31 MET). Diets were formulated to be isonitrogenous, isocaloric, and contain equal amounts of LYS and THR. Coastal bermudagrass hay was individually fed at 0.75% BW (as fed). Growth measurements, body weight, rump fat, and plasma were obtained every 7 d. The final 4 days consisted of total collection of urine and feces. Feed, fecal, and urine samples were analyzed for nitrogen content and nitrogen balance was calculated. Urine was analyzed for urea and ammonia concentration. Plasma was analyzed for urea concentration. Grain, hay, and fecal samples were analyzed for nutrient composition.

Data were analyzed using the PROC MIX procedure of SAS. Linear, quadratic, and cubic effects were tested in the form of contrasts. There was no influence of

treatment on growth measurements, nitrogen balance, or urinary urea or ammonia. Intake of LYS and THR (g/d) did not differ among treatments ($P = 0.78$ and $P = 0.38$ respectively). Plasma urea nitrogen (PUN) was influenced by treatment ($P = 0.005$) exhibiting quadratic ($P = 0.04$) and cubic ($P = 0.002$) effects. An unexpected peak in PUN was observed with 0.27 MET. Upon analysis, 0.20 MET contained more lysine than formulated, and 0.27 MET contained the least lysine. Treatments 0.20 MET and 0.31 MET contained more threonine compared to formulations. These differences may explain unexpected values of PUN concentration. Results suggest future studies that more closely isolate methionine as the only dietary variable are necessary to better explain the methionine requirements of weanling horses.

DEDICATION

This thesis is dedicated to my family. Thank you for your encouragement and understanding during this process. Mom, your love and support, even from a thousand miles away, made this possible. Dad, thank you for helping me remember what is really important and always keeping things in perspective. Cristina, thank you for supporting me throughout this process and understanding my absence while Sam is young. I truly could not have done this without each one of you.

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Thank you to all of the graduate and undergraduate students who helped throughout the project. I cannot thank you enough for your assistance during total collections and 3 hour long feed shifts.

I would like to extend my gratitude to Cargill Animal Nutrition for providing the support required to complete the project. This was an amazing opportunity that helped me realize my interest in equine research.

Thanks also go to Dr. Carey, for his willingness to help begin my nitrogen analysis. You did not hesitate to allow me to have access to your lab and helped me learn to operate your nitrogen analyzer, and I am very grateful.

Dr. Tryon Wickersham, thank you for your guidance and support throughout my lab work. You believed in me even when I sometimes didn't, and your encouragement throughout the process helped me more than you know. Thank you.

Finally, I would like to thank Dr. Josie Coverdale. You have made several unbelievable opportunities available to me, and for this I cannot thank you enough. Thank you for your guidance in the classroom, on the farm, and everywhere in between.

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CHAPTER I

INTRODUCTION

Essential amino acids must be provided to the horse for protein synthesis and growth to occur. Accurate requirements for essential amino acids are important when formulating rations to avoid growth limiting deficiencies, waste, possible toxicity, and increased nitrogen load on the environment from over supplementation. Because the exact nutrient requirements for optimal equine growth are unclear, feed formulations used in the industry provide nutrients in excess rather than risk creating limitations.

When investigating amino acid requirements in the weanling horse it is important to consider the digestibility of protein and the location of digestion. Weanlings have reduced capability for fermentation in the large intestine compared to a mature horse and rely mainly on the upper tract for digestion of protein; therefore, the amino acid profile of the concentrate portion of the diet has more influence than the forage source (Gibbs and Potter, 2002). While the dietary requirement for lysine has been established (Ott et al., 1981), there is little information regarding the remaining essential amino acid requirements of the young growing horse (NRC, 2007). Lysine is considered the first limiting amino acid in equine diets and threonine has been suggested to be the second limiting amino acid, but the requirement has not been established (Graham et al., 1994). Methionine may be limiting due to its important roles in methyl group donation,

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free radical defense, and other crucial portions of protein structure and folding pathways (Brosnan and Brosnan, 2006). Methionine is the second limiting amino acid in other monogastric species at various stages of growth (Mavromichalis et al., 1998). Additionally, methionine is the first limiting amino acid in milk protein and is present in low concentrations in several commonly used protein sources for growing horse rations; therefore, it is likely to be limiting without supplementation (Becker et al., 1955). Methionine requirements can be indirectly determined by evaluating plasma urea nitrogen (PUN), N retention, and directly determined by physical growth measurements or carcass quality characteristics (Balogun and Fetuga, 1981). It can be expected that PUN will decrease with increasing inclusion of an essential amino acid until the requirement is met, at which point a plateau, or break-point, will be reached. Break-point analysis of N retention can also be used to determine amino acid requirements, and higher quality protein sources create increased N retention allowing for greater protein deposition or growth (Chung and Baker, 1992). Conversely, compensation by over supplementing methionine, especially in the crystalline form, may negatively impact growth, and has been shown to reduce intake and growth rate in pigs (Chung and Baker, 1992). Therefore, it is important to determine a dietary requirement for equine rations, especially during various stages of growth. When investigating methionine requirements, interactions between the remethylation and transsulfuration pathways make it difficult to isolate methionine from the other sulfur containing amino acids. Therefore, when dietary methionine concentrations are altered in the diet, total sulfur amino acids are evaluated (Brosnan and Brosnan, 2006).

CHAPTER II

REVIEW OF LITERATURE

Protein and Amino Acids

All animals require nutrients for maintenance, growth, production, work, and reproduction. These nutrients include water, protein, carbohydrates, lipids, vitamins, and minerals. These nutrients are required for proper nutrition and play important roles within the body; however protein is a major component of body tissue and is second in concentration only to water (Frape, 2004). A protein is composed of chains of individual amino acids linked together by peptide bonds. It is these individual amino acids that are used to form body tissues, enzymes, antibodies, and hormones, and must be present in order for growth or production to occur. There are 30 common amino acids, of these, 10 are considered essential amino acids. Essential amino acids cannot be synthesized within the body; therefore they must be consumed from dietary protein sources (Ellis and Hill, 2005). The remaining 20 amino acids are considered nonessential and can be synthesized within the body using carbon skeletons derived from other amino acids (NRC, 1998).

Amino acids are classified based on essentiality, structure, charge, and polarity. The basic structure of an amino acid is an amine group, a carboxyl group, and a side chain. The structure and composition of the side chain greatly affects the behavior and role of the amino acid, and classifies it as neutral, acidic, basic or heterocyclic, with neutral amino acids further breaking down into the classifications of aliphatic, aromatic, and sulfur based amino acids (Ellis and Hill, 2005). These individual amino acids link

together by means of peptide bonds to form proteins, which also have varying structure classifications that affect their role and behavior.

Protein structure is divided into quaternary, tertiary, secondary, and primary forms. Primary protein structure is the most simple, and is a sequence of amino acids. Primary proteins are most susceptible to enzymatic digestion and will be more quickly broken down. Secondary structure involves hydrogen bonding which twists chains of amino acids into either a coiled α helix or a folded β sheet. Tertiary structure involves layers or groups secondary structures using hydrogen and disulfide bonds. Quaternary structure is the most complex, with several tertiary structures linked together (Ellis and Hill, 2005). Most dietary proteins are in quaternary form; therefore, in order for dietary protein sources to provide the necessary amino acids the large complex structure must be gradually broken down and unwound (Frape, 2004). This gradual disruption of the complex protein structure is the first step in protein digestion. An animal cannot absorb the whole proteins in the diet; instead, the animal must break the peptide bonds to absorb free amino acids or peptides. The process of protein digestion begins in the stomach and is completed in the small intestine.

Protein Digestion - Stomach

Protein digestion begins in the stomach of all monogastric animals. In the horse, the stomach comprises 10% of the total gastrointestinal tract and contains three different regions: esophageal, cardiac, fundic, and pyloric (Frape, 2004). The saccuss caecus within the esophageal region contains a substantial microbial community that contributes to starch digestion (Ellis and Hill, 2005). The cardiac glandular region secretes mucus

and bicarbonate which help protect the stomach lining. Digesta then moves to the fundic region which contains parietal and chief cells that secrete HCl and pepsinogen, respectively. Digesta then moves to the pyloric region where the pH reaches less than or equal to 2. The acidic environment created by the release of HCl causes denaturation of quaternary and tertiary protein structures by disrupting the hydrogen bonds (Frape, 2004). The release of HCl also converts pepsinogen to its active form, pepsin. Pepsin is the first actual enzyme involved in protein digestion, and is a specific endopeptidase which cleaves bonds on the amino side of aromatic amino acids. The action of HCl and pepsin within the stomach digests dietary protein to smaller peptides and free amino acids before moving to the small intestine. The extent of protein digestion within the stomach is limited by the rate of passage, with slower passage allowing more time for the enzyme to act (Frape, 2004). As previously discussed, in the horse the stomach comprises only 10% of the total GI tract; therefore, rapid passage through the stomach commonly occurs.

Protein Digestion - Small Intestine

Digesta enters the small intestine through the pyloric sphincter. In a 500 kg horse the small intestine is 21 to 25 m in length and has a capacity of 40 to 50 L; therefore, the rate of passage through the small intestine is slower than the stomach allowing more time for digestion and absorption to occur (Frape, 2004). The small intestine is the primary site for digestion of proteins and absorption of the products of protein digestion, and is divided into three sections: the duodenum, jejunum, and ileum. The duodenum is the major site of protein digestion and absorption of amino acids and small peptides

(Ellis and Hill, 2005). The small intestine is lined with a thin layer of mucosa, which is the layer of tissue separating intestinal content, or lumen, from the blood. The mucosal layer is composed of villi, crypts, and lacteals with each villus lined with microvilli to make up a brushy border (Frape, 2004). The villi and microvilli increase the surface area of the small intestine to increase digestive and absorptive capabilities. Enterocytes are epithelial cells that compose the brush border and are the main site of nutrient absorption (Frape, 2004). The small intestine has a neutral or somewhat alkaline pH which is much higher than the acidic pyloric region of the stomach. This elevated pH helps facilitate active transport and enzyme activity, and most of the nutrients contained in feed are digested and absorbed prececally in the small intestine (Ellis and Hill, 2005). Intestinal mucus and brush border enzymes are secreted from within the small intestine. Mucus secreted from the Brunner's glands helps lubricate and buffer the duodenal wall.

Brush border enzymes include sucrase, lactase, isomaltase, maltase, aminopeptidase, dipeptidase, and enterokinase and are secreted from the crypts of the intestinal surface (Frape, 2004). These brush border enzymes are active at the villus and aid in carbohydrate and protein digestion. Aminopeptidases cleave at the N-terminal amino acid while dipeptidases cleave dipeptides, which are two amino acids bonded together. Bile produced in the liver enters the small intestine through bile ducts in the duodenum and aids in the emulsification of fats. Additionally, pancreatic enzymes are also secreted into the duodenum via the pancreatic duct to aid in protein, starch, and fat digestion (Frape, 2004). These secretions include bicarbonate, amylase, lipase, nuclease,

and zymogens. Zymogens are secreted inactively and activated in the lumen of the small intestine by enterokinase and trypsin.

The products of protein digestion within the small intestine are di- or tripeptides and free amino acids (Frape, 2004). Whole protein absorption only occurs in the newborn within 12 to 24 hours of birth to allow for absorption of antibodies. After 24 hours of age, only di- or tripeptides and free amino acids can be absorbed, with only free amino acids entering the bloodstream (Ellis and Hill, 2005). Absorption of amino acids depends on the individual amino acid and the type of carrier they require. Amino acid carriers exhibit affinity towards certain amino acids. Larger amino acids, neutral amino acids, and essential amino acids are absorbed faster (Frape, 2004). Facilitated transport of amino acids depends on the concentration gradient, and transport of some amino acids is coupled to the sodium gradient through sodium/potassium ATPase. The majority of protein is absorbed as peptides in a sodium/hydrogen exchange mechanism coupled to the hydrogen gradient which is created by the concentration of sodium. Once absorbed into the enterocyte the peptides are broken down into free amino acids where they can then enter the bloodstream by diffusion and sodium independent carriers (Frape, 2004). Absorbed amino acids travel to the liver where they are deaminated for energy use or transaminated to other non-essential amino acids for the synthesis of protein, tissue, enzymes, hormones and other metabolites (Frape, 2004).

Protein Digestion - Large Intestine

The large intestine, referred to as the hindgut of the horse, is composed of the cecum, colon, and rectum. The hindgut functions to digest structural carbohydrates

through fermentative digestion and is a major site of absorption of water, VFAs, and minerals (Ellis and Hill, 2005). There is no enzyme secretion into the large intestine; however, it provides an environment capable of supporting bacterial and protozoal digestion, which in the horse primarily functions to digest structural carbohydrates from the forage portion of the diet (Frape, 2004). The hindgut of the horse serves essentially the same function as the ruminant forestomach (Ellis and Hill, 2005).

Ruminant protein digestion differs from the equine as the rumen microbes use dietary protein and resynthesize it into microbial crude protein, which is what is actually absorbed by the animal. Dietary protein is broken down into free amino acids, ammonia, VFAs, and CO_2 (Ellis and Hill, 2005). Peptides and free amino acids are rapidly absorbed by microbes. All amino acids are resynthesized from ammonia and carbon skeletons, including all essential amino acids. Therefore, a ruminant can exist on limited dietary sources of protein because of the use of non-protein-nitrogen sources (Ellis and Hill, 2005). A healthy microbial population synthesizes microbial crude protein when it is supplied with adequate amounts of nitrogen and rapidly fermentable carbohydrates to use as carbon skeletons.

Equines have the ability to synthesize microbial crude protein in the hindgut; however, absorption of this protein is limited due to the small intestine preceding the cecum in the gastrointestinal tract (Frape, 2004). While the microbes rapidly utilize and absorb the non-protein-nitrogen, the large intestine itself absorbs only VFAs, ammonia, and limited amounts of free amino acids, making the contribution of non-protein-nitrogen to horses minimal (Frape, 2004). Furthermore, young equines have limited

fermentation capabilities in the hindgut due to the slow development of the large intestine, and more closely resemble a true monogastric model in their protein digestion capabilities (Frape, 2004). Therefore, dietary protein quality and digestibility are extremely important during these younger stages of growth.

Protein Quality

Ideal Protein Concept

The quality of dietary protein is determined by its amino acid profile and digestibility. Of the 30 total amino acids, 20 can be synthesized by the horse from other amino acids through transamination; however, 10 of the amino acids are unable to be synthesized by the animal or cannot be synthesized at a sufficient rate to permit optimal growth and must be provided in the diet (NRC, 1998). These amino acids are considered essential. A protein source that comes close to meeting an animal's essential amino acid requirement and is easily digested is considered a high quality protein (Ott et al., 1979). A commonly used concept in ration formulation is that of an optimal dietary profile of essential amino acids that corresponds to the needs of the animal (Wang and Fuller, 1989). This is often referred to as the ideal protein concept. An ideal protein would contain all of the essential amino acids at exact amounts required by the animal and result in no deficiency or excess.

Dietary deficiencies in amino acids can limit growth and nitrogen retention (Bender, 1965). Conversely, excess dietary protein is degraded to ammonia and excreted as urea in the urine (Wang and Fuller, 1989). This excess excretion increases water loss and possibly interferes with acid-base balance during exercise (Graham-

Thiers et al., 1999). Furthermore, environmental concerns related to nitrogen excretion are increasing, placing greater importance on waste management (Lawrence et al., 2003). Improving the quality of a protein source results in more efficient utilization, and decreases nitrogen loss to the environment (Staniar et al., 2001). Formulating rations to mimic the ideal protein creates a higher quality protein source and improves efficiency.

An understanding of the ideal amino acid profile at a specific stage of life is required to evaluate the quality of a protein source (Wang and Fuller, 1989). Estimations of the ideal protein in other monogastric species are based off of previous amino acid removal studies that determine a requirement at a stage of production. In rats, removal of non-limiting amino acids exhibited no effect on N retention (Bender, 1965). This removal concept was utilized to calculate a dietary amino acid profile in which all amino acids were equally limiting by removing a portion of each amino acid and evaluating changes in N retention to determine the ideal amino acid pattern (Wang and Fuller, 1989). To date, amino acid removal studies have not been performed in equines, therefore alternate methods of determining requirements and evaluating quality of protein sources must be used. Amino acid ratios in muscle tissue correlate with the determined dietary requirements for poultry and swine (Buttery and Lindsay, 1980). Therefore, current amino acid requirement values for equines are based on ratios found in muscle tissue and mare's milk (Bryden, 1991).

Crude Protein Concentration and Digestibility

Although existing equine research lacks a defined ideal protein and most amino acid requirements are unknown, existing information includes the effect of varied levels

of dietary crude protein (CP) and protein digestibility on growth. Horses fed diets higher in CP (increasing by at least 3% CP) had greater dry matter intake with tendencies toward greater gain in body weight, height, and girth (Jordan and Myers, 1972; Ott et al., 1979). Additionally, it is expected that diets containing lower overall levels of CP also contain lower concentrations of the essential amino acids (Jordan and Myers, 1972). Foals fed a diet containing 15.5% CP gained height 32% faster than those fed a diet containing 11.9% CP. The authors suggested this was most likely caused by decreased amino acid intake with the lower crude protein diet (Jordan and Myers, 1972). Horses fed a higher quality protein source, such as soybean meal, compared to a non-protein nitrogen source (urea), exhibited increased growth and feed efficiency (Godbee and Slade, 1981). This further illustrates the role of protein quality in the diet of young growing horses.

The digestibility of the protein source and the location of digestion become especially important in the equine. Hindgut digestion of protein in horses has minor bearing on overall protein digestion because the small intestine, which is the site of absorption of amino acids and small peptides, precedes the hindgut. Additionally, protein quality is of increased importance in young horses due to increased requirements during the tissue synthesis that occurs during growth (Yoakam et al., 1978, Gibbs and Potter, 2002). Greater digestibility of a protein, especially foregut digestibility, results in greater amino acid absorption and a larger amino acid pool available for growth and other essential functions (NRC, 2007). Additionally, protein sources in equine rations are often selected for factors unrelated to the protein needs of the horse, and instead are

selected for convenience and price rather than quality (Hintz et al., 1971). Growing horses cannot maximize potential for growth on poor quality diets and may be permanently stunted (Yoakam et al., 1978). For example, foals exhibited greater gains in bodyweight, height, and girth when fed a more digestible protein source (soybean meal) compared to a less digestible source (brewers dried grains) (Ott et al., 1979). Milk proteins are an extremely high-quality protein source, as they very nearly match the animal's requirements and are easily digested in the small intestine. Young horses fed a milk-based protein source gained 58% more body weight, consumed 14% more feed, and were 20% more efficient in converting feed to gain when compared to those fed a linseed meal based protein source (Hintz et al., 1971). An alternative to using naturally occurring high quality proteins in rations is to supplement a protein source with crystalline amino acids. The addition of the crystalline amino acids brings the overall amino acid profile of the ration closer to the ideal.

Crystalline Amino Acids

The addition of crystalline amino acids to low-quality protein sources can improve the overall quality of the ration and result in increased efficiency and growth (Ott et al., 1979; Wang and Fuller, 1989; Graham et al., 1994). A crystalline amino acid is a free amino acid that is not bound to a protein. Crystalline amino acids may be isolated from naturally occurring sources, or artificially synthesized. For example, methionine can be produced from the reaction of acrolein with methyl mercaptan in the presence of a catalyst or through the formation of its intermediates such as acrolein, methylthiol, and hydrocyanic acid (Fong, et al., 1981). All amino acids contain a chiral

carbon atom, and therefore can exist as stereo isomers. These structures are referred to as D or L isomers, with L isomers occurring naturally and D isomers most often found in bacterial cells. A mixture of both D and L isomers is referred to as a racemic mixture (Voet et al., 2006). Different species utilize D and L isomers with varying efficiency. For example, D- isomers of the branched-chain amino acids (leucine, isoleucine, and valine) are utilized well by chicks but poorly by rats, and the D-isomer of phenylalanine, tyrosine, and methionine are utilized well by both rats and chicks (Baker, 1986). No information exists regarding utilization of D and L isomers in horses.

Crystalline amino acids pass more rapidly through the stomach and are more easily absorbed when compared to protein- bound amino acids (Rolls et al., 1972). Lysine was the first free amino acid to be utilized in this context (Batterham, 1979). Wang and Fuller (1989) indicated that amino acids in the crystalline form are essentially 100% digestible in pigs. Because of the great variability of amino acid profiles in natural protein sources, utilization of these protein sources results in unavoidable excess in supply of several amino acids while it may not satisfy minimum requirements for other amino acids. When using natural protein sources, it is more beneficial to formulate rations to be slightly in excess in order to avoid deficiencies of other amino acids (Wang and Fuller, 1989). Deficiencies in amino acids can result in limited protein synthesis, and therefore limited growth, while extreme excesses of amino acids can reduce intake and growth rate (Chung and Baker, 1992).

The use of natural protein sources supplemented with crystalline amino acids is optimal, as it more closely mimics the ideal protein and amino acid pattern required by

the animal. Fortification of a ration with crystalline amino acids resulted in increased voluntary feed intake and daily gain in pigs between 5 and 20 kg (Chung and Baker, 1992). Supplementation of crystalline amino acids, such as methionine, increased N retention in steers (Greenwood and Titgemeyer, 2000). Young horses fed a low quality protein source based on linseed meal with additional lysine had higher ADG and nitrogen retention compared to those fed only linseed meal (Hintz et al., 1971). Supplemental lysine and threonine reduced muscle mass loss in aged horses (Graham-Theirs and Kronfeld, 2005). The addition of lysine and threonine to a ration also increased body weight gain and gain:feed in yearling horses (Graham et al., 1994). Therefore, supplementation of crystalline amino acids can improve the overall quality of a ration allowing for more efficient utilization and optimal growth.

Determining Amino Acid Requirements

Amino acid requirements can be indirectly determined by evaluating plasma urea nitrogen (PUN), N retention, and directly determined by physical growth measurements or carcass quality characteristics (Balogun and Fetuga, 1981). It can be expected that PUN will decrease with increasing inclusion of an essential amino acid until the requirement is met, at which point a plateau will be reached. This is referred to as a single-slope break-point analysis (Campbell et al., 1997). For example, increasing dietary levels of methionine in weanling pigs showed appreciable decreases in plasma urea nitrogen (Balogun and Fetuga, 1981). Nitrogen retention is calculated by subtracting the amount of N excreted in urine and feces from the total N intake. Break-point analysis of N retention can also be used to determine amino acid requirements.

Higher quality protein sources increase N retention, which allows for greater protein synthesis and growth (Chung and Baker, 1992). Supplementation of crystalline amino acids, such as methionine, increases N retention (Greenwood and Titgemeyer, 2000). The additional methionine improved the amino acid balance of the diets fed in this study, which resulted in increased tissue synthesis and therefore increased N retained.

Limiting Amino Acids in Horses

The amino acid profile of proteins varies among sources (Gibbs and Potter, 2002). Lysine is the first-limiting amino acid in diets based on bermudagrass, alfalfa hay, and several other ingredients commonly used in equine rations (Breuer and Golden, 1971, Ott et al., 1981, Gibbs and Potter, 2002, Staniar et al., 2001) Lysine is considered the first limiting amino acid in most feeding programs due to its frequency in body proteins, such as muscle tissue and milk, and limited content in most protein sources (Ott et al., 1979). In equines, lysine is the only amino acid with a known requirement, and is also the most important factor affecting growth (Ott and Kivipelto, 2002). Through regression analysis, several studies determined the dietary requirement for lysine in weanling horses, which is 33 to 42 g/d or 4.3% of the total CP requirement (Breuer and Golden, 1971; Ott et al., 1979; Ott and Kivipelto, 2002). There is little information regarding the remaining essential amino acid requirements of the young growing horse.

The second and third limiting amino acids for horses are unknown, but existing research suggests it is likely threonine or methionine. Graham et al. (1994) suggested that threonine is the second limiting amino acid in horses based on observations of increased bodyweight and girth gain in horses with 0.1% added threonine to a corn, oat,

and soybean meal based concentrate with bermudagrass hay. However, this study also altered levels of lysine across dietary treatments, which eliminates threonine as the sole variable, making it difficult to conclude that additional dietary threonine alone increased growth. Furthermore, this inconclusive data was unable to provide a recommendation for dietary threonine for the growing horse (Graham et al., 1994). An additional study investigated the effect of supplemental lysine and threonine on growth in young horses and found a positive effect on growth with increased threonine intake (Staniar et al., 2001). However, this study utilized group feeding, and observation of horses during feeding revealed that some horses consumed supplement in concentrations greater or less than assumed, making it impossible to determine individual intake. Furthermore, there was no influence of dietary threonine concentration on PUN concentrations or body weight. A lack of physiological response to treatment, and discrepancies with dietary intake data prevent the authors from determining an accurate recommendation for dietary threonine in the horse (Staniar et al., 2001). Therefore, although threonine is commonly referenced as the second limiting amino acid in horses, there is insufficient data to support this claim.

Methionine is the second limiting amino acid in corn/soybean meal based diets in nursery pigs (Mavromichalis et al., 1998). Additionally, methionine is the first limiting amino acid in milk protein (Becker et al., 1955) and is present in low concentrations in several commonly used protein sources for growing horse rations; therefore, it is also likely to be limiting without supplementation and also does not yet have a known requirement in the horse.

Sulfur Containing Amino Acids

One unique subdivision of amino acids is the sulfur containing group, which includes methionine and cystine. The sulfur containing amino acids have been studied in depth due to their roles in methyl group donation, free radical defense, and other crucial roles in protein structure and folding pathways. Methionine is one of the 10 essential amino acids. Methionine and cystine are often considered together as the sulfur containing amino acids as they are virtually impossible to isolate and study independently due to transsulfuration. During the metabolism of methionine, it may be converted to cysteine and taurine, which are other sulfur based amino acids, and also acts as the primary donor of methyl groups. Methionine also provides the start code for the transcription of most eukaryotic protein synthesis (Brosnan and Brosnan, 2006).

Metabolism

The initial step of methionine metabolism is the formation of S-adenosyl-L-methionine in an ATP consuming reaction using the enzyme methionine adenosyl transferase. This reaction is extremely high energy and uniquely removes all 3 phosphates from the consumed ATP due to the presence of a sulfonium ion. S-adenosyl-L-methionine (SAM) is required for the synthesis of carnitine, creatine, epinephrine, purines, and nicotinamides. Additionally, SAM can provide the sulfur required for biotin and lipoic acid synthesis (Brosnan and Brosnan, 2006). S-adenosyl-L-methionine may be thought of as the active form of methionine, and donates its methyl group to other amino acid residues, DNA, RNA, and many more. This methyl donation is possible because of its positive sulfonium ion which allows it to join with nucleophiles. There are

numerous SAM- dependent methyltransferases, with 60 identified in mammals and several that are still unknown (Stipanuk, 2004). Numerous enzymes require SAM, with only ATP having a higher number of enzymes that require it. The product of these methyltransferase reactions is S-adenosylhomocysteine (SAH), which acts as an inhibitor to the methyltransferases. The SAH is then hydrolyzed by S-adenosylhomocysteine hydrolase to form adenosine and homocysteine. Homocysteine can then be re-methylated and converted back to methionine by methionine synthase in the tissue or betaine:homocysteine methyltransferase in the liver. This process is known as transmethylation, or the methionine cycle, which is illustrated in Figure 1.

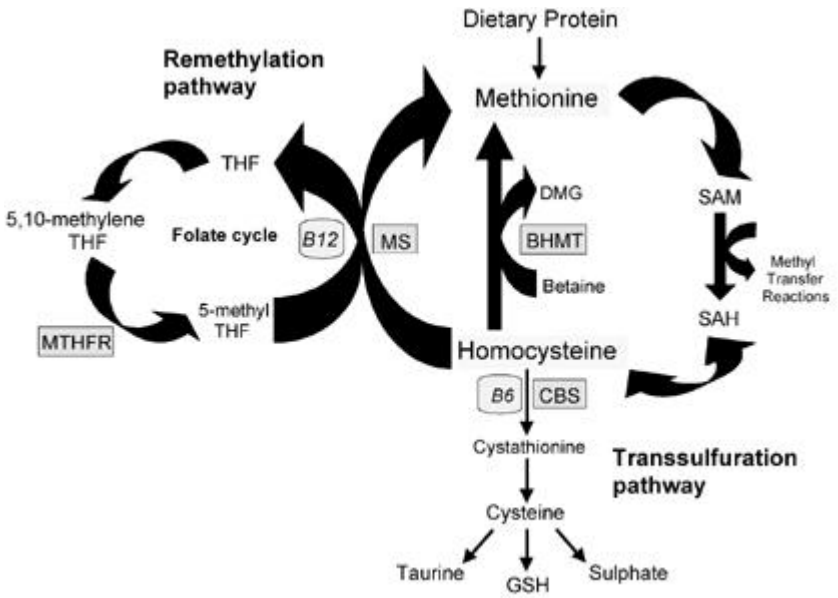

Figure 1. Transsulfuration and remethylation pathway (Mosharov et al., 2000).

The actual catabolism of methionine is performed in the transsulfuration pathway, which irreversibly converts methionine to homocysteine, then cysteine. This pathway occurs in the liver, pancreas, intestine, and kidney (Brosnan and Brosnan, 2006). Homocysteine is converted to cystathione by cystathione β-synthase, which removes the β-OH group from the serine. The cystathione and water are taken to cysteine by cystathione γ- lyase. This reaction also produces an α-ketobutyrate which can be further metabolized to succinyl CoA and enter the citric acid cycle, and ammonium which is usually excreted in the urine.

Sparing Effect of Cysteine

The transsulfuration pathway also allows cysteine to have a sparing effect on methionine. Although this pathway is irreversible, the presence of cysteine in the diet allows for more flux through the methionine cycle, where homocysteine is remethylated to methionine instead of converting to cysteine. The exact extent of this sparing effect is debatable, with many conflicting studies. Mosharov et al. (2000) was unable to prove an exact effect of cysteine on methionine, but concluded that increased dietary cysteine increased the fraction of homocysteine that undergoes remethylation versus transsulfuration and can be evaluated using a single tracer ($NaHCO_3$). Kurpad et al. (2004) found an approximate 30% sparing rate of cysteine for methionine requirements of Indian men; however, they were unable to determine an actual dietary requirement. Patients consuming a minimal methionine diet with excess cysteine exhibited up to a 64% sparing rate of methionine (DiBuono et al., 2003). It is also suspected that glutathione, which is formed from cysteine and can provide a storage form for cysteine,

may act as an uncontrollable variable in these studies that makes it more difficult to determine the actual sparing effect of dietary amounts of cysteine (Kurpad et al., 2004). Further studies need to be conducted that consider all possible dietary variables and use multiple tracers to more closely examine the flux through the transmethylation and transsulfuration pathways (Storch et al., 1990).

Fates of Cysteine in the Body

Cysteine, whether it is produced from methionine in the transsulfuration pathway or consumed in dietary proteins, has many possible fates (Figure 2). The cysteine sulfinate pathway provides the primary source for taurine formation. Cysteine sulfinate removes CO_2 by decarboxylation and converts to hypotaurine, which then becomes taurine (Gropper et al., 2008). Taurine is the most abundant free amino acid found in animal tissue, where it has many functions such as an antioxidant, neurotransmitter, or membrane stabilizer. Taurine is also important in the retina for normal healthy vision (Gropper et al., 2008). Cysteine is also required for the synthesis of glutathione, an important antioxidant. Glutathione can also serve as a storage form for cysteine, and release cysteine into the bloodstream when concentrations are low (Stipanuk, 2004).

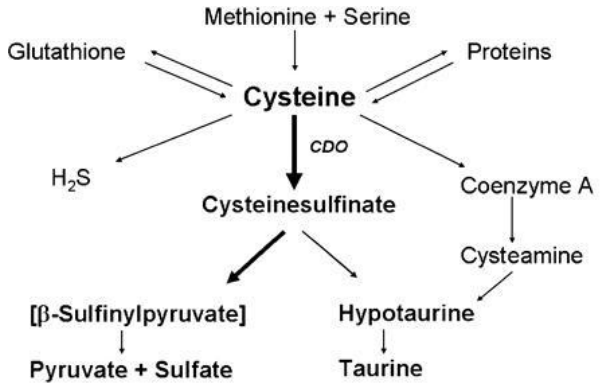

Figure 2. Metabolic fates of cysteine. (Stipanuk Lab, 2009)

Cysteine, the free amino acid, is potentially toxic and may be spontaneously catabolized in the gastrointestinal tract. Cystine is composed of 2 cysteine molecules linked by a disulfide bond, which is more stable in the gastrointestinal tract and can be reduced to 2 cysteine molecules once inside a cell. In cases of cysteine deficiency, cystine cannot be added to intravenous solutions because it is insoluble, and cysteine is potentially toxic, therefore glutathione is used as a source of cysteine (Cho et al., 1984). Additionally, muscle degradation can provide an endogenous source of cysteine during a deficient state by degrading its glutathione (Cho et al., 1984). Furthermore, cysteine possesses the unique ability to form disulfide bonds, which makes it necessary for many folding and structural protein pathways. For example, the hormone insulin is a protein with cysteine cross-linking which increases its molecular stability.

Metabolic Regulation

Both transmethlyation and transsulfuration pathways have several regulatory points and necessary cofactors that control flux. Recycling of homocysteine to methionine makes it difficult to estimate rate of flow through these pathways (Stipanuk, 1986), however new methods are being developed to analyze the cycles. One such method is the Storch-Young model, which labels C-1 and the terminal methyl group with an isotope tracer, then evaluates CO_2 to measure the oxidation of methionine (Hoffer, 2002). Because the end products of the transsulfuration pathway are sulfate and taurine, which are excreted in the urine, the presence of free sulfate in urine provides an index for sulfur amino acid intake (Stipanuk, 2004). The average rate of the transsulfuration pathway is influenced by the amount of dietary methionine (Hoffer, 2002).

There are 3 influential methyl transferases that regulate production of homocysteine and methyl group metabolism. These 3 enzymes are guanidinoacetate methyltransferase (GAMT), phosphatidylethanolamine N-methyltransferase (PEMT), and glycine N-methyltransferase (GNMT) (Williams and Schalinske, 2007). guanidinoacetate methyltransferase consumes up to 70% of all of methyl groups derived from S-adenosylmethionine. Glycine N-methyltransferase regulates SAM:SAH, which acts as a method of conserving methyl groups for SAM-dependent reactions and then disposes of them when in excess (Williams and Schalinske, 2007). An index of potential for transmethylation is SAM:SAH. For example, when SAH levels are elevated there will be a decrease in transmethylation (Williams and Schalinske, 2007). Phosphatidylethanolamine N-methyltransferase regulates homocysteine balance by

catalyzing reactions that donate SAM's methyl group to phosphatidylethanolamine, producing phophatidylcholine. Therefore SAM acts as an allosteric feedback activator of methionine adenosyltransferase and cystathione β-synthase, and when dietary methionine is present in large amounts, SAM activates enzymes to catabolize excess methionine. The SAH is inhibited by its products, homocysteine and adenosine, and creates a buildup of SAH and SAM (Stipanuk, 1986). Large dietary quantities of cysteine inhibits the activity of cystathione- β-synthase, allowing homocysteine to remethylate instead of catabolize, which may provide an explanation for cysteine's methionine sparing ability.

Homocysteine sits at an important regulatory branching point where it can either remethylate or enter the transsulfuration pathway. The K_ms of transsulfuration enzymes for substrates are higher than that of the remethylation enzymes. This means that when homocysteine is present in low concentration remethylation will spare methionine, and when homocysteine is in high concentration it will proceed to transsulfuration (Stipanuk, 2004). There is also evidence that suggests oxidative stress may encourage flux through the transsulfuration pathway, with one study reporting that oxidative stress stimulated cystathione β-synthase of the transsulfuration pathway to encourage conversion of cystathione to cysteine and then glutathione in an effort to self correct depleted anti-oxidant pools (Chung and Baker, 1992). This suggests that methionine and cysteine may play an important and previously unknown role in the maintenance of cellular antioxidant balance.

The hormone insulin and the diseased state of diabetes affect the enzyme betaine-homocysteine S-methyltransferase (BHMT), one of the two major enzymes involved in the remethylation pathway (Ratnam et al., 2006). Insulin acts as an inhibitor and causes a decrease in BHMT, which is a zinc metalloenzyme that transfers methyl groups from betaine to homocysteine to produce dimethylglycine and methionine. The mechanism of insulin in this inhibition remains unknown (Ratnam et al., 2006). Another hormone, hydrocortisone, increases the activity of BHMT in the liver, and thyroxine decreases BHMT activity. Also, glucocorticoids regulate the expression of glycine N-methyltransferase (GNMT) which controls the SAM:SAH (Ratnam et al., 2006).

Unique Requirements for Metabolism

The transsulfuration pathway exhibits a unique requirement for the vitamins B_6 and B_{12}. Both cystathione β-synthase and cystathione γ- lyase are pyridoxal phosphate dependent enzymes, which is derived from vitamin B_6 (Stipanuk, 2004). Additionally, SAM requires vitamin B_{12} , and is only the second known enzyme (after methylmalonyl mutase) with this requirement (Stipanuk, 1986). Deficiencies in vitamins B_6 and B_{12} limit enzymes and create increased concentrations of their reactants. This limits overall metabolism of the sulfur amino acids.

Requirements and Toxicities

Although the importance of sulfur amino acids is clear and a great deal of research exists studying the general biochemical pathways of transmethlyation and transsulfuration and enzyme deficiencies, there is comparatively less nutritional data on sulfur amino acid utilization among different species of livestock. Although cysteine was

found to supply almost half of the total sulfur amino acid requirement of rats, cats, dogs, and pigs, experiments performed on growing steers found cysteine was unable to spare methionine (Campbell et al., 1997; Heger et al., 2007). Methionine is the most limiting amino acid for growing cattle when the protein source is coming from microbial crude protein and when consuming a soybean hull based diet (Lambert et al., 2002). Nitrogen retention of cattle increases with methionine supplementation, but methionine synthase activity decreased. Taurine increased in the liver with supplemented methionine, but concentrations of methionine and serine in the liver were not affected. Also the liver concentrations and activity of cystathione β synthase were unchanged with methionine supplementation (Lambert et al., 2002). In sheep, methionine supplementation resulted in a higher rate of transsulfuration (Liu et al., 2000).

The amino acids methionine and cysteine themselves are toxic when consumed at levels 5 times greater than the requirement, with methionine creating a decrease in growth of young animals and cysteine acting as a strong reducing agent (Baker, 2006). It has been suggested that the free sulfhydryl group of cysteine is what causes its toxicity (Dilger et. al., 2006). Pigs were more susceptible to toxicity symptoms and growth depression from excess dietary cysteine than excess methionine (Dilger et. al., 2006). The overall mechanisms responsible for these toxicity problems are still unknown. Different species have shown very different responses to elevations of dietary methionine and cysteine. For example, excess cysteine resulted in high mortality rate among chicks. In contrast, pigs only expressed depressed weight gain and feed consumption (Dilger et. al., 2006). Pigs were more susceptible to toxicity and growth

depression from excess dietary cysteine than excess methionine (Dilger et. al., 2006). The toxicity levels in cattle, sheep, and horses have not been evaluated. It is important to note that the bioavailability of the sulfur amino acids is unknown for many common feed ingredients (Chung and Baker, 1992). Without knowing the digestibility of a component of a ration there is no way to determine the amount of methionine and cysteine that will be available for use by the animal. This highlights the need for research to determine availability of methionine in commonly used protein sources, toxic levels, and requirements of the sulfur amino acids in species where it has yet to be determined, such as equines.

CHAPTER III

INFLUENCE OF METHIONINE ON GROWTH AND NITROGEN BALANCE IN WEANLING QUARTER HORSES

Introduction

Requirements for essential amino acids (AA) are important when formulating rations to avoid deficiencies or toxicities. Exact requirements for equines are unclear; therefore rations are formulated to provide nutrients in excess rather than risking deficiency. A requirement for lysine has been established, but little information exists for the remaining AA (Ott et al., 1981). Methionine (MET) may be limiting due to its role in methyl group donation and portions of protein structure and folding pathways (Brosnan and Brosnan, 2006). Methionine is the second limiting AA in other monogastric species during growth and is first limiting in milk protein (Mavromichalis et al., 1998). Additionally, its concentration is low in commonly used protein sources for equine rations. Therefore, it is likely to be limiting without supplementation (Becker et al., 1955).

Amino acid requirements can be determined by evaluating changes in plasma urea nitrogen (PUN), N retention, and physical growth (Balogun and Fetuga, 1981). Plasma urea N will decrease with increasing inclusion of an essential AA until the requirement is met and a break-point is reached. Break-point analysis can also be used to determine AA requirement when measurements of N retention and growth are made (Chung and Baker, 1992). If excessive amounts of AA are supplemented, especially in crystalline form, reductions in intake and growth rate have been reported in pigs (Chung

and Baker, 1992). Therefore, it is important to determine a dietary requirement for equine rations, especially during stages of growth. When investigating MET requirements, interactions between the remethylation and transsulfuration pathways make it difficult to isolate MET from the other sulfur containing AA. Therefore, when met concentrations are altered in the diet, total sulfur AAs are evaluated (Brosnan and Brosnan, 2006). The objective of this trial is to determine the effect of increasing dietary levels of MET on growth and nitrogen balance in weanling horses.

Materials and Methods

Care, handling, and sampling of animals were approved by the Texas A&M University Animal Care and Use Committee.

Horses and Treatments

Twenty-four Quarter Horse weanlings of similar breeding from the Texas A&M Horse Center were used in a complete randomized block design. Prior to starting the experiment, all horses were blocked by age into 4 groups (n = 6) to be weaned (average age of weaning 120 ± 10d) and adapted to solid feed over a 21 d period. During adaptation horses were fed a commercial diet (SafeChoice, Cargill Animal Nutrition, Elk River, MN) and coastal bermudagrass hay. Within the 21 d adaptation, intakes reached the targeted amount for the experimental period of 1.5% BW (as fed) concentrate and 0.75% BW (as fed) hay. Weanlings were housed by block in dry lots (25 m x 25 m) with free access to water.

The experimental period was divided into two phases totaling 56 d. Phase 1 consisted of determination of intake and physical growth measurements and lasted 52 d. Phase 2 allowed for determination of N balance via a total collection of feces and urine over the final 4 d of the experiment. During Phase 1, horses were fed twice daily in individual stalls (3.0 m × 3.0 m). Weanlings were given 3 hrs to consume grain and hay per feeding, and refusals were weighed and recorded.

At the beginning of Phase 1, weanlings were randomly assigned within block to 1 of 4 dietary treatments. Treatments consisted of increasing concentrations of methionine in a pelleted concentrate (Table 1, Table 2) fed at 1.5% BW (as fed) and formulated as follows: basal diet containing 0.20% methionine (Table 3; 0.20 MET), basal + 0.03% methionine (0.23 MET), basal + 0.07% methionine (0.27 MET), and basal + 0.11% methionine (0.31 MET). All diets were formulated to be isocaloric, isonitrogenous, and contain equal amounts of lysine and threonine (Table 2). All horses were also individually offered the same coastal bermudagrass hay provided during the adaptation period (0.46% Lys, 0.16% Met, 0.36% Thr) at 0.75% BW (as fed). Weanlings remained on treatment diets for both phases of the trial totaling 56 d, with BW measured every 7 d and intake adjusted accordingly. Body weights were taken using a digital platform scale *(CAS Corp. Seoul, Rep. of Korea)*.

Table 1. Composition of basal pelleted concentrate fed to weanling Quarter horses.

Ingredients[1]	%
Oats- whole	44.87
Oat by-product	19.91
Wheat midds	13.63
Wheat by-product	8.43
Soy hulls	5.00
Soy oil	2.75
Ca Carb	1.26
Mono-dical P	1.08
Pellet binder	1.00
Salt	0.71
Bentonite	0.50
L-Lysine HCl	0.35
L-Threonine	0.22
Selenium (0.06%)	0.08
Trace mineral premix	0.05

[1]Composition of concentrate remained constant across dietary treatments with the exception of methionine.

Table 2. Nutrient composition of diets fed to weanling Quarter horses.

Item	Treatment[1]				
	0.20% MET	0.23% MET	0.27% MET	0.31% MET	Hay[2]
DM, %	89.66	89.74	90.00	89.96	85.44
CP, %	11.39	11.39	11.39	11.39	9.86
ADF, %	13.75	15.36	16.49	14.41	32.21
NDF, %	29.21	31.41	32.43	31.34	65.39
Fat, %	5.45	5.45	5.45	5.45	-
Ca, %	0.85	0.85	0.85	0.85	-
P, %	0.70	0.70	0.70	0.70	-
Mg, %	0.19	0.19	0.19	0.19	-
K, %	0.55	0.55	0.55	0.55	-
Cl, %	0.59	0.59	0.59	0.59	-
Na, %	0.30	0.30	0.30	0.30	-
DE, Mcal/kg	3.97	3.99	3.97	3.97	4.02

[1]Dietary treatment consisted of a basal pelleted concentrate (whole oats, oat byproducts, wheat midds) with various concentrations of methionine (MET).
[2] Hay consisted of coastal bermudagrass (*Cynodon dactylon*).

Table 3. Formulation of lysine, threonine, and methionine concentration in pelleted dietary treatments fed to weanling Quarter horses.

	Treatment[1]			
Formulation (DM)	0.20% MET	0.23% MET	0.27% MET	0.31% MET
Lysine, %	0.78	0.78	0.78	0.78
Threonine, %	0.62	0.62	0.62	0.62
Methionine, %	0.20	0.23	0.28	0.31

[1]Dietary treatment consisted of a basal pelleted concentrate (whole oats, oat byproducts, wheat midds) with increasing levels of methionine (MET).

Phase 1: Measurements and Samples

Daily feed intake of both concentrate and hay was determined during Phase 1 to calculate DMI, ADG, and feed efficiency (F:G). Blood samples were collected every 7 d prior to morning feeding via jugular venipuncter into evacuated tubes containing sodium heparin (Becton-Dickinson, Franklin Lakes, NJ.). Samples were immediately placed on ice until centrifugation at 2700 × g for 20 min. Plasma was harvested and stored at -20 °C for subsequent determination of plasma urea concentration. Physical growth measurements were recorded every 7 d, and included wither and hip height, circumference of the forearm and gaskin at its widest point, body length, heart girth, and rump fat. Rump fat measurements were obtained via ultrasound images on the left hip at a point 5 cm dorsal of halfway between the first coccygeal vertebrae and the ischium (Westervelt et al., 1976) using an ultrasound instrument (Aloka SSD-500V, Aloka Inc., Tokyo, Japan).

Phase 2: Total Fecal and Urine Collection

Determination of N balance by total collection of urine and feces occurred during Phase 2 on d 52 through 56 of the experiment. Weanlings were housed in the same stalls used for feeding and restricted to a 1.5 × 3.0 m area with rubber mats covering the floor. Urine collection harnesses were fitted to each animal and urine was collected into a polyethylene jug using vacuum (GAST Manufacturing, Michigan). Weanlings were continuously observed throughout Phase 2 which allowed immediate collection of feces from the rubber mats after defecation and insured that urine harnesses were correctly fitted to avoid sample loss. Collected fecal samples were stored in closed plastic containers for later processing. Fecal and urine storage containers were emptied and processed every 6 hr. Total fecal weight was recorded (Bastrop Scale Co Inc., Bastrop TX) and a 10% subsample was stored at -20 °C for subsequent analysis. Total urine volumes were also recorded and a 10% subsample acidified using HCl and stored at -20 °C for subsequent analysis of N, urea, and ammonia. Grain and hay samples were collected for each day of collection for determination of daily N intake.

Sample Analysis

Nitrogen

Nitrogen content of the grain, hay, fecal, and urine samples was determined using a Rapid N Cube Nitrogen Analyzer (Elementar Analysensysteme, Hanau, Germany) to allow calculation of N balance. Grain and hay samples collected for each day of total collections were ground through a 1 mm screen, (Wiley Mill, Thomas Scientific, Swedesboro, NJ) and analyzed to determine daily N intake. Fecal and urine samples

were thawed and homogenized, and wet sample aliquots of 250 mg were used to determine daily N excretion. Following analysis, N balance was calculated by determining total g N consumed and subtracting total g N excreted.

Urea and Ammonia

Urea and ammonia concentrations were determined colorimetrically using a spectrophotometer (Beckman-Coulter, DU 730, Life Science UV/Vis Spectrophotometer) and following the methods of Marsh et al. (1965) and Broderick and Kang (1980). Plasma samples collected during Phase 1 were analyzed for urea N concentration while urine samples were analyzed for both urea N and ammonia concentrations.

Nutrient Analysis

Grain, hay, fecal, and urine samples were analyzed for nutrient content. Fecal samples were thawed and homogenized. Half of the total sample was dried in a forced air oven (Lindberg/Blue M, Asheville, NC.) at 60 °Celsius for 72 hours then ground through a 1mm screen (Thomas Scientific, Swedesboro, NJ.). All dried samples and urine samples were composited for each collection prior to nutrient analysis then analyzed for DM, OM, CP, ADF, NDF and GE. The DM of grain, hay, and fecal samples was determined by drying for 24 h at 105°C in a forced air oven (Lindberg/Blue M, Asheville, NC.), and OM was determined as loss in dry weight upon combustion for 8 h at 450°C in a muffle furnace. Crude protein was calculated as N × 6.25. Grain, hay, and fecal samples were analyzed for NDF and ADF with a fiber analyzer (model 200, Ankom Technology, Fairport, NY). Heat-stable alpha-amylase was used during NDF

determination of grain samples due to starch content. Gross energy content was determined on dried and ground samples of grain, hay, and feces through bomb calorimeter using a Par 6300 oxygen-bomb calorimeter (Parr Instrument CO., Moline, IL).

Statistical Analysis

Data were analyzed using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Linear, quadratic, and cubic effects were tested in the form of contrasts. Main effects tested were treatment, day, and treatment by day interaction. Probabilities less than 0.05 were considered statistically significant and 0.10 considered a trend towards significance.

Results

Intake

Target intakes of 1.5% BW (as fed) concentrate and 0.75% BW (as fed) hay were achieved. The NRC (2007) estimates growing horses will consume 2.5% BW per d in dry matter, but this likely over estimates the actual feed intake necessary to meet minimum requirements. Horses readily consumed all diets with only minor refusals of dietary treatments. Weanlings consumed nutrients at or exceeding current recommendations (NRC, 2007). Daily CP g/d exceeded the requirement of 1,204 - 1,217 g/d with values ranging from 1,719 - 2,588 g/d. Total daily lysine intakes exceed the requirement of 28.8 - 29.1 g for weanlings (estimated mature BW of 500 kg) by at least 5 g. Recommendations for daily DE intake were also exceeded by at least 4 Mcal/d (NRC, 2007). There was no effect of dietary treatment on average g/d intake of nitrogen

(Table 5) , lysine, or threonine (Table 4; $P = 0.52$ and $P = 0.38$, respectively). Dietary intake of methionine increased ($P = 0.01$) from 7.79 to 11.01 g/d MET as per experimental design.

Table 4. Formulation vs. analysis of lysine, threonine, and methionine content in pelleted dietary treatments and intake of amino acids (g/d) fed to weanling Quarter horses.

Item	Treatment[1]				SEM	Diet *P*-value
	0.20% MET	0.23% MET	0.27% MET	0.31% MET		
Formulation (DM)						
Lysine, %	0.78	0.78	0.78	0.78	-----	-----
Threonine, %	0.62	0.62	0.62	0.62	-----	-----
Methionine, %	0.20	0.23	0.28	0.31	-----	-----
Analysis (DM)						
Lysine, %	0.91	0.81	0.81	0.79	-----	-----
Threonine, %	0.66	0.56	0.58	0.61	-----	-----
Methionine, %	0.23	0.25	0.28	0.32	-----	-----
Intake (g/d)[2]						
Lysine	30.36	27.65	27.35	26.89	1.80	0.52
Threonine	21.86	18.99	19.46	20.83	1.30	0.38
Methionine	7.79	8.36	9.38	11.01	0.54	< 0.01

[1]Dietary treatment consisted of a basal pelleted concentrate (whole oats, oat byproducts, wheat midds) with increasing levels of methionine (MET).
[2]Intake (g/d) concentrate treatment.

Nitrogen Balance

There was no influence of dietary treatment on N intake ($P = 0.96$) or N excretion ($P = 0.99$), resulting in no influence of treatment on calculated N retention values ($P = 0.72$; Table 5). Increasing dietary methionine concentrations also did not

influence urinary excretion of urea or ammonia ($P = 0.31$ and $P = 0.58$, respectively). Apparent digestibility was within 15% of the estimated total tract digestibility of N for mature horses according to the NRC (2007). The slightly decreased apparent digestibility could be due to the difference in variety of coastal bermudagrass hays, laboratory differences, or differences between the mature versus growing model.

Table 5. Effect of increasing dietary levels of methionine on nitrogen balance measurements in weanling Quarter horses.

Measurement	Treatment[1]				SEM	Diet *P*-value[2]
	0.20% MET	0.23% MET	0.27% MET	0.31% MET		
N Intake (g/d)	103.71	101.38	100.34	99.69	6.31	0.96
N Excretion (g/d)	74.02	74.63	73.26	72.62	5.55	0.99
Fecal N (g/d)	34.42	36.67	36.13	35.70	2.97	0.94
Urine N (g/d)	39.60	37.96	37.13	36.92	3.66	0.94
Retained N (g/d)	29.69	26.75	27.08	27.07	2.32	0.72
Urine Urea (mM)	4.94	3.91	5.83	4.99	0.69	0.31
Urine NH_3(mM)	3.78	2.47	3.23	4.65	1.35	0.59
Apparent Digestibility N (%)	66.57	63.92	64.14	64.31	1.58	0.52

[1]Dietary treatment consisted of a basal pelleted concentrate (whole oats, oat byproducts, wheat midds) with increasing levels of methionine (MET).
[2]Linear, quadratic, and cubic P-values ≥ 0.21

Growth

There was no influence of increasing dietary methionine level ($P \geq 0.25$) on any of the growth measurements observed (Table 6). However, the growth measurements obtained and gain are similar or exceed those found in previous studies (Ott et al., 1981; Graham et al., 1994; Gibbs and Potter, 2002). Rump fat measurements decreased with

age, resulting in greater lean gain versus fat gain, and an overall loss of rump fat. Previous studies, reporting fat gain as a percentage of total body fat determined using a prediction equation, found fat gains of up to 22.6% (Graham et al., 1994). However, the horses used were yearlings and were fed to voluntary intake up to 2% BW/d grain (As fed). Weanlings used in the current study were limit-fed at 1.5% BW/d grain (As fed) and were in a more rapid stage of growth, which may explain the overall loss of fat.

Table 6. Effect of increasing dietary levels of methionine on growth measurements of weanling Quarter horses expressed as gain over 56 d.

	Treatment[1]					
Measurement	0.20% MET	0.23% MET	0.27% MET	0.31% MET	SEM	Diet *P*-value[2]
BW (kg)	39.80	38.00	35.65	37.00	2.28	0.64
ADG (kg/d)	0.71	0.68	0.64	0.66	0.04	0.64
Wither Height (cm)	5.03	5.57	4.60	5.44	0.77	0.79
Hip Height (cm)	4.83	6.05	5.86	5.54	0.79	0.69
Body length (cm)	6.52	10.04	10.91	9.01	2.27	0.54
Heart girth (cm)	12.59	11.42	9.81	10.27	1.35	0.50
Forearm (cm)	1.72	3.02	1.77	1.95	0.51	0.25
Gaskin (cm)	2.02	2.12	1.81	2.28	0.37	0.82
Rump fat (mm)	-0.33	-0.17	-0.35	-0.09	0.12	0.34
Feed:gain	6.22	6.68	6.77	6.55	0.30	0.58

[1]Dietary treatment consisted of a basal pelleted concentrate (whole oats, oat byproducts, wheat midds) with increasing levels of methionine (MET).
[2]Linear, quadratic, and cubic P-values $\geq$ 0.09.

Plasma Urea Nitrogen

Weekly plasma urea N (PUN) concentrations decreased with age as expected during normal growth ($P < 0.001$). Plasma urea nitrogen concentrations were also influenced by dietary MET concentration ($P = 0.005$) and data exhibited a quadratic effect ($P = 0.05$; Figure 3). Concentrations of PUN decreased from 0.20 MET to 0.23 MET as expected with additional dietary methionine, and 0.31 MET remained at a similar PUN concentration as 0.23 MET. Plasma urea N concentration of 0.27 MET was greater than expected, which creates the quadratic response. Concentrations of PUN were greater for 0.27 MET than values for 0.20 MET, 0.23 MET, and 0.31 MET ($P < 0.01$). Urine urea and ammonia concentrations were also elevated for 0.27 MET when evaluating the least square means (Table 5). While these values are not statistically significant ($P = 0.31$, $P = 0.59$, respectively), they suggest the PUN results are not simply an artifact of the analysis.

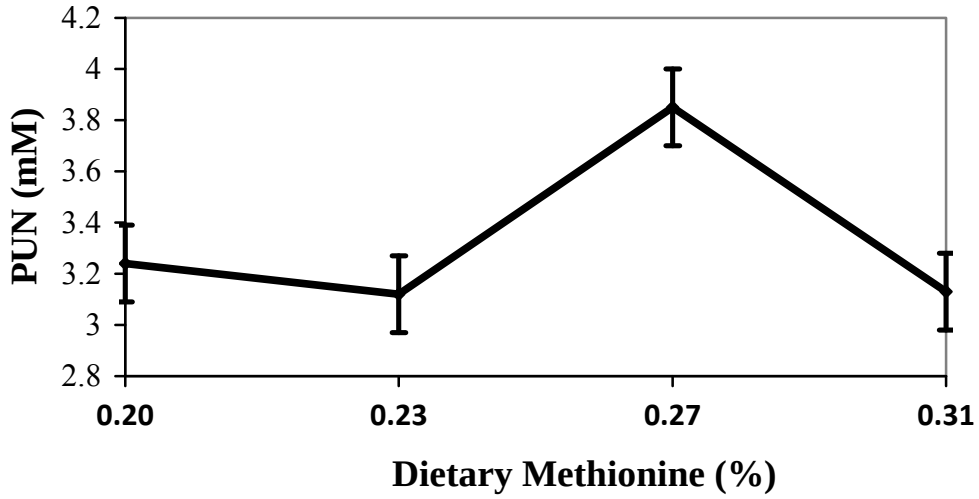

Figure 3. Effect of increasing levels of dietary methionine on plasma urea nitrogen concentration of weanling quarter horses. Methionine supplementation exhibited a quadratic response in PUN concentration ($P \leq 0.05$), and created differences in PUN concentration between treatments ($P \leq 0.01$).

Discussion

Data collected during this study fall within the normal range of growth, N retention and urea concentrations reported in previous research in horses of a similar age (Ott et al., 1981; Graham et al., 1994; Gibbs and Potter, 2002). Growth measurements obtained are similar or exceed those found in previous studies (Ott et al., 1981; Graham et al., 1994). This study fed a similar base ration to that of Ott et al. (1981) and Graham et al. (1994) but with slight variations in amino acid profiles. These studies report similar growth gains; however, they evaluated growth in yearlings Quarter horses. Therefore gains reported in the current study would logically exceed their values as weanlings are in a more rapid stage of growth. For example, growth measurements from this study

expressed as gain over 56 d compare very closely to those found by Graham et al. (1994) in yearlings over 112 d or over 196 d by Ott et al. (1981). There was no effect of increasing dietary inclusion of methionine on the physical growth measurements reported; however, this can be expected in a study of such short duration. Additionally, previous studies have indicated that energy plays a first-limiting role to growth and protein is limiting only when energy is supplied in sufficient quantities (Ott and Asquith, 1986). Dietary treatments in this study exceeded recommended values of DE and CP for growth (NRC, 2007).

Plasma urea N concentration decreases as the quality of dietary protein increases in animals fed at or below protein requirements (Eggum, 1970). Conversely, use of low-quality proteins and feeding excess dietary protein above the requirement will increase PUN concentrations. There is also a reduction in the crude protein requirement as horses age and as the rate of growth diminishes (Staniar et al., 2001). In this study, PUN concentrations decreased with increasing levels of methionine in the diet, indicating that the addition of crystalline methionine improved the amino acid profile of the diet. However, an unexpected peak in PUN was observed with 0.27 MET (Figure 1). Upon closer analysis, the mean g/d intake of lysine and threonine was not equal across treatments, with 0.20 MET having elevated lysine and threonine and 0.27 MET having a low g/d intake of lysine and threonine (Table 1). While these differences in lysine and threonine were not significant ($P = 0.78$ and 0.38 respectively), it is possible that the slight variance in amino acid profile and g/d amino acid consumption could explain

some of the results seen in PUN concentrations and the similar values seen in urine urea and ammonia.

These results compare to those of Graham et al. (1994) in a study evaluating effect of supplemental threonine on the growth of yearling horses. Their study reported that lysine percentage varied across dietary treatments, and resulted in the ration formulated to contain the greatest percentage of threonine also containing the greatest percentage of lysine. The addition of a second variable made it difficult for the authors to isolate the effect of threonine on growth. However, Graham et al. (1994) still provided a recommended percentage inclusion of threonine based on the highest bodyweight and girth gains observed in horses fed the highest dietary level of threonine. This implies that the addition of threonine improved the amino acid balance of the diets fed in this study, which resulted in the growth responses.

In this study, it is difficult to determine an exact dietary methionine requirement based solely on the PUN data. The PUN response of 0.27 MET cannot easily be explained in addition to the lack of dietary influence on N balance to reinforce PUN results. The profile of amino acids absorbed may have differed between treatment groups due to the variation of digestibility of protein sources within the small intestine (Potter et al., 1992). However, all treatments consisted of the same basal diet with only the only variable being the addition of crystalline methionine. To further attempt to prevent variation due to feed ingredients, all ration components were purchased in bulk quantities. All treatments were then mixed prior to the start of the experiment to maintain a more consistent base ration and greater consistency within treatments as well.

CHAPTER IV

SUMMARY

In summary, when closely evaluating the data, the methionine requirement of the concentrate portion of the ration likely falls between 0.23 MET (8.36 g MET) and 0.31 MET (11.02 g MET) because of the initial decrease in plasma urea concentration from 0.20 MET to 0.23 MET followed by a similar concentration in 0.31 MET and excluding the response of 0.27 MET. In future studies, longer trial duration may improve observations in increasing levels of methionine on physical growth measurements. Additionally, beginning the trial at a younger age may help to capture horses at a slightly more rapid stage of growth where they may be more sensitive to variations in amino acid profile due to further increases in protein synthesis. Further studies to more closely determine the dietary methionine requirement of weanling horses would be beneficial.

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