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

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Abstract

Current equine threonine requirement estimates do not account for probable use of threonine to maintain gut health and mucin synthesis. The objective of this study was to determine if threonine supplementation (+Thr) would increase whole-body protein synthesis (WBPS) in weanling colts (Study 1) and adult mares (Study 2). Both studies used a crossover design, where each of six animals were studied twice while receiving the isonitrogenous diets. The basal diets contained lower threonine levels (Basal) than the threonine (+Thr) supplemented diets. Threonine intakes in mg/kg BW/day were as follows: 79 (Basal) and 162 (+Thr) for Study 1 and 58 (Basal) and 119 (+Thr) for Study 2, in comparison to the NRC estimated requirements of 81 and 33 mg/kg BW/day for weanling and mature horses, respectively.

Following 5 days of adaptation, blood samples were taken before and 90 min after the morning concentrate meal. The next day, whole-body phenylalanine kinetics were determined using a 2 h primed, constant infusion of [^{13}C]sodium bicarbonate followed by a 4 h primed, constant infusion of [$1\text{-}^{13}\text{C}$]phenylalanine. Most plasma amino acid (AA) concentrations were elevated post-feeding ($P<0.01$). Lysine and valine plasma concentrations were lower ($P<0.10$), while methionine, threonine, and glycine plasma concentrations were greater ($P<0.10$) 90 min post concentrate meal feeding with +Thr in both studies. Phenylalanine flux, intake, oxidation and non-oxidative disposal were similar between treatments ($P>0.05$). These findings suggest that supplementation of a single AA can affect the metabolism of several AAs and threonine was not a limiting AA in these diets.

Keywords: Amino acids; Horse; Phenylalanine kinetics; Protein synthesis; Threonine

Introduction

Little is known about limiting amino acids in common equine diets or the indispensable amino acid requirements of horses. It has been suggested that the horse's requirements for amino acids may follow the ratio of concentrations of amino acids in the body. Because muscle tissue makes up the majority of the horse as reviewed by Kearns et al. (2002), this has been used to suggest amino acid requirement ratios (Bryden, 1991). To date, there have been no studies to estimate threonine requirements in horses and only estimates based on tissue composition. In some equine diets lysine has been identified as the first limiting amino acid (Hintz et al., 1971; Ott et al., 1981). Threonine (Graham et al., 1994) and methionine (Winsco et al., 2011; Graham-Thiers et al., 2012) have also been suggested as potentially limiting amino acids in yearling and weanling horse diets, respectively.

Supplementation of lysine and threonine together has been shown to increase subjective muscle mass scores and decrease plasma urea nitrogen in mature and aged horses (Graham-Thiers and Kronfeld, 2005). However, in both the Graham et al. (1994) and Graham-Thiers and Kronfeld (2005) studies, which suggested that threonine may be a limiting amino acid, threonine was provided at levels well above the requirement estimated using muscle amino acid composition (Bryden, 1991) in all treatments. The NRC (2007) suggested requirement of threonine for yearlings is 70 mg/kg BW/day, although increased girth circumference was associated with increased threonine intake from 94 to 113 mg/kg BW/day (Graham et al., 1994). Using muscle tissue to estimate requirements does not take into account other uses of amino acids or the fact that muscle protein amino acid composition is not necessarily representative of the composition of other body proteins. For example, the use of threonine by the gastrointestinal tract is considerable in other species.

In the gut, threonine is oxidized and used for mucin production, which varies with diet. Relatively high rates of threonine oxidation in the splanchnic tissues have been demonstrated in piglets (Schaart et al., 2005) and human infants (~70%) (van der Schoor et al., 2007), although this value is less for human adults (~18%) (Chapman et al., 2013) and adult mini-pigs (~37%) (Remond et al., 2009). Ileal losses of threonine through mucin also contribute to threonine usage in humans (Gaudichon et al., 2002) and intestinal mucin production is considered a major metabolic fate for threonine (Wu, 2009). For rats (Fahim et al., 1987), swine (Fogg et al., 1996), and humans (Robertson et al., 1996), threonine makes up a considerable portion of the mucin proteins. Intestinal mucin synthesis is also influenced by diet constituents, such as fiber (Satchithanandam et al., 1990; Ito et al., 2009). In swine, feedstuffs containing greater amounts of hemicellulose are associated with greater endogenous threonine losses (Myrie et al., 2008). Therefore, increased dietary hemicellulose would be expected to increase threonine requirements in animals. Horses can be fed diets with forages that have a range of acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents, as reviewed by Pagan (1997), but the effect of inclusion of various types of forage in equine diets on threonine use or requirement has not been investigated.

In addition to use for mucin and muscle protein synthesis, other major functions of threonine include immune function, protein phosphorylation, and glycine synthesis, as reviewed by Wu (2009). Glycine is also a precursor for serine synthesis. Threonine supplementation has resulted in greater plasma levels of glycine and serine in pigs (Edmonds and Baker, 1987). Since

the metabolism of amino acids is intertwined, threonine supplementation may have consequences beyond increasing protein synthesis.

In a previous study (Tanner et al., 2014), we found that non-oxidative disposal of an indicator amino acid, phenylalanine, was greater in the diet that had a greater content of crude protein (4.1 g/kg bodyweight (BW)/day) and threonine (88 mg/kg BW/day). The phenylalanine non-oxidative disposal results indicated that whole body protein synthesis was limited in the weanlings receiving the diet with lower protein (3.1 g/kg body weight (BW)/day) and threonine (67 mg/kg BW/day) content (Tanner et al., 2014). Given the amino acid profiles of the diets compared to NRC recommendations, it is possible that threonine was the limiting amino acid in the diet that supported lower levels of whole-body protein synthesis.

As using muscle composition may underestimate threonine requirements and fiber may increase the need for threonine, we investigated in two studies the effect of dietary threonine supplementation to diets, including grass forages, on whole-body protein synthesis in horses. The objective was to determine if threonine supplementation (+Thr) would increase whole-body protein synthesis (WBPS) in weanling colts (Study 1) and adult mares (Study 2) fed typical equine diets. We hypothesized that threonine supplementation would increase WBPS in both studies.

Materials and methods

Animals, housing, and feeding

Study 1

All procedures used in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC 2012–0991, granted 15 August 2012). Six weanling Thoroughbred colts of similar age (176 ± 30 days) and weight (269 ± 24 kg) were obtained from University of Kentucky's Maine Chance Farm. During both the 7 day adaptation period and days they were not being sampled, the weanlings were kept in individual dry lot pens and were brought into individual sawdust bedded stalls ($3.7 \text{ m} \times 3.7 \text{ m}$) twice daily for concentrate feeding. Horses remained in stalls while catheterized on the days that isotope infusions were performed. Bodyweights were taken weekly through the adaptation and study periods on a livestock scale (TI-500, Transcell Technology).

Diets were designed to meet or exceed the 2007 Nutrient Requirements of Horses recommendations (NRC, 2007). Each weanling received each of two diets in a crossover design, which consisted of a concentrate (Buckeye Growth, Buckeye Nutrition) at 1.7% of BW, timothy hay cubes (Premium Timothy Hay Cubes) at 1% BW, and an amino acid supplement. Supplements were isonitrogenous amounts of crystalline threonine (+Thr; 83 mg/kg BW/day) or glutamate (Basal; 103 mg/kg BW/day) top dressed onto the concentrate portion of the feed. The analyzed composition of the diets before the amino acid supplements were added is listed in Table 1. Feed was collected weekly throughout the study and sent to Dairy One Cooperative¹ for proximate analysis and was analyzed for amino acid content as described below.

¹ See: <http://dairyone.com/> (accessed 25 September 2015)

Study 2

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

Each mare on Study 2 received each of two diets in a randomly determined order, which consisted of a low threonine formulated concentrate (Table 2) at 0.8% of BW, chopped timothy (Totally Timothy, Lucerne Farms) at 1.2% of BW, and an amino acid supplement. Supplements were isonitrogenous amounts of threonine (+Thr; 72 mg/kg BW/day) or glutamate (Basal; 89 mg/kg BW/day). The analyzed composition of the diets before the amino acid supplements were added is listed in Table 3. Feed collection and analysis were completed as described for Study 1.

Study designs and procedures

Both studies were conducted as crossover designs, where each horse received both diets in a randomly determined order. All horses received each treatment diet for 5 days prior to sampling with no additional washout between treatments. Growing animals can be studied using phenylalanine kinetics while receiving potentially deficient diets and the risk of detrimental effects associated with prolonged nutrient restriction is minimized due to the short adaptation period (< 3 days) required for this method (Moehn et al., 2004b; Elango et al., 2009).

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

On day 7, two jugular vein catheters were inserted, one for blood sampling and one for isotope infusion (Urschel et al., 2012). Whole-body phenylalanine kinetics were determined using 2 h primed ($7.1 \mu\text{mol/kg BW}$), constant ($6 \mu\text{mol/kg BW/h}$) infusions of [^{13}C]sodium bicarbonate, to measure whole-body CO_2 production (Coggan et al., 1993; Moehn et al., 2004a), followed immediately by 4 h primed ($13 \mu\text{mol/kg BW}$), constant infusions of [$1\text{-}^{13}\text{C}$]phenylalanine ($9 \mu\text{mol/kg BW/h}$), to measure phenylalanine oxidation to CO_2 and phenylalanine flux. Validation was previously performed in mature horses and resulted in a rapid rise to plateau isotopic enrichment in the plasma phenylalanine (Urschel et al., 2012). The horses wore cordless infusion pumps (J-1097 VetPro Infusion Pump, Jorgensen Laboratories) that were attached to surcingles to infuse the isotope and allow the horses to move freely in their stalls.

Beginning 90 min prior to the start of isotope infusion procedures 1/24 of the morning meal was given every half-hour during infusion in order to reduce fluctuations in phenylalanine oxidation (Mohn et al., 2003; Moehn et al., 2004a). Meals fed prior to the [$1\text{-}^{13}\text{C}$]phenylalanine infusion were top-dressed with non-isotopic phenylalanine to ensure that phenylalanine entering the system during the measurement of CO_2 production and [$1\text{-}^{13}\text{C}$]phenylalanine oxidation to CO_2 were as equivalent as possible. Breath samples were collected every 30 min beginning 30

min prior to the start of the [¹³C]sodium bicarbonate infusion until the end of the [1-¹³C]phenylalanine infusion into gas impermeable bags using a modified equine Aeromask (Urschel et al., 2009). Blood samples were collected every 30 min beginning 30 min prior to the start of the [1-¹³C]phenylalanine infusion until the end of the infusion procedures.

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

Sample analyses

Blood sample processing

All blood samples were collected into heparinized Vacutainers (Becton-Dickinson) and promptly centrifuged at 1,500 g for 10 min at 4 °C. Plasma was removed and frozen at -20 °C until the time of analysis.

Amino acid analyses

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

Plasma metabolite concentrations

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

Isotope enrichment

The amount of $^{13}\text{CO}_2$ relative to $^{12}\text{CO}_2$ in breath samples collected during the phenylalanine and bicarbonate infusions was determined immediately after sampling using an infrared isotope analyzer (IRIS-2; Wagner Analysen Technik Vetriebs).

The relative concentrations of [1- ^{13}C]phenylalanine to unlabeled phenylalanine, known as the phenylalanine isotopic enrichment, in the plasma samples was determined by Metabolic Solutions using a previously described method (Matthews et al., 1990), which is described in brief elsewhere (Tanner et al., 2014).

Calculations and statistics

Calculation of whole-body phenylalanine kinetics was completed as previously described (Tanner et al., 2014). All data were analyzed using SAS (version 9.1.3, SAS Institute), using the MIXED procedure unless otherwise noted. In all cases, differences were considered significant at $P < 0.05$. The phenylalanine kinetics parameters were analyzed using a one-way analysis of variance with treatment as the fixed effect and horse as the random effect. Plasma amino acid and urea concentration data were analyzed using a repeated measures analysis, with treatment,

time, and time $\times$ treatment interaction as fixed effects and horse as the random effect. The variance-covariance matrix was selected based on the lowest value of the Schwarz Bayesian criterion. In all cases, when differences were significant, means were separated using the pdiff option with the Tukey-Kramer adjustment.

Results

All horses completed both treatments in their respective studies. For Study 1, dietary threonine intake was 162 mg/kg BW/day on the +Thr treatment and 79 mg/kg BW/day on the Basal treatment. Compared to the NRC dietary threonine requirement estimate of 81 mg/kg BW/day for 6-month old weanlings with an expected mature BW of 500 kg only the +Thr treatment provided threonine above the recommendation. All other indispensable amino acid requirements were met with Study 1 diets, with the exception of histidine. For Study 2, dietary threonine intake was 119 mg/kg BW/day on the +Thr treatment and 58 mg/kg BW/day on the Basal treatment, which is well above the NRC dietary requirement estimate of 33 mg/kg BW/day for adult horses at maintenance. All other indispensable amino acid requirements were met with Study 2 diets. However, these NRC requirements are only estimates based on muscle protein amino acid composition.

Plasma urea concentrations

Plasma urea concentrations were greater at 90 min post concentrate meal feeding than pre-feeding ($P < 0.05$) in both studies. There was no difference in plasma urea concentrations due

to treatment for Study 1 (Table 4), but for Study 2 there was a tendency for a treatment effect with higher concentrations with the Basal treatment ($P = 0.07$; Table 5).

Plasma amino acid concentrations

There were significant effects of time, diet, and the interaction of time by diet on plasma amino acid concentrations in both studies ($P < 0.05$; Tables 4 and 5). Generally, plasma amino acid concentrations were greater at 90 min post-feeding when compared to baseline values. Lysine and valine plasma concentrations post feeding were lower with threonine supplementation ($P < 0.10$), while methionine and threonine plasma concentrations were greater with threonine supplementation ($P < 0.10$) in both studies. Notably, plasma glycine concentrations increased both pre and post feeding with threonine supplementation in both studies and glycine was the only dispensable amino acid to significantly respond to treatment ($P < 0.01$). Plasma threonine concentrations increased with both supplementation and meal feeding and threonine was the only amino acid to have a significant interaction between treatment and time ($P < 0.05$) for the plasma concentrations for both studies.

Phenylalanine kinetics

Phenylalanine flux, intake, oxidation and non-oxidative disposal were similar between treatments (Tables 6 and 7; $P > 0.05$). Carbon dioxide production was also similar between treatments ($P > 0.10$) for both studies.

Discussion

Although it has been suggested that threonine is a limiting amino acid in some equine diets (Graham et al., 1994; Graham-Thiers and Kronfeld, 2005), our studies did not find that supplementing threonine to the typical equine diets fed in both studies increased whole body protein synthesis, suggesting that there was either no threonine limitation or there was a limitation in another indispensable amino acid in the unsupplemented (Basal) diets, for either weanling or mature horses. It is unlikely that an amino acid other than threonine was limiting as indispensable amino acid requirements according to the NRC were met in all diets, with the exception of histidine in the weanling diets. Histidine is an unlikely limiting amino acid in equine diets (Tanner et al., 2014). Due to threonine's use in the gut, we expected that the actual threonine requirement would be greater than the recommendation based on muscle tissue, especially because horses consume diets that are high in fiber compared to other non-ruminant species. Instead, our findings from Study 1 indicate that the current threonine requirement estimate for weanlings (NRC, 2007) is adequate. The current threonine requirement for mature horses was not evaluated in Study 2 as threonine intakes for both treatment diets were above the NRC recommendation (NRC, 2007).

There was no difference between treatments for any of the phenylalanine kinetics data ($P > 0.10$) for either study. However, rates of non-oxidative phenylalanine disposal in Study 1 were similar to those previously measured in weanling Thoroughbreds ($\sim 58 \mu\text{mol/kg}\cdot\text{h}$) (Tanner et al., 2014). Rates of non-oxidative disposal for the adult mares were lower than those of the weanlings, which would be expected due to the additional protein accretion associated with growth. The rates of non-oxidative disposal measured for the mares were similar to those

reported for mature Arabian geldings ($\sim 35 \mu\text{mol/kg}\cdot\text{h}$) (Urschel et al., 2012). Overall, our phenylalanine kinetics data were in line with previously published data in both mature and growing horses, but did not indicate that threonine was limiting when the horses were receiving the Basal diet in either study.

In yearlings, when threonine intake was increased from 94 to 113 mg/kg BW/day, girth circumference increased (Graham et al., 1994). The study that found improved muscle mass scores when aged and mature horses were supplemented with threonine and lysine had a smaller difference in threonine intakes between the basal and supplemented groups (range 65-102 mg/kg BW/day) (Graham-Thiers and Kronfeld, 2005) than did the mares in Study 2 (range 58-119 mg/kg BW/day), where differences in whole-body protein synthesis were not found. Perhaps the differences observed in that previous study were due to differences in lysine intake and not threonine intake (Graham-Thiers and Kronfeld, 2005). Interestingly, both studies suggesting that threonine may be limiting in some equine diets provided threonine at levels above the NRC (2007) suggested requirements (33 mg/kg BW/day for mature horses and 70 mg/kg BW/day for yearlings) (Graham et al., 1994; Graham-Thiers and Kronfeld, 2005), indicating that the threonine requirement may be greater than the amount suggested by using the muscle amino acid ratios put forth by Bryden (1991).

Previous studies that suggest threonine could be limiting in equine diets have used grass hays (coastal bermudagrass and an orchardgrass/timothy mix) and concentrates based heavily on corn and oats (Graham et al., 1994; Graham-Thiers and Kronfeld, 2005). Our studies used timothy grass hay and the concentrates were based on wheat (Study 1) and soy hulls (Study 2).

Very little is known about the digestibility of amino acids in various feedstuffs in horses; however, threonine digestibility is known to vary according to feedstuff for swine (NRC, 2012). Standard ileal digestibilities for corn (77%) and oats (75%) are greater than those for soy hulls (62%) and wheat middlings (73%) in swine (NRC, 2012), indicating that threonine may have been less digestible in the concentrates used in the current study than in the previous studies (Graham et al., 1994; Graham-Thiers and Kronfeld, 2005). However, if this was the case it would have been expected that threonine would be more likely to be limiting in diets where the threonine digestibilities are lower. Therefore difference in digestibility of threonine provided by the concentrates does not explain why threonine was implicated as a limiting amino acid in previous studies, but not by the studies presented here. However, an estimation of the availability of threonine in each complete diet used in the present study based on swine data is not possible due to the lack of data on forage amino acid digestibility in swine (NRC, 2012).

One possible reason that we did not find threonine to be limiting in either of our diets could be due to increased digestibility of threonine in the forages that were chosen for our diets relative to the forages used in previous studies. Although forages with greater NDF content also have lesser crude protein (CP) digestibility in horses (Gibbs, et al., 1988), neither of the previous studies that identified threonine as a limiting amino acid reported dietary NDF composition (Graham et al., 1994; Graham-Thiers and Kronfeld, 2005). Therefore, it is unclear as to whether differences in findings between the previous (Graham et al., 1994; Graham-Thiers and Kronfeld, 2005) and current studies may have been due to differences in dietary NDF composition.

Additional research on the availability of threonine from feedstuffs is needed to put better into context why no differences in whole-body protein synthesis were detected even when a previous study apparently provided greater levels of threonine in the basal treatment and there was still a response of increased girth circumference to the additional threonine supplementation (Graham et al., 1994).

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

Ileal losses in mucin need to be measured as opposed to fecal mucin, as gut microbes are known to digest mucins, as reviewed by Derrien et al. (2010). Fecal bacterial populations are noted to differ between herbivores, omnivores, and carnivores (Endo et al., 2010). It is possible that the loads of mucin degrading microbes differ between omnivores and herbivores and mucin

secretion is lesser in herbivores as a result. With lower amounts of mucin production the need for threonine in the gut would be reduced. Mucin protein compositions in other species contain high percentages of threonine. For example, intracellular mucin in rats is 31% threonine (Fahim et al., 1987) and polymeric colonic mucin in swine is 15% threonine (Fogg et al., 1996) compared to equine muscle protein being only ~5% threonine (Lorenzo and Pateiro, 2013). The amino acid composition of equine mucin has not yet been measured. In humans fed protein-free diets, ileal threonine losses (Fuller et al., 1994) are 28% of the WHO (2007) requirement. It is possible that the Basal diets provided more threonine than required by the horses, despite our expectations.

Although threonine supplementation did not increase whole-body protein synthesis, it did affect plasma concentrations of other amino acids. Some of these differences can be explained if the supplemental threonine was saturating degradation pathways. Threonine catabolism is known to take place through two pathways in mammals (Dale, 1978; Bird and Nunn, 1983). One pathway is known to create glycine, while the other is known to produce α -ketobutyrate. Plasma α -ketobutyrate levels were not measured, as characterizing threonine catabolism in the horse was not the objective of the study. Glycine plasma concentrations most likely responded to treatment because glycine can be produced from threonine. Glycine is converted to serine when it is catabolized and although we did not see greater serine plasma concentrations when the colts were supplemented with threonine, we did see an increase in plasma serine concentrations in the adult mares. In the adult mares, plasma lysine and valine concentrations were lower with threonine supplementation. Lower plasma lysine concentrations in response to supplemental threonine has been described in other species as well (Rosell and Zimmerman, 1984; Faure et al., 2005); however, in the absence of differences in whole-body protein synthesis or degradation

between treatments in the present study, the mechanisms underlying these changes in indispensable amino acid concentrations are unclear and will require additional research.

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

Conclusions

Given that our studies found no differences in whole-body protein synthesis when threonine was supplemented, the findings indicate that the threonine supplementation for weanlings receiving this commercial concentrate and a quality grass forage or adult horses at maintenance on a diet composed of 60% grass forage and 40% concentrate is most likely not warranted. However, threonine supplementation can affect amino acid metabolism and additional research is needed to determine how these changes in metabolism affect long term growth and athletic performance. Additional research is also needed to determine amino acid requirements in horses.

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Tables

Table 1: As-fed nutrient composition of the feeds used in both diets fed to the weanlings.

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

^a Growth Pellet, Buckeye Nutrition, Dalton, OH, USA

^b *n* = 6 samples for each feed

Table 2: Composition of low threonine concentrate used in Study 2 on an as-fed basis ^a.

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

^a The concentrate was mixed by Buckeye Nutrition specifically for this study.

^b Dynamate is a potassium and magnesium sulfate product made by The Mosaic Company

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

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

Table 3: As-fed nutrient composition of the feeds used in both diets fed to the adult mares.

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

^a *n* = 6 of each feed

Table 4: Study 1. Plasma amino acid concentrations (mmol/L) in weanling Thoroughbred colts ($n = 6$) at baseline and 90 min post concentrate meal feeding when supplemented isonitrogenous amounts of either threonine (+Thr) or glutamate (Basal) ¹.

	Basal		+Thr		SEM	<i>P</i> -values ²		
	Baselin e	90 min	Baselin e	90 min		Treatment	Time	Treatment × time
Indispensable								
Histidine	31 ^a	69 ^b 12	34 ^a	69 ^b	4	NS	<0.0001	NS
Isoleucine	59 ^a	1 ^b 19 ^b ,	56 ^a	100 ^b	6	0.07	<0.0001	NS
Leucine	95 ^a	9 [*] 18 ^b ,	87 ^a	161 ^b	10	0.03	<0.0001	NS
Lysine	64 ^a	2 [*] b,	59 ^a	154 ^b	8	0.06	<0.0001	NS
Methionine	23 ^a	44 [*] 10 ^b ,	30 ^a	60 ^b	3	0.0003	<0.0001	0.10
Phenylalanine	56 ^a	3 [*] 13 ^b ,	55 ^a	90 ^b	4	0.09	<0.0001	NS
Threonine	77 ^{a,*}	7 [*]	178 ^a	351 ^b	20	<0.0001	<0.0001	0.01
Tryptophan	2	4 27	9	10	5	NS	NS	NS
Valine	166 ^a	8 ^b	161 ^a	243 ^b	12	0.10	<0.0001	NS
Dispensable								
Alanine	118 ^a	25 0 ^b	136 ^a	259 ^b	8	NS	<0.0001	NS
Arginine	69 ^a	18 9 ^b	68 ^a	166 ^b	8	NS	<0.0001	NS
Asparagine	46 ^a	10 5 ^b	49 ^a	99 ^b	4	NS	<0.0001	NS
Aspartate	55	62	59	58	4	NS	NS	NS
Citrulline	77 ^a	10 3 ^b	80 ^a	105 ^b	4	NS	<0.0001	NS
Glutamate	79	83	76	82	5	NS	NS	NS
Glutamine	610 ^a	95 9 ^b	654 ^a	971 ^b	20	NS	<0.0001	NS
Glycine	595 ^{a,*}	69 ^b , 1 [*]	643 ^a	730 ^b	11	0.002	<0.0001	NS
Ornithine	33 ^a	20 65 ^b	31 ^a	57 ^b	3	NS	<0.0001	NS
Proline	90 ^a	34 7 ^b	90 ^a	191 ^b	7	NS	<0.0001	NS
Serine	246 ^a	5 ^b	249 ^a	338 ^b	10	NS	<0.0001	NS

Taurine	27 ^a	40 ^b 12	28 ^a	44 ^b	3	NS	0.0002	NS
Tyrosine	67 ^a	0 ^b	66 ^a	119 ^b	5	NS	<0.0001	NS
Urea (mmol/L)	2.7 ^a	3.1 ^b	2.8 ^a	3.2 ^b	0.2	NS	<0.0001	NS

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

^{a,b} Different superscripts within a row indicate a significant effect of time ($P < 0.05$)

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

² NS, non-significant

Table 5: Study 2. Plasma amino acid concentrations (mmol/L) in adult Thoroughbred mares ($n = 6$) at baseline and 90 min post concentrate meal feeding when supplemented with isonitrogenous amounts of either threonine (+Thr) or glutamate (Basal) ¹.

	Basal		+Thr			<i>P</i> -values ²		
	Baseline	90 min	Baseline	90 min	SEM	Treatment	Time	Treatment × time
Indispensable								
		b,						
Histidine	63 ^{a,*}	80 [*]	72 ^a	86 ^b	3	0.01	<0.001	NS
Isoleucine	54 ^a	63 ^b	52 ^a	56 ^b	2	NS	0.01	NS
Leucine	114 ^a	137 ^b	112 ^a	126 ^b	6	NS	0.01	NS
		b,						
Lysine	76 ^a	109 [*]	70 ^a	91 ^b	4	0.01	<0.001	NS
Methionine	31 [*]	40 [*]	47	53	4	0.0002	0.09	NS
Phenylalanine	75 ^a	83 ^b	76 ^a	82 ^b	2	NS	<0.001	NS
		b,						
Threonine	90 ^{a,*}	101 [*]	497 ^a	638 ^b	32	<0.001	0.02	0.05
Tryptophan	12	12	11	12	0	NS	0.06	NS
		b,						
Valine	149 ^a	168 [*]	144	154	5	0.05	0.0003	NS
Dispensable								
Alanine	221 ^a	259 ^b	220	248	9	NS	0.0001	NS
Arginine	83 ^a	120 ^b	83 ^a	106 ^b	4	0.08	<0.001	NS
		b,						
Asparagine	41 ^{a,*}	57 [*]	49 ^a	62 ^b	3	0.02	<0.001	NS
Aspartate	25	29 [*]	22	22	1	0.0001	NS	NS
Citrulline	87 ^{a,*}	114 ^b	97 ^a	119 ^b	4	0.05	<0.001	NS
Glutamate	87 [*]	84 [*]	71	70	3	<0.001	NS	NS
Glutamine	424 ^a	551 ^b	457 ^a	543 ^b	19	NS	<0.001	NS
Glycine	555 [*]	557 [*]	660	676	16	<0.001	NS	NS
Ornithine	46 ^a	54 ^b	46 ^a	51 ^b	2	NS	0.01	NS
Proline	96 ^a	136 ^b	103 ^a	127 ^b	5	NS	<0.001	NS
		b,						
Serine	224 ^{a,*}	259 [*]	286 ^a	319 ^b	9	<0.001	0.0004	NS
Taurine	43 [*]	44 [*]	46	48	1	0.03	NS	NS
		b,						
Tyrosine	76 ^{a,*}	90 [*]	87 ^a	104 ^b	4	0.002	0.0004	NS
Urea		12.						
(mmol/L)	11.3 ^a	0 ^b	10.7 ^a	11.4 ^b	0.6	0.07	0.03	NS

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

^{a,b} Different superscripts within a row indicate a significant effect of time within a diet ($P < 0.05$)

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

² NS, non-significant

Table 6: Study 1. Whole-body phenylalanine kinetics in weanling Thoroughbred colts ($n = 6$) that were supplemented with isonitrogenous amounts of either threonine (+Thr) or glutamate (Basal) ^a.

	Basal	+Thr	Pooled SE	<i>P</i> -value
Phenylalanine flux [$\mu\text{mol/kg}\cdot\text{h}$]	61	62	3	0.56
Carbon dioxide production [$\mu\text{mol/kg}\cdot\text{h}$]	21238	20734	1317	0.71
Phenylalanine entering the free phenylalanine pool [$\mu\text{mol/kg}\cdot\text{h}$] ^b				
Phenylalanine from dietary intake	24.5	23.0	1.0	0.18
Phenylalanine from protein breakdown	36.1	39.5	3.0	0.30
Phenylalanine leaving the free phenylalanine pool [$\mu\text{mol/kg}\cdot\text{h}$] ^b				
Phenylalanine oxidation	12.2	11.5	2.2	0.74
Non-oxidative phenylalanine disposal	48.4	51.0	3.1	0.42

^a Values are least squares means $\pm$ SE, as determined using a one-way analysis of variance

^b The following stochastic model of phenylalanine kinetics was used:

Flux = rate of phenylalanine entry = rate of phenylalanine leaving;

Rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown;

Rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal;

A 50% rate of splanchnic phenylalanine extraction was assumed when calculating the amount of dietary phenylalanine reaching general circulation

Table 7: Study 2. Whole-body phenylalanine kinetics in mature Thoroughbred mares ($n = 6$) that were supplemented with isonitrogenous amounts of either threonine (+Thr) or glutamate (Basal) ^a.

	Basal	+Thr	Pooled SE	<i>P</i> -value
Phenylalanine flux [$\mu\text{mol/kg}\cdot\text{h}$]	52	50	3	0.96
Carbon dioxide production [$\mu\text{mol/kg}\cdot\text{h}$]	14588	14864	491	0.98
Phenylalanine entering the free phenylalanine pool [$\mu\text{mol/kg}\cdot\text{h}$] ^b				
Phenylalanine from dietary intake	30.4	30.3	0.1	0.73
Phenylalanine from protein breakdown	21.9	20.0	3.3	0.97
Phenylalanine leaving the free phenylalanine pool [$\mu\text{mol/kg}\cdot\text{h}$] ^b				
Phenylalanine oxidation	11.3	11.0	1.4	0.99
Non-oxidative phenylalanine disposal	41.0	39.2	2.6	0.96

^a Values are least squares means $\pm$ SE, as determined using a one-way analysis of variance

^b The following stochastic model of phenylalanine kinetics was used:

Flux = rate of phenylalanine entry = rate of phenylalanine leaving;

Rate of phenylalanine entry = phenylalanine intake + phenylalanine release from protein breakdown;

Rate of phenylalanine leaving = phenylalanine oxidation + non-oxidative phenylalanine disposal;

A 50% rate of splanchnic phenylalanine extraction was assumed when calculating the amount of dietary phenylalanine reaching general circulation