1 **Short Communication**

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4 **Splanchnic extraction of phenylalanine in mature mares was not affected by threonine**
5 **supplementation**
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Abstract

This study determined splanchnic extraction of phenylalanine at two intakes of threonine. Six Thoroughbred mares were supplemented with isonitrogenous amounts of either threonine or glutamate. Dietary threonine intakes were 119 (+Thr) and 58 (Basal) mg/kg/day, respectively. Each horse received each diet twice and each was studied once with an oral and once with an intravenous (IV) infusion of [1-¹³C]phenylalanine. A 2-h primed, constant IV infusion of [¹³C]sodium bicarbonate and a 4-h primed, constant infusion of [1-¹³C]phenylalanine, either orally or IV, were used to measure isotopic enrichments. Phenylalanine kinetics were not affected by diet ($P > 0.05$). Splanchnic extraction of phenylalanine was $26 \pm 5\%$ and $27 \pm 3\%$ for the +Thr and Basal supplemented diets, respectively. These values will improve accuracy of future equine indicator amino acid oxidation studies.

Keywords: Amino acids; Horse; Phenylalanine kinetics; Splanchnic extraction

Isotopic amino acid (AA) methodologies, as reviewed by Elango et al. (2012), have been adapted for use in the horse (Urschel et al., 2012; Tanner et al., 2014). In previous equine studies using this method, estimates of splanchnic extraction from other species have been used (Urschel et al., 2012; Tanner et al., 2014), but gastrointestinal differences make equine specific values desirable.

Knowing the splanchnic extraction of the indicator AA is of limited use if that value changes as the level of the test AA changes. Therefore, in this study we examine two levels of dietary threonine. Threonine was chosen as it could be second limiting, after lysine, in some equine diets (Graham et al., 1994). The objectives were to measure splanchnic extraction of phenylalanine at two different levels of threonine intake and determine whether the oral administration of [1-¹³C]phenylalanine would create measureable amounts of isotope in the collected breath and plasma samples.

All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC 2012-0925 granted 1 March 2012). Six Thoroughbred mares (12 ± 3 years; 564 ± 23 kg) from the University of Kentucky's Maine Chance Farm were used. During the 7-day adaptation period and days they were not being sampled, the mares were turned out during the day with muzzles. Horses were fed individually and allowed additional time overnight to consume the forage.

Each mare received each of two diets, which consisted of a concentrate (Table 1) at 0.8% of bodyweight (BW), chopped timothy (Totally Timothy, Lucerne Farms) at 1.2% BW, and an

AA supplement. Supplements were isonitrogenous amounts of crystalline threonine (+Thr; 72 mg/kg/day) or glutamate (Basal; 89 mg/kg/day) that were top-dressed onto the concentrate. Glutamate was chosen as it is a dispensable AA that is not directly metabolically related to threonine. Weekly feed samples were sent to Dairy One Cooperative Inc.¹ for proximate analysis and analyzed for AA content.

Each horse was studied with an intravenous (IV) infusion and the oral administration of [1-¹³C]phenylalanine (Fig. 1) while on each diet, with the horses receiving the treatments in a random order. One horse was unable to complete the IV infusion of [1-¹³C]phenylalanine when fed the +Thr diet due to a catheter problem. Horses received supplementation for 6 days before sampling. On day 7, jugular vein catheters were inserted (Urschel et al., 2012). Horses that received the infusion of [1-¹³C]phenylalanine IV had two catheters, one for blood sampling and one for infusion while horses that received the infusion of [1-¹³C]phenylalanine orally only had one inserted.

Whole-body phenylalanine kinetics were determined using a 2-h primed (7.1 µmol/kg), constant (6 µmol/kg/h) IV infusion of [1-¹³C]sodium bicarbonate, to measure whole-body CO₂ production, followed immediately by a 4-h primed (13 µmol/kg), constant administration of [1-¹³C]phenylalanine (9 µmol/kg/h), either IV or orally, to measure phenylalanine oxidation to CO₂ and phenylalanine flux. Cordless pumps (J-1097 VetPro Infusion Pump, Jorgensen Laboratories)

¹ See: <http://dairyone.com/> (accessed 26 September 2015)

were attached to surcingles for IV isotope infusion. Oral administration of [1-¹³C]phenylalanine, including the prime dose, was top-dressed onto feed with saline every half-hour.

The horses readily consumed the [1-¹³C]phenylalanine in saline on their feed. Beginning 90 min before isotope infusion procedures, 1/24 of the morning meal was given every half-hour during infusion (Mohn et al., 2003). Meals fed before the [1-¹³C]phenylalanine infusion were top-dressed with non-isotopic phenylalanine. Breath samples were collected every 30 min beginning 30 min before 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 into heparinized Vacutainers (Becton-Dickenson) every 30 min beginning 30 min before the start of the [1-¹³C]phenylalanine infusion through the rest of infusion procedures. Following isotope infusion procedures, the catheters were removed and the horses were allocated to the next treatment at their afternoon concentrate meal.

Sample analyses to determine total feed AA concentrations and isotopic enrichments were conducted as previously described (Tanner et al., 2014). Blood and breath phenylalanine enrichment plateaus were used to calculate phenylalanine kinetics also as previously described (Tanner et al., 2014). Splanchnic extraction was calculated using the following formula (Kriengsinyos et al., 2002):

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

where Q_{IV} is phenylalanine flux when the $[1-^{13}C]$ phenylalanine isotope was administered IV and Q_O is the phenylalanine flux when the $[1-^{13}C]$ phenylalanine isotope was administered orally.

Data were analyzed using SAS (version 9.3, SAS Institute), using the MIXED procedure. The phenylalanine kinetics parameters were determined using a one-way analysis of variance with treatment as the fixed effect and horse as the random effect. In all cases, when differences were significant, means were separated using the Tukey-Kramer adjusted pdiff option.

Composition of the diet without the AA supplements is listed in Table 2. Total dietary threonine intake was 119 mg/kg/day on the +Thr diet and 58 mg/kg/day on the Basal diet, which is above the NRC (2007) dietary requirement estimate of 33 mg/kg/day for adult horses at maintenance.

The oral administration resulted in stable plateaus in both breath $^{13}CO_2$ and blood ^{13}C -phenylalanine enrichment, confirming the doses and length of oral administration. Phenylalanine intake and CO_2 production (Table 3) did not differ between treatments ($P > 0.10$). Phenylalanine kinetics (Table 3) were not affected by diet ($P > 0.10$), although phenylalanine flux, oxidation and non-oxidative disposal values were greater when the isotope was administered orally ($P < 0.05$). The differences in flux between the oral and IV infusions allowed for the calculation of phenylalanine splanchnic extraction of $26 \pm 5\%$ and $27 \pm 4\%$ for the +Thr and Basal diets, respectively.

Splanchnic extraction of phenylalanine in other species ranges from 19% (Kriengsinyos et al., 2002) to 47% (Volpi et al., 1999), with most values between 20% and 30% (Volpi et al., 1999; Cvitkovic et al., 2004; Gooding et al., 2013). For future studies in horses using phenylalanine kinetics, splanchnic extraction of phenylalanine will no longer have to be estimated. Both the splanchnic extraction value for phenylalanine and the less invasive oral administration of [1-¹³C]phenylalanine method will be useful in future phenylalanine kinetic and indicator AA oxidation studies in horses.

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171 **Table 1**172 Composition of the formulated concentrate 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

173 ^a The concentrate was mixed by Buckeye Nutrition specifically for this study.174 ^b Dynamate is a potassium and magnesium sulfate product made by The Mosaic Company175 ^c The proprietary vitamin/mineral premix was designed specifically for inclusion in Buckeye
176 Nutrition feeds and is manufactured by Trouw Nutrition177 ^d The proprietary trace mineral premix was designed specifically for inclusion in Buckeye
178 Nutrition feeds and is manufactured by Trouw Nutrition

179 **Table 2**

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

	Timothy			Concentrate		
<i>Overall nutrient composition</i>						
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
<i>Amino acid composition (g/100g feed)</i>						
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

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Table 3

Whole-body phenylalanine kinetics in mature Thoroughbred mares that were receiving either 72

mg/kg/day of threonine supplement (+Thr) or an isonitrogenous amount of glutamate (Basal; 89

mg/kg/day) when [1-¹³C]phenylalanine was administered either intravenously (IV) or orally (O) ^c

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

^{a,b} Different superscripts within a row indicate statistically significant differences ($P < 0.05$)

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

^d 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

Figure legend

Fig.1. Schematic showing differences in how isotopic phenylalanine used in kinetics studies is metabolized differently based on the route of administration. Orally ingested phenylalanine must pass through the splanchnic tissue before entering the general plasma phenylalanine pool, where some of it is used by those tissues. In contrast, isotopic phenylalanine infused IV directly enters the phenylalanine plasma pool. Unlabeled phenylalanine may also enter the plasma pool through degradation of body proteins. Phenylalanine leaves the plasma pool when it is used for synthesis of body proteins or converted to tyrosine in the liver. Tyrosine is also used for body protein synthesis and is released from body protein when degradation occurs. A negligible amount of tyrosine is converted to other metabolites, such as dopamine. Excess tyrosine not used for either protein synthesis or other metabolites undergoes oxidation with the labeled carbon from [1-¹³C]phenylalanine expelled as ¹³CO₂ in the breath.

Figure 1