Dietary phenylalanine requirements are similar in small, medium, and large breed adult dogs using the direct amino acid oxidation technique¹

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ABSTRACT: We have previously determined phenylalanine (**Phe**) requirements in mature dogs; however, little information is available on differences of Phe minimum requirements on different breed sizes. The objective of this study was to determine Phe requirements in adult dogs of three different breed sizes using the direct AA oxidation (**DAAO**) technique. In total, 12 adult dogs were used, four Miniature Dachshunds (5.3 ± 0.6 Kg BW; 1.8 ± 0.1 years old; mean $\pm$ SD), four Beagles (8.3 ± 0.7 Kg BW; 6.7 ± 0.2 years old; mean $\pm$ SD), and four Labrador Retrievers (34.9 ± 2.2 Kg BW; 4.4 ± 1.4 years old; mean $\pm$ SD). A basal Phe-deficient diet with excess of tyrosine (**Tyr**) was formulated. Dogs were randomly fed the basal diet supplemented with increasing levels of Phe; the Phe content in the final experimental diets was 0.24, 0.29, 0.34, 0.44, 0.54, 0.64, and 0.74%. After 2 d of adaptation to the experimental diets, dogs underwent individual DAAO studies. During the DAAO studies, total daily feed was divided in 13 equal meals; at the sixth meal, dogs were fed a bolus of L-[1-¹³C]-Phe

(9.40 mg/kg BW), and thereafter, L-[1-¹³C]-Phe (2.4 mg/kg BW) was supplied with every meal. Total production of ¹³CO₂ (F¹³CO₂) during isotopic steady state was determined by enrichment of ¹³CO₂ in breath samples and total production of CO₂ measured using indirect calorimetry. The mean requirement for Phe and the 95% confidence interval (**CI**) was determined using a two-phase linear regression model. To account for differences in feed intake, requirements were expressed in mg.kg BW⁻¹.d⁻¹. The mean requirement for Phe were 41.9, 41.3, and 42.6, and upper 95% CI of Phe requirements were 57.3, 58.4, and 64.8 mg.kg BW⁻¹.d⁻¹ for Miniature Dachshunds, Beagles, and Labrador Retrievers, respectively. The mean requirement and the upper 95% CI for the pooled data (all dogs) was 45.3 and 55.4 mg.kg BW⁻¹.d⁻¹, respectively. In conclusion, the Phe requirements for different breeds were similar among dog breeds studied. However, Phe recommendations proposed in this study are lower than those proposed by NRC and AAFCO (mg.kg BW⁻¹.d⁻¹).

Key words: adult dogs, direct AA oxidation, phenylalanine requirement

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INTRODUCTION

The AA recommendations for pet food in North America are issued by AAFCO (2010) and in Europe by FEDIAF (2013). The recommendations made by AAFCO and FEDIAF are scaled

up recommendations from [NRC \(2006\)](#) to account for different digestibility and AA bioavailability of different ingredients. The [NRC \(2006\)](#) recommendations are based on animal characteristics (g/kg BW), on a gross dietary composition basis (g/kg), and on a dietary energy basis (g/Mcal; [Morris and Rogers, 1994](#)).

In [NRC \(2006\)](#), different terminology is used to refer to essential AA requirements. The minimal requirement (**MR**) represents the lowest level of AA intake at which nitrogen balance is maintained. The adequate intake (**AI**) is defined as the concentration or amount presumed to be needed when no MR has been scientifically investigated. Finally, the required allowance (**RA**) is the dietary concentration of the nutrient which supports a specific life stage, considering the bioavailability of the nutrient in common quality feed ingredients ([NRC, 2006](#)).

The MR recommendations for the requirements of indispensable AA for growing puppies are based on empirical data ([Milner, 1979](#); [Burns and Milner, 1982](#)), but there is a lack of information on maintenance AA requirements for adult dogs. [NRC \(2006\)](#) states that: “No individual dose-response peer-reviewed reports could be found for the minimal requirements of any of these amino acids -including Phe- for maintenance on dogs.” [Shoveller et al., \(2017\)](#) determined phenylalanine (**Phe**) requirements for adult dogs. However, there are vast phenotypic differences among dog breeds that may contribute to differences in macronutrient metabolism, potentially modifying AA requirements that have not been accounted for. Accurate determination of essential AA requirements is necessary to ensure proper dietary supply, reducing excess of nutrient excretion, and increasing sustainability of pet food industry ([Swanson et al., 2013](#)).

Studies in humans, pigs, and recently in dogs have validated the use of carbon oxidation techniques to determine AA requirements at a whole body-tissue level ([Bross, et al. 1998](#); [Moehn, et al., 2004](#); [Pencharz and Ball, 2003](#); [Shoveller et al., 2017](#)). Comparison of AA recommendations based on nitrogen balances or carbon oxidation techniques suggests that nitrogen balance underestimates AA requirements by ~40% ([Zello et al., 1995](#); [Elango et al., 2008](#)). The discrepancy is because carbon oxidation techniques are more sensitive to changes in AA intakes, and therefore result in much higher requirements than nitrogen balance-based methods ([Zello et al., 1995](#); [Elango et al., 2008](#)). The objective of the present study was to determine dietary Phe requirements of different dog breed sizes using direct AA oxidation (**DAAO**). We hypothesized

that the mean Phe requirements for adult dogs can change among different breed and it is greater than the current [NRC \(2006\)](#) recommendation.

MATERIALS AND METHODS

Animals and Housing

The present experiment was approved by the Procter and Gamble Pet Care's Institutional Animal Care and Use Committee (**IACUC**). A total of 12 dogs were used, four adult spayed Miniature Dachshunds (5.3 ± 0.6 kg BW; 1.8 ± 0.1 yr old, mean $\pm$ SD), four adult spayed Beagles (8.3 ± 0.7 kg; 6.7 ± 0.2 yr old, mean $\pm$ SD), and four neutered Labradors (34.9 ± 2.2 kg; 4.4 ± 1.4 yr old, mean $\pm$ SD). All dogs resided at Procter and Gamble Pet Care (Lewisburg, OH) and were considered healthy based on a general health evaluation by a licensed veterinarian prior to the study. During the study, dogs were pair-housed in kennels (2.4×2.4 m) in a temperature-controlled building (22°C) and with a lighting schedule of 12 h:12 h light:dark. Dogs received similar amounts of daily socialization, exercise, and regular veterinary care as previously reported ([Shoveller et al., 2017](#)).

Diets and Study Design

A basal diet was formulated to meet or exceed requirements for all essential AA according to [NRC \(2006\)](#); previously reported in [Shoveller et al., 2017](#)). The extruded kibble basal diet was fed twice daily (0700 and 1300 h) during 14 d prior to the beginning of the experiment (adaptation period) in amounts known to maintain their individual BW. After the 14-d adaptation period to the basal diet, a test diet (similar to basal diet but without added crystalline Phe; final Phe = 0.24%; [Table 1](#)) was fed to the dogs at 16 g/kg of BW for Miniature Dachshunds and Beagles and at 14 g/kg of BW for Labradors for 2 d prior to each DAAO study. The test diets were supplemented with one of seven Phe (Skidmore Sales & distributing, West Chester Township, OH) solutions (0, 1.67, 3.33, 6.67, 10.00, 13.33, and 16.67 g/L) at 4.8 mL/kg BW for Miniature Dachshunds and Beagles dogs and at 4.2 mL/kg BW for Labradors. To maintain similar nitrogen content among all solutions, alanine was added (8.99, 8.09, 7.19, 5.39, 3.6, 1.8, and 0 g/L for solution 1 to 7, respectively). The final Phe contents in the test diet plus the dressing solution were 0.24, 0.29, 0.34, 0.44, 0.54, 0.64, and 0.74% (experimental diets). After the 2-d adaptation period to the experimental diet ([Moehn](#)

Table 1. Analyzed nutrient content of the test diet

Nutrient	Analyzed content
Metabolizable energy, kcal/kg (calculated) ¹	3,730
DM, %	93.0
CP, %	10.69
Arg, %	1.16
His, %	0.522
Ile, %	0.656
Leu, %	0.906
Lys, %	0.694
Met, %	0.487
Cys, %	0.947
Phe, %	0.243
Tyr, %	0.412
Thr, %	0.685
Trp, %	0.170
Val, %	0.560

Arg = arginine; Cys = cysteine; His = histidine; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; Phe = phenylalanine; Thr = threonine; Trp = tryptophan; Tyr = tyrosine; Val = valine.

¹Calculated metabolizable energy based on modified Atwater values.

et al., 2004), the DAAO study combined with indirect calorimetry was conducted. After each DAAO study, dogs returned to the basal diet for 4 d before feeding the test diet with a different dressing solution and conducting the next DAAO study. This 7-d feeding regime was repeated seven times. Dogs were assigned to the experimental treatments randomly, and no dog received the same order of treatments. After completion of the study, all dogs were fed each of the seven experimental diets. Blood samples (3 mL) were collected from the jugular vein in serum vacutainers (Becton & Dickinson) at the end of each IAAO study and represent fed state serum AA concentrations. Throughout the whole study, dogs had access to fresh water via an automatic watering system. All dogs consumed their entire daily diet offerings (basal or test diets), negating the need to collect and measure food refusals.

Direct AA Oxidation Studies

The day when the DAAO study was conducted, dogs were moved to individual respiration calorimetry chambers. After 30 min of gas equilibration, three fasting respiration/indirect calorimetry measurements were taken over three consecutive 25-min periods to determine resting volume of CO₂ and O₂ produced (VCO₂; VO₂) by each dog (Table 2). Dogs were then fed (time 0) their corresponding feed allowance divided in 13 equal meals; the first 3 meals were fed every 10 min to induce a fed state, and the other 10 meals were fed every 25 min. The

total amount of feed fed during the DAAO study was based on BW measured the same day in the morning after 18 h of fasting (16 g/kg of BW for Miniature Dachshunds and Beagles and 14 g/kg BW for Labradors). Background enrichment was determined by the collection of CO₂ samples over three consecutive 25-min periods. The next meal (95 min after first feeding) contained a priming dose (9.40 mg/kg BW) of L-[1-¹³C]-Phe (99%; Cambridge Isotope Laboratories, Inc., Tewksbury, MA). To maintain the supply of L-[1-¹³C]-Phe, the following seven meals contained constant doses (2.40 mg/kg BW) of L-[1-¹³C]-Phe for all dogs. Expired CO₂ was collected over the last eight 25-min periods. Overall, during each DAAO study, each dog spent ~6.3 h inside the calorimetry chamber. Table 2 presents the overall timeline of each DAAO study.

Sample Collection and Analysis

Nitrogen content in the basal diet was analyzed with a LECO analyzer (LECO Corporation, MI). Amino acid content in the test diet was analyzed using AOAC method 999.12 (AOAC International, 2000). Calorimetry data were collected automatically using Qubit calorimetry software (Customized Gas Exchange System and Software for Animal Respirometry; Qubit Systems Inc.). Measured VCO₂ during fasting and fed states were averaged over the collection periods to obtain a mean fasting and fed VCO₂ for each dog. Background and enriched samples of CO₂ were collected by trapping subsamples of expired CO₂ in 1 M NaOH. The NaOH solution was subsampled and stored at room temperature until further analysis. The enrichment of ¹³C in breath CO₂ captured in NaOH solution was measured by continuous-flow isotope ratio mass spectrometry (20/20 isotope analyzer; PDZ Europa Ltd., Cheshire, UK). Enrichment of CO₂ samples was expressed above background samples (APE).

Calculations

The rate of ¹³CO₂ released from Phe oxidation per kg of BW (F¹³CO₂, mmol.kg⁻¹.h⁻¹) was calculated using the following equation: F¹³CO₂ = (FCO₂)(ECO₂)(44.6)(60)/[(BW)(1.0)(100)], in which FCO₂ is the average production of CO₂ during the isotope steady state phase (mL/min); ECO₂ is the average ¹³CO₂ enrichment in expired breath at isotopic steady state (APE, %); and, BW is the weight of the dog (kg). The constants 44.6 (mmol/mL) and 60 (min/h) convert the FCO₂ to micromoles per hour;

Table 2. Feeding, isotope dosing, and sampling schedule on the day of each DAAO study

	Fasting state			Fed state															
Time, min	-75	-50	-25	0	10	20	45	70	95	120	145	170	195	220	245	270	295		
Food ¹				◆	◆	◆	◆	◆	◆	◆	◆	◆	◆	◆	◆	◆			
				Background						Isotope steady state phase									
L-[1- ¹³ C]-Phe ²									♣*	♣	♣	♣	♣	♣	♣	♣			
Samples																			
Indirect cal- orimetry ³		♠	♠	♠			♠	♠	♠										
Breath ⁴							♥	♥	♥	♥	♥	♥	♥	♥	♥	♥	♥		
Blood																	✓		

Symbols denote dogs receiving the respective procedure during that time period. DAAO = direct amino acid oxidation; Phe = phenylalanine.

¹The food offered to each dog consisted of a Phe-deficient test diet top-dressed with 1 of 7 isonitrogenous Phe-alanine solutions to deliver total dietary Phe concentrations of 0.24, 0.29, 0.34, 0.44, 0.54, 0.64, and 0.74% (as-fed basis). Each meal represented one-thirteenth of the daily feed intake for each dog.

²Priming dose of L-[1-¹³C]-Phe was started with the sixth meal (time: 95 min) followed by continuous L-[1-¹³C]-Phe supply through the remaining 200 min of the study. Both priming and continuous isotope dosing solutions were top-dressed on the dry kibble meal.

³Three 25-min measures of respiratory gases were obtained prior to feeding to calculate resting volume of CO₂ produced (VCO₂). Starting at 45 min, VCO₂ was measured in 25-min intervals for the duration of the study.

⁴Three 25-min breath collections for ¹³CO₂ background breath samples were obtained at 45, 70, 95 min (fed state) before the isotope dosing. Breath samples were then collected every 25 min for the duration of the study.

the factor 100 changes APE to a fraction; and, the 1.0 is the retention factor of CO₂ in the body due to bicarbonate fixation as reported previously (Shoveller et al., 2017). Resting and fed energy expenditure (EE) was calculated based on VO₂ and VCO₂ based on modified Weir equation (Weir, 1949). Energy expenditure (kcal/d) was expressed in relation to BW and metabolic BW (BW^{-0.75}) for all dog breed sizes.

Body Composition Determination

Lean body mass (LBM) was determined during the 7-wk study using an X-Ray Bone Densitometer (Model Delphi A, Hologic Inc., MA) on Beagles and Labradors dogs. Accurate prediction of LBM was not possible in Miniature Dachshunds due to their unique body conformation. During scans, anesthetized dogs were positioned in sternal recumbency with the cranial aspect of ante brachium placed on the table and phalanges pointing caudally. Body mass composition (i.e., mineral, fat, lean, and water contents) was determined in the left and right arms and legs, trunk, and head (data not shown). Whole body composition was determined by the sum of all regions measured on individual dogs.

Statistical Analysis

The effect of Phe content in the test diet on F¹³CO₂ was analyzed using PROC MIXED of SAS (v. 9.4; SAS Institute Inc., Cary, NC) with diet as a fixed effect and dog as a random effect. The estimate of the mean Phe requirement and the upper 95%

confidence intervals (CI) for individual dog breeds and pooling data from all breeds were derived by breakpoint analysis of the F¹³CO₂/kg BW using a two-phase linear regression model as previously reported (Shoveller et al., 2017). Differences in AA concentration in blood were determined using orthogonal contrast comparing each dietary Phe content against the lowest Phe diet (0.24%). The mean Phe requirements were also calculated with Phe concentration in serum data using the two-phase linear regression model. Within different breed sizes, BW, and the calorimetry data were analyzed using PROC MIXED of SAS (v. 9.4; SAS Institute Inc.) with diet as a fixed effect and dog as a random effect. The same data were also pooled per dog breed and were compared using breed as a fixed effect and dog as a random variable. Results were considered statistically significant at $P \leq 0.05$, and a trend when $P \leq 0.10$.

RESULTS

All animals remained healthy throughout the study. There were no differences in BW, resting EE (REE), and respiratory quotient (RQ) among dietary treatments ($P > 0.10$; Table 3). Body weight and LBM were different ($P \leq 0.05$) among all breeds (Table 4). Lean body mass as percentage of BW was higher for Beagles compared to Labradors ($P \leq 0.05$). Resting EE per unit of metabolic BW was higher for Beagles compared to Miniature Dachshunds ($P \leq 0.05$), but fed EE was not different among breeds ($P > 0.10$). Fasting RQ was higher ($P \leq 0.05$) for Miniature Dachshunds compared to

Table 3. Body weight, REE, and RQ for Miniature Dachshunds, Beagles, and Labrador Retrievers fed diets containing graded levels of Phe

	Dietary Phe, ¹ %							Pooled ANOVA	
	<u>0.24</u>	<u>0.29</u>	<u>0.34</u>	<u>0.44</u>	<u>0.54</u>	<u>0.64</u>	<u>0.74</u>	SEM ²	<i>P</i> value
	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4		
Miniature Dachshunds									
BW, kg	5.26	5.32	5.3	5.3	5.32	5.32	5.36	0.29	NS
REE, kcal/kg BW	42.3	39.6	39.6	39.3	43.4	41.0	40.2	3.5	NS
RQ	0.81	0.81	0.83	0.82	0.87	0.82	0.83	0.02	NS
Beagles									
BW, kg	7.98	8.03	8.05	8.04	7.99	8	7.97	0.42	NS
REE, kcal/kg BW	46.8	50.2	46.6	65.4	68.8	48.5	47.8	11.0	NS
RQ	0.77	0.76	0.76	0.76	0.76	0.76	0.76	0.01	NS
Labrador Retrievers									
BW, kg	35.0	34.9	35.2	35.2	35.2	35.0	35.1	1.1	NS
REE, kcal/kg BW	27.5	30.0	29.5	28.6	29.7	33.7	29.9	2.6	NS
RQ	0.85	0.85	0.85	0.85	0.85	0.83	0.85	0.01	NS

Phe = phenylalanine; REE = resting energy expenditure, RQ = respiratory quotient.

¹Phe content in the diet is in as-fed basis.

²SEM based on *n* = 4 for each level of Phe in the diet.

Table 4. Body weight, LBM, and indirect calorimetry data of different size dogs used in the present study

	Miniature Dachshunds	Beagles	Labrador Retrievers	Pooled ANOVA	
	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	SEM ¹	P value
BW, kg	5.63 ^c	8.04 ^b	35.0 ^a	1.06	<0.001
LBM, kg	—	6.68 ^b	26.14 ^a	0.26	<0.001
LBM, % BW	—	84.0 ^b	74.6 ^a	2.4	0.002
REE, Kcal/BW ^{0.75, 2}	61.9 ^b	91.2 ^a	72.7 ^{ab}	8.1	0.001
FEE, Kcal/BW ^{0.75}	103.6	101.9	99.5	18.3	0.968
Fasting RQ ²	0.83 ^a	0.76 ^b	0.77 ^b	0.02	<0.001
Fed RQ	0.85 ^b	0.87 ^a	0.85 ^b	0.01	<0.001
VCO ₂ , L/min	2.61 ^b	3.61 ^{ab}	10.40 ^a	0.57	<0.001
VO ₂ , L/min	3.05 ^b	4.11 ^{ab}	12.22 ^a	0.70	<0.001

¹SEM based on *n* = 4 for each dog breed.

²LBM = lean body mass, FEE = fasting energy expenditure, REE = resting energy expenditure, RQ = respiratory quotient, VCO₂ = resting volume of CO₂ produced, VO₂ = resting volume of O₂ produced.

other breeds, but fed RQ was higher for Beagles (*P* ≤ 0.05).

All dogs reached isotopic steady state at every intake of Phe (data not shown). Production of ¹³C (*F*¹³CO₂) from the oxidation of L-[1-¹³C]-Phe at the highest level of dietary Phe (0.74%) was removed from the model in Miniature Dachshunds as it was considerably lower than with previous Phe concentration. The breakpoint of the two-phase linear regression of *F*¹³CO₂, the mean Phe requirement, was at 0.262% Phe in the diet (as-fed basis) with an upper 95% CI of 0.358% (Fig. 1A). For Beagles, the mean Phe requirement was 0.258% of diet (as-fed basis) with an upper 95% CI of 0.365% of diet (as-fed basis; Fig. 1B). The oxidation of L-[1-¹³C] Phe (*F*¹³CO₂) for Labrador Retrievers resulted in a mean

Phe requirement of 0.304% of diet (as-fed basis) with an upper 95% CI of 0.463% (Fig. 1C). For ease of comparison with other studies, estimated 95% CI of Phe requirements are 41.9, 41.3, and 42.6 mg.kg⁻¹ BW.d⁻¹ for Miniature Dachshunds, Beagles, and Labrador Retrievers, respectively. When pooling data from all dogs, the mean Phe requirement was 0.263% with an upper 95% CI of 0.343% (Fig. 2A). When these data were expressed as Phe intake (mg/kg BW), to account for the different feed intakes among breeds, the mean Phe requirement in the two-phase linear regression was 45.3 mg/kg BW with an upper 95% CI of 55.4 mg/kg BW (Fig. 2B).

Concentrations of Phe and tyrosine (Tyr) in blood serum were not different among dietary

Figure 1. Production of $^{13}CO_2$ from the oxidation of orally administered L-[1- ^{13}C]-Phe in adult dogs fed diets with increasing levels of Phe. Miniature Dachshunds (A), Beagles (B), Labrador Retrievers (C). $n = 4$ at each level of dietary Phe. Dash lines represent mean Phe requirement; dotted lines represent 95% confidence interval. Phe = phenylalanine.

treatments in Miniature Dachshunds at any level of dietary Phe ($P > 0.10$; Table 5). Phe concentration was greater ($P \leq 0.05$) at the highest level of dietary Phe compared to the lowest level, and Tyr concentration increased ($P \leq 0.05$) in the last three highest levels of dietary Phe compared to the lowest dietary level of Phe in Beagles. In Labrador Retrievers, Phe in serum was greater at the highest three concentrations of dietary Phe ($P \leq 0.05$), and Tyr greater only at the highest level of dietary Phe ($P \leq 0.05$) when

Figure 2. Production of $^{13}CO_2$ from the oxidation of orally administered L-[1- ^{13}C]-Phe in adult dogs fed diets with increasing levels of dietary Phe (A) or Phe intake (B). Pooled data from Miniature Dachshunds, Beagles, and Labrador Retrievers. Dash lines represent mean Phe requirement; dotted lines represent 95% confidence interval. Phe = phenylalanine.

compared to the 0.24% Phe diet. When pooling data from all breeds, Phe in blood serum was greater at the highest three dietary Phe levels ($P \leq 0.05$) and Tyr greater only at the highest level of dietary Phe ($P \leq 0.05$). When using Phe concentration in serum for the two-phase model, the mean Phe requirement was 0.361% for Miniature Dachshunds; the corresponding upper 95% CI was 0.628%. No breakpoint was detected for Beagles or Labrador Retrievers (data increased linearly only; $P \leq 0.05$). The pooled data resulted in a breakpoint of 0.296% and 48.6 mg/kg BW with a 95% CI of 0.488% and 67.1 mg/kg BW when Phe concentration was expressed as percentage in the diet or intake, respectively (data not shown).

DISCUSSION

The purpose of the present study was to determine the Phe requirements in adult dogs of three different breeds using the DAAO technique. The estimated mean requirement of Phe of adult dogs

Table 5. Serum Phe and Tyr concentrations in Miniature Dachshunds, Beagles, Labrador Retrievers, and all dogs pooled fed diets containing increasing levels of Phe

Dietary Phe, %	0.24	0.29	0.34	0.44	0.54	0.64	0.74	SEM ¹
	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	<i>n</i> = 4	
Miniature Dachshunds								
Phe, μ M	114.6	110.0	95.9	115.8	143.0	147.5	137.1	15.4
Tyr, μ M	61.4	51.9	43.9	50.2	77.0	67.1	80.8	10.2
Beagles								
Phe, μ M	96.3	122.5	118.9	140.7	140.0	135.3	168.2*	16.1
Tyr, μ M	55.4	79.3	77.2	72.6	93.2*	106.7*	122.5*	10.4
Labrador Retrievers								
Phe, μ M	101.1	128.4	115.4	135.1	144.9*	142.3*	156.1*	13.1
Tyr, μ M	73.6	65.8	70.8	85.9	94.9	92.8	101.2*	9.6
	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12	<i>n</i> = 12	
All dogs (pooled data)								
Phe, μ M	101.1	128.3	115.4	135.1	144.9*	142.3*	156.1*	13.1
Tyr, μ M	73.6	65.8	70.8	85.9	94.9	92.8	101.2*	9.6

Phe = phenylalanine; Tyr = tyrosine.

¹SEM, *n* = 4 at each level of dietary Phe for Miniature Dachshunds, Beagles, and Labrador Retrievers; *n* = 12 at each level of dietary Phe for pooled data.

*Significantly different ($P \leq 0.05$) when compared to the lowest level of dietary Phe.

is not different among the different breeds of dogs studied. To account for variability in the population, we compare the estimated 95% CI with the MR recommended in [NRC \(2006\)](#). The upper 95% CI is similar to the currently recommended MR level by NRC in percentage basis (g/100g DM). However, [NRC \(2006\)](#) also presents AA requirement in mg/kg BW^{0.75} units to account for the diversity of phenotypes in dog breeds. As we have considered specific dog breeds, we expressed Phe requirements in mg/kg BW, and the NRC recommendations were modified using the average BW for each dog breed ([Table 6](#)). The estimated upper 95% CI was lower than MR recommended by [NRC \(2006\)](#) for Miniature Dachshunds and Beagles, but higher for Labrador Retrievers. Current recommendations made by NRC are based on extrapolated data from growing animals using mainly nitrogen balance or growth performance studies ([Zello et al., 1995](#); [NRC, 2006](#); [Elango et al., 2008](#)). Therefore, empirical data to correct or validate such recommendations are necessary. Moreover, there is a lack of information on AA requirements among different sizes of dogs. Indeed, [Shoveller et al. \(2017\)](#) recently determined Phe requirements in dogs using the DAAO technique, but only used dogs of medium size (~21 kg), not multiple breed sizes. It should be noted that in the present study only four observations per breed were used, and there was a high variability associated with the breakpoint analysis. Further studies are necessary to validate the requirements suggested herein. To our knowledge,

this is the first dose–response study exploring dietary Phe requirements in adult dogs of different breeds and/or sizes.

Nitrogen balance and growth performance measured at different levels of intake of the test AA are the most common techniques for determining AA requirements empirically ([Milner, 1979](#); [Morris, 2004](#)). [Milner et al. \(1984\)](#) is the only study that determined Phe requirements in dogs (immature Beagles) using different levels of dietary Phe. In that study, the Phe requirement was estimated at 51.8 mg/kg of BW for maximal nitrogen balance. However, to extrapolate these data for determining AA requirements in adult dogs are inaccurate. The nitrogen balance technique applied to mature dogs lacks sensitivity as the protein pool changes at minimal rates ([Moughan, 1995](#)). Therefore, estimated requirements drawn from studies using nitrogen balance or growth performance should be considered carefully. [Elango et al. \(2008\)](#) reported an increase, from 25 to 42 mg.kg BW⁻¹.d⁻¹, of Phe requirements in adult humans with the use of the DAAO when compared to nitrogen balance technique. [Shoveller et al. \(2017\)](#) have recently determined Phe requirements in adult dogs, and there was an increase of Phe requirement of 40% compared to [NRC \(2006\)](#) recommendations. In the present study, Phe requirements calculated by DAAO and AA concentration in blood are lower than those suggested in [NRC \(2006\)](#) for Miniature Dachshunds and Beagles. Moreover, when comparing the 95% CI for Labrador Retrievers to that suggested in [Shoveller et al. \(2017\)](#), our estimations are

Table 6. Dietary Phe requirements for adult dogs at maintenance as recommended by AAFCO, FEDIAF, NRC, and the present study

Units of Phe requirement	AAFCO ¹	FEDIAF ²	NRC ³		All dogs (pooled data)		Miniature Dachshunds		Beagles		Labrador Retrievers	
			MR	RA	MR	CI	MR	CI	MR	CI	MR	CI
g/100 g DM	0.44	0.54	0.36	0.45	0.263	0.343	0.262	0.358	0.258	0.365	0.304	0.463
g/Mcal ME	1.18	1.05	0.90	1.13	0.705	0.920	0.702	0.960	0.692	0.979	0.815	1.241
mg/kg BW	66.0	75.0			45.3	55.4	41.9	57.3 (77.9) ⁴	41.3	58.4 (71.3)	42.6	64.8 (49.3)

CI = confidence interval; MR = minimal requirement; Phe = phenylalanine; RA = recommended allowance.

¹Association of American Feed Control Officials Manual, 2010.

²European Pet Food Industry Federation Nutritional guidelines for complete and complementary pet food for cats and dogs, 2013.

³Nutrient requirements of dog and cats. [National Research Council, 2006](#).

⁴Values in parentheses represent NRC MR for Phe in adult dogs at maintenance converted from mg/kg BW^{0.75} to mg/kg BW using the average BW of individual breeds.

~25 % lower, suggesting that previous results could be overestimated. The test diets used in [Shoveller et al. \(2017\)](#) and the present experiment were formulated to be the same. The analyzed CP and AA content, however, was higher in the diet used by [Shoveller et al. \(2017\)](#). The higher content is likely the result of the innate variability in AA content in chicken meal ([Wang and Parsons, 1998](#)). The higher AA content in the diet used by [Shoveller et al. \(2017\)](#) may explain the higher requirement reported in their study. Higher protein content in the diet will enhance catabolic pathways ([Das and Waterlow, 1974](#)) falsely increasing ¹³CO₂ and the Phe requirements. Alternatively, in dogs and cats, the black-hair coat intensity can be affected by levels of Phe + Tyr in the diet ([Yu et al., 2001](#); [Anderson et al., 2002](#); [Biourge and Sergheraert, 2002](#)) and may also contribute to the difference in AA requirements between these two studies.

The statistical analysis of serum AA concentrations in the fed state from all breeds also supports the estimated requirements determined with F¹³CO₂ data. The high level of variation in serum AA among dietary treatments is likely due to the differences in food intake, but additionally physiological differences among dogs and dog breed in the absorption and metabolism of the aromatic AA. Theoretically, concentration of most essential AA in blood should remain low and constant at different levels of AA intake below the requirement; above the requirement, AA concentration in blood should increase proportionally. However, this data set is associated with more variability as no mean Phe requirement could be determined for Beagles and Labrador Retrievers, and the Phe requirement estimated for Miniature Dachshunds, using serum AA concentration, was higher and more variable than Phe requirements using the DAAO technique. Thus, only the Phe requirement estimated with

the whole data set ($n = 12$) should be considered, and determining Phe requirement for individual breeds using the DAAO technique is justified in the current study.

When using DAAO for determining AA requirements, it is essential that other metabolic responses are not affected by the different levels of the test AA intake ([Moehn et al., 2004](#)). In the present study, within breed sizes, REE and RQ were not altered by the different concentration of dietary Phe, validating the results obtained with the DAAO technique used. There were differences among breeds for the indirect calorimetry parameters as would be expected given breed differences. Differences in absolute LBM can be explained by differences in BW, but the percentage of LBM was higher for Beagles compared to Labrador Retrievers. The latter is of interest as the lean tissue is more metabolically active compared to fat tissue with respect to AA metabolism ([Patterson et al., 2002](#)); it should be considered, however, that all Beagles used in the present study were females and all Labradors were castrated males potentially confounding the differences in LBM relative to BW. The fasting state period started no sooner than 17 h post the last feeding, and the RQ values should represent fat oxidation among all breeds. However, the higher RQ for Miniature Dachshunds may indicate a preferential protein catabolism along with greater carbohydrate oxidation as energy sources during the fasting state resulting in a greater RQ. It is also possible carbohydrates reserves were not totally depleted during the 17-h fasting period. The latter could explain the small increment in RQ values in fasting vs. fed states in Miniature Dachshunds when compared to Beagles and Labrador Retrievers. Given the higher RQ during fasting and the smaller increase when fed, metabolism of macronutrients (carbohydrates, fat, and protein) in Miniature

Dachshunds differs compared to medium and large dog breeds. This may indicate potential variation in AA requirements among breeds for other AA different than Phe.

In conclusion, the estimates of the Phe requirement were not different among breeds studied for mean requirement. When Phe requirements are expressed in mg/kg BW, the 95% CI was lower to the MR for Phe recommended by [NRC \(2006\)](#), and suggestions from [AAFCO \(2010\)](#), and [FEDIAF \(2013\)](#). Although, in the present study, there were no differences in mean Phe requirements among breeds, RQ, and REE differ among dog breeds indicating different macronutrient utilization. Research determining other AA requirements among different breeds in adult dogs is warranted.

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Conflict of Interest. A.K.S. and L.F. were employees of the Procter & Gamble Co.; L.F. is now employed by Mars, Pet Care, A.G. is now employed by Simmons Pet Food, and A.K.S. is now faculty at the University of Guelph. W.D.M. has no conflicts of interest.

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