The effect of graded concentrations of dietary tryptophan on canine behavior in response to the approach of a familiar or unfamiliar individual

James R. Templeman, Gary M. Davenport, John P. Cant, Vern R. Osborne, and Anna-Kate Shoveller

Abstract

Tryptophan (Trp) is an indispensable dietary amino acid that supports the modulation of mood and behavior in mammalian species through its role in the serotonergic pathway. This study assessed the behavior patterns of 36 healthy, female adult mixed-breed hound dogs fed a control diet (tryptophan = 0.18% as-fed) or 1 of several experimental diets with graded concentrations of tryptophan (0.05%, 0.1%, and 0.15% of the total diet) supplemented on top of the 0.18% basal level. At baseline, and every 8 weeks throughout the 24-week period, behavioral parameters were evaluated for each dog in response to the approach of an individual familiar to the dogs and another individual who was unfamiliar to the dogs. Differences in behavior scores (activity, $P = 0.0197$; distance, $P = 0.0358$; confidence, $P < 0.0001$; and ear position, $P < 0.0001$) between the unfamiliar and familiar individuals supported the efficacy of the behavioral ethogram used. No consistent and significant differences in behavior were observed, however, among dogs fed the control diet and those fed an experimental diet with any level of tryptophan supplementation. Future research should consider the tryptophan-to-large-neutral-amino-acid ratio and not just tryptophan concentrations, seek to understand the variation in tryptophan requirements among breeds, and look to utilize additional markers of serotonin status.

Résumé

Le tryptophane (Trp) est un acide aminé alimentaire indispensable qui supporte la modulation de l'humeur et le comportement chez des espèces animales via son rôle dans le cycle sérotonergique. La présente étude a évalué les patrons de comportement de 36 chiennes adultes en santé de race mélangée de type chien de chasse nourries avec une diète contrôlée (tryptophane = 0,18 %, tel que nourri) ou 1 de plusieurs diètes expérimentales avec des concentrations graduées de tryptophane (0,05 %, 0,1 %, et 0,15 % de la diète totale) ajoutées en plus du niveau de base de 0,18 % de la diète. Au temps 0, et à toutes les 8 semaines pendant la période de 24 semaines de l'étude, les paramètres de comportement furent évalués pour chaque chien en réponse à l'approche d'un individu familier aux chiens et un autre individu qui n'était pas familier aux chiens. Les différences dans les pointages de comportement (activité, $P = 0,0197$; distance, $P = 0,0358$; confiance, $P < 0,0001$; et position des oreilles $P < 0,0001$) entre l'individu familier et le non-familier supporte l'efficacité de l'éthogramme de comportement utilisé. Aucune différence constante ou significative dans le comportement ne fut observée, toutefois, parmi les chiens nourris la diète témoin et ceux nourris avec une diète expérimentale quelque soit le niveau de Trp ajouté. Des recherches ultérieures devraient considérer le ratio tryptophan/gros acide aminé neutre et pas seulement les concentrations de tryptophane, essayer de comprendre la variation dans les besoins de tryptophane parmi les races, et voir à utiliser des marqueurs additionnels du statut de la sérotonine.

(Traduit par Docteur Serge Messier)

Introduction

Tryptophan (Trp) is an indispensable amino acid for domestic canines *(Canis lupus familiaris)*. It is recommended that dogs consume at least 1.1 gram of Trp per kilogram of dry matter food, assuming a dietary energy density of 4000 kcal of metabolizable energy (ME) per kilogram, to maintain basic health (1). Supplementing Trp in excess of this requirement is being investigated as a means of increasing production of serotonin, a cerebrospinal fluid neurotransmitter involved in regulating mood (2–5). Serotonin is synthesized in the brain by tryptophan hydroxylase, a saturable, rate-limiting enzyme (6,7). As this enzyme is only about 50% saturated under normal physiological conditions, it is likely that increased availability of the Trp substrate influences the serotonergic pathway and leads to greater synthesis of serotonin (8–11).

Tryptophan, and ultimately the serotonergic system, has been shown to influence behaviors related to anxiety, stress, fear, and aggression in a number of mammalian subjects (12–15). However, Trp competes with other large neutral amino acids (LNAAs), including tyrosine, phenylalanine, leucine, isoleucine, and valine, for transport across the blood-brain barrier. In most protein-containing foods, Trp has the lowest concentration of all the LNAAs in contrast to its

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Table I. Ingredients and chemical composition of nutritionally complete and balanced dry diet containing graded concentrations of supplemental tryptophan (Trp) at 0% (control), 0.05%, 0.1%, and 0.15%.

Item	Control	05Trp	10Trp	15Trp	
Ingredient composition, % (as-fed)					
Dried chicken protein	24.9	24.5	24.5	24.4	
Corn meal	20.9	20.9	20.8	20.7	
Grain sorghum	20.9	20.8	20.8	20.7	
Barley flour	11.4	11.5	11.7	11.8	
Fishmeal	6.9	6.9	6.9	6.9	
Chicken fat	4.7	4.8	4.8	4.8	
Beet pulp	3.4	3.4	3.4	3.4	
Chicken digest (flavor)	2.0	2.0	2.0	2.0	
Egg product	1.0	1.0	1.0	1.0	
Brewer's yeast	1.0	1.0	1.0	1.0	
Salt	0.50	0.50	0.50	0.50	
Vitamin premix	0.40	0.40	0.40	0.40	
Mineral premix	0.37	0.37	0.37	0.37	
Potassium chloride	0.39	0.39	0.39	0.39	
Choline chloride	0.23	0.23	0.23	0.23	
Dicalcium phosphate	0.23	0.21	0.20	0.19	
$_{DL}$-Methionine	0.02	0.02	0.02	0.02	
Tryptophan	—	0.05	0.10	0.15	
Chemical composition (as-fed)					
Dry matter (%)	91.4	92.9	92.7	91.9	
Crude protein (%)	27.6	27.8	27.5	27.2	
Acid hydrolyzed fat (%)	15.9	16.2	16.1	15.7	
Crude fiber (%)	1.9	2.1	2.3	2.3	
Ash (%)	6.5	6.8	6.8	6.6	
Gross energy (Cal/g)	4746	4825	4784	4706	
Calcium (%)	1.22	1.21	1.24	1.12	
Chloride (%)	0.697	0.879	0.875	0.875	
Copper (mg/kg)	21.2	21.9	22.3	22.5	
Iron (mg/kg)	380	371	374	366	
Magnesium (mg/kg)	938	956	957	939	
Manganese (mg/kg)	54.9	56.9	56.0	47.9	
Phosphorus (%)	0.95	0.97	0.99	0.91	
Potassium (%)	0.61	0.61	0.62	0.65	
Sodium (%)	0.379	0.377	0.375	0.394	
Zinc (mg/kg)	217	224	212	230	
Amino acids, % (as-fed)					AAFCO[X]
Tryptophan	**0.18**	**0.23**	**0.29**	**0.36**	**0.15**
Arginine	1.77	1.46	1.69	1.55	0.47
Histidine	1.23	0.68	0.48	2.06	0.17
Isoleucine	1.07	0.96	1.04	1.02	0.35
Leucine	1.98	1.79	2.06	2.00	0.63
Lysine	1.53	1.37	1.48	1.09	0.58
Phenylalanine	1.10	0.98	1.10	1.07	0.41
Theonine	1.09	0.93	1.12	1.01	0.44
Tyrosine	0.87	0.67	0.66	0.82	0.27
Valine	1.19	1.10	1.19	1.08	0.45
Cysteine	0.27	0.32	0.30	0.40	0.29
Methionine	0.44	0.37	0.46	0.53	0.30
Alanine	2.14	1.58	1.97	2.05	
Aspartate	2.03	1.90	2.18	2.00	

Table I. Ingredients and chemical composition of nutritionally complete and balanced dry diet containing graded concentrations of supplemental tryptophan (Trp) at 0% (control), 0.05%, 0.1%, and 0.15% *(continued)*.

Item	Control	05Trp	10Trp	15Trp
Glutamate	3.59	3.34	3.91	3.57
Glycine	2.03	1.79	2.14	1.80
Hydroxy-proline	0.58	0.50	0.62	0.51
Proline	1.80	1.46	1.60	1.63
Serine	1.34	1.01	1.21	1.11
Taurine	0.21	0.12	0.14	0.14
Total LNAA[Y]	6.21	5.5	6.05	5.99
Trp:LNAA ratio[Z]	0.0290:1	0.0418:1	0.0479:1	0.0601:1
Molar Trp/LNAA	0.0196	0.0282	0.0322	0.0408

LNAA — large neutral amino acids; 05Trp — supplemented with tryptophan at 0.05% of the total diet; 10Trp — supplemented with tryptophan at 0.1% of the total diet; 15Trp — supplemented with tryptophan at 0.15% of the total diet; MR — minimum requirements.

[X] AAFCO — Association of American Feed Control Officials; % as-fed based on 92% DM, for adult canine maintenance, presuming a caloric density of 4000 kcal ME/kg.

[Y] LNAA — sum of leucine, valine, isoleucine, phenylalanine, and tyrosine.

[Z] Result is based on calculated values for appropriate amino acids.

estimated dietary requirement (11,16). Therefore, in most cases, when the protein component of a diet is increased, it effectively lowers the Trp: LNAA ratio and the potential for Trp to be transported across the blood-brain barrier decreases. Indeed, high-protein dog foods are a current market trend that further escalates the need for research that links Trp intake, which increases serotonergic brain activity, with the modulation of mood and behavior in dogs. Such research is currently limited to observing the effects of supplementing dietary Trp in subjects predisposed to or already diagnosed with behavioral issues, such as aggression, hyperactivity, or anxiety (12,15,17).

The objective of this study was to investigate and evaluate changes to the behavior of healthy, socialized, adult mixed-breed hounds fed a nutritionally complete and balanced diet, formulated with graded concentrations of tryptophan [0.05% (05Trp), 0.1% (10Trp), and 0.15% (15Trp) of the total diet] over a 24-week period. Behavioral characteristics that were elicited by the dogs in response to a familiar approaching individual (FAI) or an unfamiliar approaching individual (UAI) were evaluated. We hypothesized that supplementing diets with Trp would improve some, but not all, canine behavioral scores and that the improvement would be most significant with the UAI groups and between the control diet and 15Trp diet. This study was designed as an extension of a larger project investigating the substitution of dried chicken protein for soybean meal in a nutritionally complete and balanced dried adult dog food (18).

Materials and methods

Animals, housing, and diet

All study protocols were designed and conducted according to guidelines specified in the Guide for the Care and Use of Laboratory Animals (19) and approved by the Institutional Animal Care and Use Committee of Procter and Gamble Pet Care.

Thirty-six domestic, spayed female, purpose-bred, mixed-breed hound dogs were used throughout the duration of this study. The dogs were an average 4.01 ± 1.56 years of age, with mean body weights of 12.56 ± 1.3 kg. All dogs were deemed healthy, based on veterinary diagnostic evaluations, and remained healthy throughout the study period (Research Animal Resources, University of Minnesota; data not shown). The dogs were individually housed in 2.23-m^2 indoor runs at the Sinclair Research Center in Colombia, Missouri and provided with drinking water *ad libitum*. All the dogs taking part in the study had been subjected to a strict socialization regimen since birth.

The ingredients and chemical composition of all the diets used in this study are listed in Table I. The control diet consisted of a dry, extruded diet formulated to be nutritionally complete and balanced for adult dogs (20). The control diet contained 0.18% Trp on an as-fed basis. The experimental diets were supplemented with graded concentrations of Trp (0.05%, 0.1%, or 0.15% of the total diet), on top of the 0.18% basal level. This resulted in final Trp concentrations of: 1.8, 2.3, and 3.6 g/kg, for the 05Trp, 10Trp and 15Trp diets, respectively. The Trp:LNAA ratios of the 05Trp, 10Trp, and 15Trp diets were 0.0418:1, 0.0479:1, and 0.0601:1, respectively. All diets were formulated to be isonitrogenous and isoenergetic in accordance with the energy and nutrient guidelines for adult dog food (20). All test diets contained supplemental $_{DL}$-methionine. No additional amino acids were supplemented with the exception of Trp (Evonik-Degussa, Evonik Industries, Essen, Germany), which was supplemented in all 3 experimental diets (05Trp, 10Trp, 15Trp). Nutrient content of each diet was determined before the start of the study using duplicates and procedures of the Association of Official Analytical Chemists (21) for dry matter (DM) (934.01), crude protein (CP) (990.03), amino acids (999.12), acid-hydrolyzed fat (954.02), fatty acids (969.33), fiber (962.09), starch (979.10), ash (942.05), phosphorus (964.06), and calcium (968.08). Dietary gross energy (GE) was

Table II. Amino acid intake from nutritionally complete and balanced dry diets containing graded concentrations of supplemental tryptophan (Trp) at 0% (control), 0.05%, 0.1%, and 0.15% fed to the dogs over 24 weeks.

Diet *(n)*	Control (n = 12)	05Trp (n = 8)	10Trp (n = 8)	15Trp (n = 8)	*P*-value	NRC RA
Total intake, g/day	1794.01 ± 85	1760.85 ± 104	1575.29 ± 104	1618.30 ± 104	NS	
AA, mg/kg (BW)/day[X]						
Tryptophan	**10.16 ± 0.67**	**13.99 ± 0.82**	**16.59 ± 0.82**	**19.80 ± 0.82**	**< 0.0001***	**23**
Arginine	102.37 ± 4.06	88.29 ± 5.00	96.38 ± 5.00	85.36 ± 5.00	0.0482*	58
Histidine	71.02 ± 3.27	40.71 ± 4.00	27.37 ± 4.00	113.43 ± 4.00	< 0.0001*	30
Isoleucine	61.89 ± 2.55	57.77 ± 3.12	59.08 ± 3.12	56.35 ± 3.12	NS	63
Leucine	114.15 ± 4.87	108.01 ± 6.00	117.30 ± 6.00	110.12 ± 6.00	NS	113
Lysine	88.45 ± 3.43	82.32 ± 4.20	84.57 ± 4.20	59.99 ± 4.20	< 0.0001*	58
Phenylalanine	63.22 ± 2.64	58.86 ± 3.24	62.67 ± 3.24	59.00 ± 3.24	NS	75
Theonine	62.88 ± 2.59	56.33 ± 3.18	63.76 ± 3.18	55.91 ± 3.18	NS	71
Valine	68.59 ± 2.83	66.34 ± 3.47	67.75 ± 3.47	59.66 ± 3.47	NS	81
Methionine	25.40 ± 1.13	22.31 ± 1.39	26.23 ± 1.39	29.22 ± 1.39	0.0129*	54
Tyrosine	52.72 ± 1.94	42.34 ± 2.35	47.17 ± 2.35	36.12 ± 2.35	NS	48
Total LNAA[Y]	370.73 ± 15.5	347.31 ± 19.0	370.56 ± 19.0	341.05 ± 19.0	NS	380
Trp:LNAA Ratio[Z]	0.0274:1	0.0403:1	0.0448:1	0.0581:1		

n — number of dogs per diet group; AA — amino acid; LNAA — large neutral amino acid; 05Trp — supplemented with tryptophan at 0.05% of the total diet; 10Trp — supplemented with tryptophan at 0.1% of the total diet; 15Trp — supplemented with tryptophan at 0.15% of the total diet; NS — not significant ($P > 0.05$); NRC — National Research Council; RA — recommended allowance.

[X] Milligram of amino acid per kilogram of canine body weight (BW) per day.

[Y] LNAA — sum of leucine, valine, isoleucine, phenylalanine, and tyrosine.

[Z] Result is based on calculated values for appropriate amino acids.

* Denotes statistical significance ($P < 0.05$).

determined using an IKA C-2000 automated bomb calorimeter (IKA Works, Wilmington, North Carolina, USA).

Treatment groups, feeding procedures, measurements, and collections

A baseline diet consisting of a nutritionally complete and balanced house diet used by the facility was fed to all dogs taking part in the study for 16 weeks preceding the experimental diet period. For the experimental diet period, the control diet group consisted of 12 dogs, while each of the Trp-supplemented diet groups consisted of 8 dogs. The test diets were fed for a 24-week period. Food was offered once daily at 0640 h. Any food left over from the previous meal was collected and weighed before the morning feeding. The daily allotment of food was based on historical data of the metabolizable energy requirements of each dog. Feed intake was measured daily and body weight was recorded weekly after a 12-hour overnight fast.

Behavioral observations

The behavioral parameters of the dogs were assessed by observing the approach of an individual the dogs were familiar with and an individual the dogs were unfamiliar with. Assessments were carried out in each dog's kennel, which consisted of a 2.23-m^2 indoor run. Behavioral patterns in response to the unfamiliar/familiar approaching individuals were recorded at weeks 0, 8, 16, and 24 between 0800 and 1200 h. All behavioral assessments were done by individuals who were blind to the diet consumed by each dog that they assessed. To ensure that the dogs had the same degree of familiarity to the approaching individuals throughout the study, the unfamiliar individual differed for each dog and at each evaluation, while the familiar individual was always the same. For the sake of uniformity, both the familiar and unfamiliar individuals were male for all dogs in all assessments.

Each assessment took about 1.5 min. For each assessment, an individual, whether familiar or unfamiliar, quietly entered the room, slowly approached the gate of the kennel without making eye contact with the dog, turned sideways to face the kennel, quietly entered the kennel while ignoring the dog, approached the dog, and reached towards the top of the dog's head as if to pat it. The approaching individual remained quiet and maintained steady movements throughout the entire procedure. The order in which the familiar and unfamiliar individuals were presented to the dogs was switched for each evaluation and the time interval between the assessments was the same for all dogs. Each dog was assessed at the same time within the 4-hour assessment period for each evaluation. On assessment days, exposure of people not a part of the study was avoided until after the assessment period had ended, (e.g., after 1200 h).

Two evaluators were trained to score behaviors using the behavioral ethogram provided in Supplementary Table I. These evaluators remained the same throughout the study. The evaluators live-scored each event and were situated in the same place for each assessment of a particular dog, remaining 2 kennel lengths behind the approaching individual at all times. The evaluators assigned the dogs a score from 1 to 10 for each of the 9 behavioral parameters observed during each 1.5 min assessment. Behavioral parameters and criteria for evaluating the dogs are provided in Supplementary Table II.

Table III. Behavior mean scores (pooled across the 24-week test period) of dogs consuming nutritionally complete and balanced dry diets containing graded concentrations of supplemental tryptophan (Trp) at 0% (control), 0.05%, 0.1%, and 0.15% (age + pen-position as covariates).

Behavior parameter	Approaching individual	Control (n = 12)	05Trp (n = 8)	10Trp (n = 8)	15Trp (n = 8)	Approaching individual P-value	Diet P-value	Diet* Approaching individual P-value
Activity level	U	6.2088	6.9490	7.0759	6.2991			
	F	7.1084	6.5632	7.352	6.8120			
	Pooled	6.6586[a,b]	6.7561[a,b,c]	7.2144[c]	6.5556[a,b]	0.0197*	0.0082*	0.0076*
Distance from individual	U	7.6729	7.1052	7.4211	7.4937			
	F	7.8150	7.4901	7.7827	8.1610			
	Pooled	7.7440	7.2976	7.6019	7.8274	0.0358*	0.2149	0.7698
Response to approach	U	7.1056	6.4472	7.4887	6.5201			
	F	7.1826	6.1472	6.7893	6.5452			
	Pooled	7.1441[a]	6.2972[b]	7.1390[a]	6.5327[b]	0.1201	< 0.0001*	0.1942
Confidence	U	4.5422	4.7615	4.6134	4.6733			
	F	5.8817	5.3416	6.0912	5.7258			
	Pooled	5.2119	5.0516	5.3523	5.1996	< 0.0001*	0.3727	0.0283*
Posture	U	8.7546	8.8331	8.8624	9.1028			
	F	9.1934	9.0033	9.4056	9.1429			
	Pooled	8.9740	8.9182	9.1340	9.1228	0.0917	0.8149	0.7341
Tail position	U	6.5398	6.3285	6.6001	6.3016			
	F	6.5953	6.1674	6.7437	5.9369			
	Pooled	6.5675[a]	6.2480[a,b]	6.6719[a]	6.1192[b]	0.5969	0.0408*	0.6609
Ear position	U	5.2265	6.0365	5.8325	5.6284			
	F	4.5926	4.8025	4.3904	4.5525			
	Pooled	4.9069[a]	5.4195[b]	5.1115[a,b]	5.0904[a,b]	< 0.0001*	0.0027*	0.0163*
Aggression	U	1.0000	1.0000	1.0000	1.0000			
	F	1.0000	1.0000	1.0000	1.0000			
	Pooled	1.0000	1.0000	1.0000	1.0000	—	—	—

U — unfamiliar; F — familiar; 05Trp — supplemented with tryptophan at 0.05% of the total diet; 10Trp — supplemented with tryptophan at 0.1% of the total diet; 15Trp — supplemented with tryptophan at 0.15% of the total diet; n — number of dogs fed each diet.

* Denotes statistical significance ($P < 0.05$) for approaching individual, diet, or diet*AI effects from pooled AI results.

[a,b,c] Denotes significant effect ($P < 0.05$) of diet (pooled UAI and FAI groups).

Statistical analysis

All data were analyzed using the mixed procedure of SAS Version 9.3 (SAS Institute, Cary, North Carolina, USA), assuming fixed effects of diets and random effects of dog. Results are reported as least square means ± SEM. Means were separated by Tukey-Kramer adjusted multiple comparison. Akaike's Information Criterion identified the best model for each analysis. Inter-observer reliability on behavioral scoring was conducted by calculating Kappa statistics using the FREQ procedure in SAS Version 9.3 (SAS Institute), comparing observation weeks within observer, and controlling for observed behavior. Statistical significance was declared at $P < 0.05$.

Body weight (BW) at time −2 wk (Y_{ijk}) was analyzed using the model:

$$Y_{ijk} = \mu + AI_i + Wk_j + AI \times Wk_{ij} + e_{ijk} \quad \textit{Equation 1}$$

where: μ is the overall mean; AI_i is the ith approaching individual effect (i = 0%, 0.05%, 0.1%, or 0.15% Trp supplementation); Wk_j is the repeated measures by week (j = 0, 8, 16, 24); $AI \times Wk_{ij}$ is the interaction between ith approaching individual and jth week; and e_{ijk} is the random error.

Food intake (Y_{ijk}) was analyzed with initial BW (in-BW_{ijk}) as a covariate:

$$Y_{jkl} = \mu + \beta \times inBW_{ijk} + AI_i + Wk_j + AI \times Wk_{ijk} + e_{ijk} \quad \textit{Equation 2}$$

where: μ is the overall mean; AI_i is the ith approaching individual effect (i = 0%, 0.05%, 0.1%, or 0.15% Trp supplementation); Wk_j is the repeated measures by week (j = 0, 8, 16, 24); $AI \times Wk_{ij}$ is the interaction between ith approaching individual and jth week; and e_{ijk} is the random error.

Behaviors (Y_{ijk}) were analyzed with age (A_{ijk}) and pen position (PP_{ijk}) as covariates:

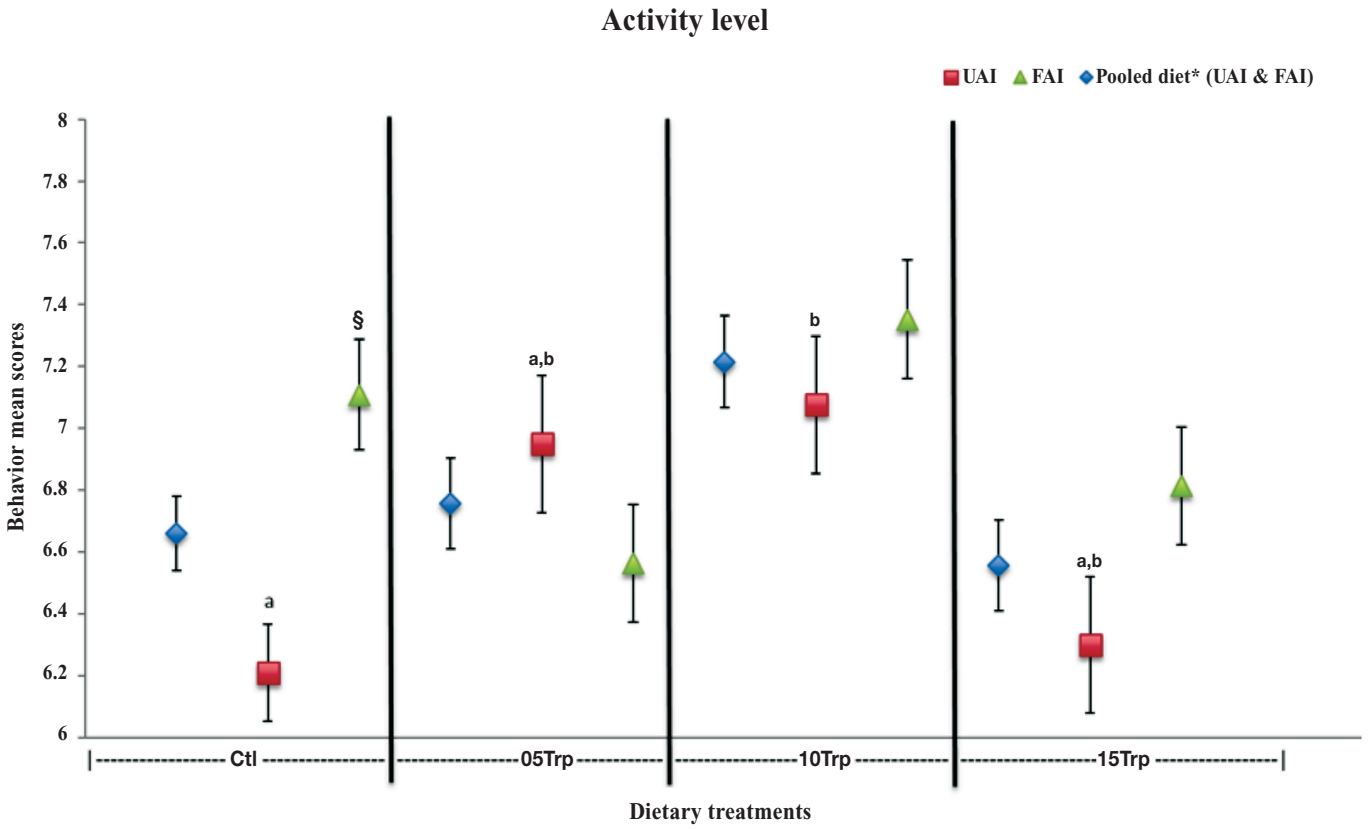

Figure 1. Mean activity level behavior scores of dogs consuming dry foods containing graded concentrations of supplemental tryptophan (Trp) at 0% (Ctl), 0.05%, 0.1%, and 0.15% pooled across the 24-week test period. Diet means (pooled UAI and FAI values; blue diamond marker), as well as discrete unfamiliar and familiar approaching individual group means (red square and green triangle markers, respectively), are depicted for each dietary treatment. Behavior score range for activity level: 1 = completely inactive; 10 = overly active.
UAI — unfamiliar approaching individual; FAI — familiar approaching individual; 05Trp — supplemented with tryptophan at 0.05% of the total diet; 10Trp — supplemented with tryptophan at 0.1% of the total diet; 15Trp — supplemented with tryptophan at 0.15% of the total diet; Ctl — control diet with 0% Trp supplemented.
*** Denotes significant effect ($P < 0.05$) for pooled diet effect (UAI and FAI).**
[a,b] Denotes significant effect ($P < 0.05$) of dietary treatment*UAI or FAI (diet*approaching individual effect).
§ Denotes difference between UAI and FAI groups within each specific dietary treatment.

$$Y_{jkl} = \mu + \beta \times A_{ijk} + [PP_{ijk}]\, AI_i + Wk_j + AI \times Wk_{ijk} + e_{ijk}$$

Equation 3

where: μ is the overall mean; AI_i is the ith approaching individual effect (i = 0%, 0.05%, 0.1%, or 0.15% Trp supplementation); Wk_j is the repeated measures by week (j = 0, 8, 16, 24); $AI \times Wk_{ij}$ is the interaction between ith approaching individual and jth week; and e_{ijk} is the random error.

Results

There was no effect of dietary treatment on body weight ($P = 0.8849$) or feed intake ($P = 0.3269$) throughout the 24-week study. For the extent of the experimental period, the average daily intake of the test diets was within 5% of the intake of the baseline diet for all dogs, regardless of their assigned test diet (data not shown). With no overall differences found in body weight, feed intake, and body composition among the 4 diets over the 24-week period (data not shown), we would not expect differences if the study was conducted for a longer period during the adult stage of life.

The approaching individual group (familiar *versus* unfamiliar) was significant ($P < 0.05$) for activity, distance, confidence, and ear position. All behavior scores are given in Table III. The scores for activity ($P = 0.0197$), distance ($P = 0.0358$), and confidence ($P < 0.0001$) were all greater in the FAI group. This indicated that the dogs were more active, closer to the evaluator, and more confident when in the presence of the familiar individual. The ear position score was greater with the UAI ($P < 0.0001$), which indicated that the ears were more erect and facing forward. The scores for response to approach, posture, and tail position were similar ($P > 0.05$) among the approaching individual groups. The average scores for these behaviors indicate that overall the dogs demonstrated amiable behavior (6.78 average response to approach), were in a standing position (9.03 average posture), and had a resting tail (6.4 average tail position).

Dietary treatment influenced ($P < 0.05$): activity, response to approach, and ear and tail position. Specifically, activity scores for the 10Trp diet were greater (increased activity) than for dogs fed the control and 15Trp diets ($P = 0.0259$ and 0.0079, respectively), yet neither of those 2 diets differed from one another or from the 05Trp diet ($P > 0.05$). Response to approach scores for dogs fed the 05Trp

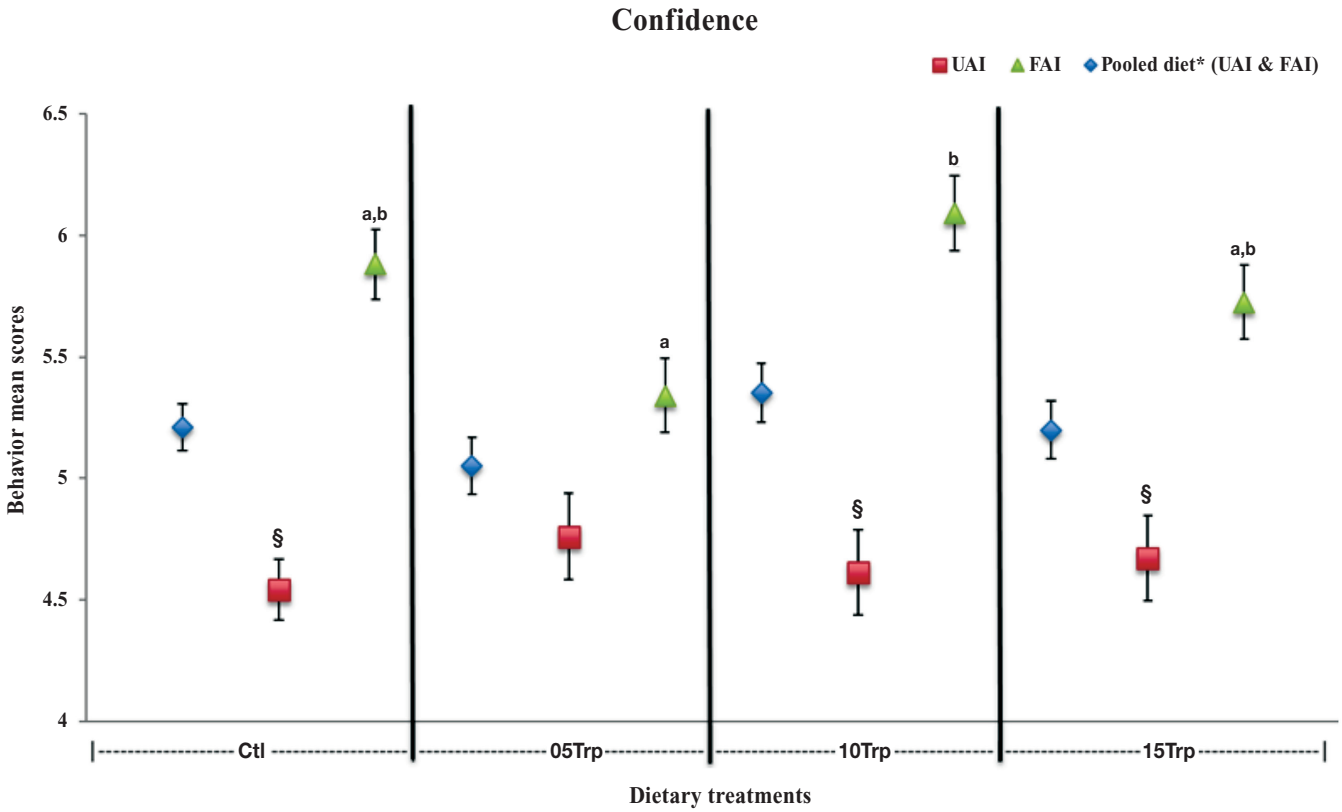

Figure 2. Mean confidence behavior scores of dogs consuming dry foods containing graded concentrations of supplemental tryptophan (Trp) at 0% (Ctl), 0.05%, 0.1%, and 0.15% pooled across the 24-week test period. Diet means (pooled UAI and FAI values; blue diamond marker), as well as discrete unfamiliar and familiar approaching individual group means (red square and green triangle markers, respectively), are depicted for each dietary treatment. Behavior score range for confidence: 1 = fearful; 10 = highly confident.

UAI — unfamiliar approaching individual; FAI — familiar approaching individual; 05Trp — supplemented with tryptophan at 0.05% of the total diet; 10Trp — supplemented with tryptophan at 0.1% of the total diet; 15Trp — supplemented with tryptophan at 0.15% of the total diet; Ctl — control diet with 0% Trp supplemented.

* Denotes significant effect ($P < 0.05$) for pooled diet effect (UAI and FAI).

[a,b] Denotes significant effect ($P < 0.05$) of dietary treatment*UAI or FAI (diet*approaching individual effect).

[§] Denotes difference between UAI and FAI groups within each specific dietary treatment.

and 15Trp diets were lower (more avoidant) than dogs fed the control diet ($P = 0.0001$ and 0.0129, respectively), but the 05Trp and 15Trp diets did not differ from one another ($P > 0.05$). Ear position scores for dogs fed the 05Trp diet were greater, indicating that their ears were more erect and engaged than dogs fed the control diet ($P = 0.001$), although neither of those diets differed from the 10Trp or 15Trp diets ($P > 0.05$).

The interaction effect of diet*approaching individual was significant ($P < 0.05$) for activity, confidence, and ear position (Figures 1–3). When approached by the unfamiliar individual, dogs fed the 10Trp diets were significantly more activity ($P = 0.0389$; Figure 1) than the dogs fed the control diet, but neither differed from dogs fed the 05Trp or 15Trp diets ($P > 0.05$). Although an overall increase in activity was noted in response to the FAI compared to the UAI group, there were no differences among dietary treatments or between any of the experimental diets and the control diet ($P > 0.05$). Dogs fed the 10Trp diet exhibited significantly more confidence-related behavior towards the familiar individual ($P = 0.0154$; Figure 2) than the dogs fed the 05Trp diet, although neither of those diet groups differed from the control or 15Trp diets ($P > 0.05$). No differences in confidence levels were observed among dogs in any of the dietary groups in response to the UAI ($P > 0.05$). When exposed to an UAI, dogs fed the 05Trp and 10Trp diets exhibited more erect and engaged ears ($P = 0.0009$ and 0.0482, respectively; Figure 3) than the dogs fed the control diet and neither diet group differed from the dogs fed the 15Trp diet. No differences in ear position ($P > 0.05$) were observed in response to exposure to the FAI.

Discussion

Under these experimental conditions, the Trp concentrations supplemented to an extruded adult dog food had little effect on the behavioral response to either familiar or unfamiliar individuals approaching a cohort of healthy, socialized, mixed-breed hounds. Differences were observed between the Trp-supplemented diets and the control diet (within the same approaching individual group) for a select number of behaviors assessed. The inconsistency and intermittent nature of these differences, along with the clear absence of any observable difference between the control diet and the diet with the highest graded Trp-supplementation (15Trp), however, prevents us from confidently concluding that the concentrations of Trp used in the present study affected the behavioral patterns of the canine cohort studied in response to the approach of a familiar or unfamiliar individual.

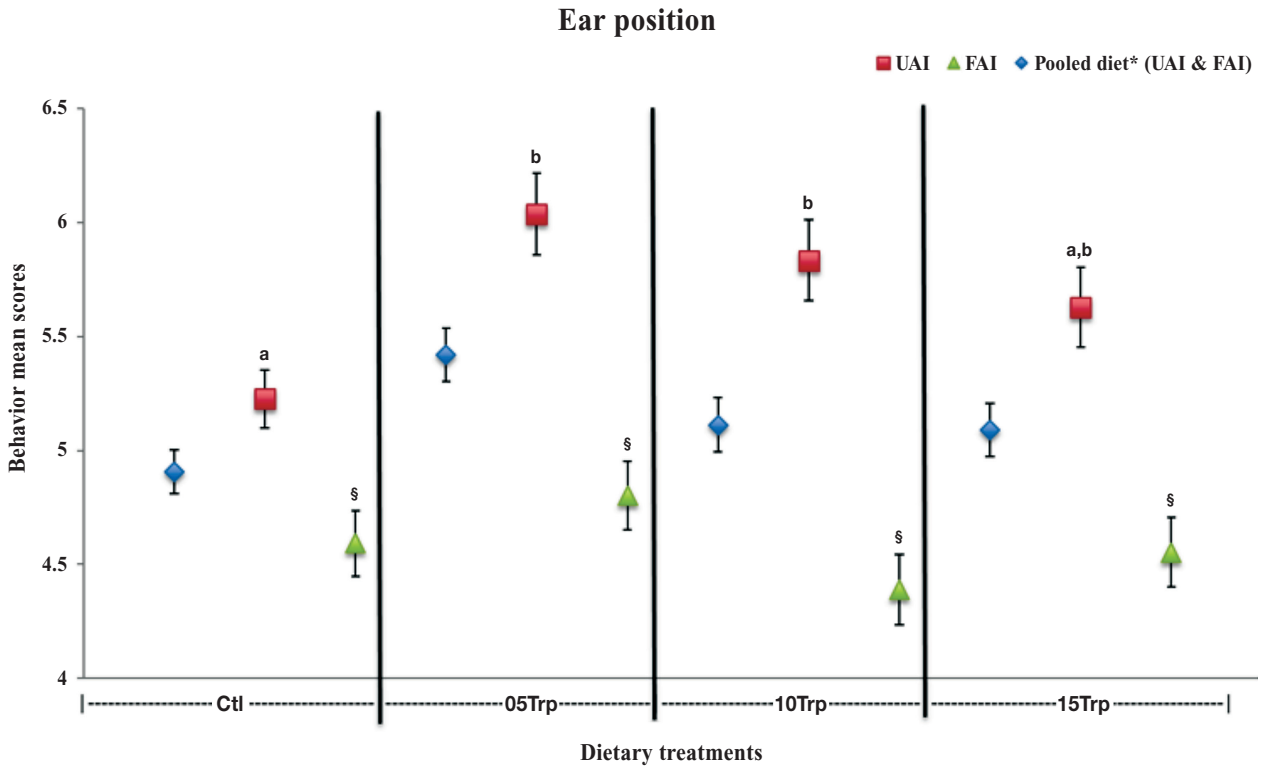

Figure 3. Mean ear position behavior scores of dogs consuming dry foods containing graded concentrations of supplemental tryptophan (Trp) at 0% (Ctl), 0.05%, 0.1%, and 0.15% pooled across the 24-week test period. Diet means (pooled UAI and FAI values; blue diamond marker), as well as discrete unfamiliar and familiar approaching individual group means (red square and green triangle markers, respectively), are depicted for each dietary treatment. Behavior score range for ear position: 1 = laid back; 5 to 6 = erect; 10 = forward.

UAI — unfamiliar approaching individual; FAI — familiar approaching individual; 05Trp — supplemented with tryptophan at 0.05% of the total diet; 10Trp — supplemented with tryptophan at 0.1% of the total diet; 15Trp — supplemented with tryptophan at 0.15% of the total diet; Ctl — control diet with 0% Trp supplemented.

*** Denotes significant effect ($P < 0.05$) for pooled diet effect (UAI and FAI).**

[a,b] Denotes significant effect ($P < 0.05$) of dietary treatment*UAI or FAI (diet*approaching individual effect).

[§] Denotes difference between UAI and FAI groups within each specific dietary treatment.

While the use of the Tukey-Kramer adjustment for multiple comparisons may be considered too conservative when evaluating behavioral data with the number of animals used, an observable dose response or consistency among significant differences between diets remained absent when implementing an alternative, more liberal multiple comparison procedure, e.g., pdiff. Differences ($P < 0.05$) in behavior scores for activity, distance, confidence, and ear position between the unfamiliar and familiar approaching individuals supported the efficacy of the behavioral ethogram used. However, as these differences were independent from the diet, this may suggest that the dogs' genetics, environment, and past experiences with familiar or unfamiliar individuals could act as a better predictor than their diet in regard to how they will react when approached.

Tryptophan was the only LNAA for which intake (mg/kg per day) differed among the different diets ($P < 0.0001$; Table II). While the Trp: LNAA ratios for the diets fed in this study were lower than the diet with the highest Trp: LNAA ratio in each of the studies previously discussed (11,17), these high ratios were achieved by supplementing Trp at the expense of crude protein. As the goal of this study was to apply the research to commercial diets, crude protein did not differ among the diets ($P > 0.05$). With consistent levels of crude protein, the Trp: LNAA ratios used for each of the dietary treatments still fell within the range of highest and lowest ratios used by DeNapoli et al (17).

When fed at the predicted nutrient requirements for adult dogs at maintenance, the suggested minimum requirements for Trp and the LNAAs translate to a minimum dietary Trp: LNAA ratio of 0.061:1 (1). Typical complete and balanced diets, such as the one used for this trial, are formulated to meet or exceed the minimum individual amino acid requirements, and not to specifically maintain the ratios at which the NRC-suggested minimum nutrient requirements generate. While all diets fell short of the 0.061: 1 Trp: LNAA ratio previously proposed (15Trp = 0.0581: 1 on an average daily intake basis; Table II), all diets fed exceeded the regulatory and scientific recommendations for Trp concentrations in the diet (AAFCO values; Table I). As such, this study is relevant to the current formulation approach of the dog food industry. Dogs may behave differently in response to the approach of a familiar or unfamiliar individual if the diets fed were formulated to the 0.061: 1 ratio (Trp: LNAA) rather than to individual requirements. This could be done by further elevating the Trp inclusion concentrations, decreasing the crude protein content of the diet, or combining both of those approaches.

Further research into the Trp: LNAA ratio is warranted. Future studies should aim to provide dietary Trp to exceed the suggested 0.061: 1 ratio.

More attention should also be paid to the animal's body weight and total dietary intake on a BW basis (Table II; NRC recommended allowance) because protein and amino acids are required on a total body protein basis. The Trp inclusion levels for the diets used in the present study exceeded the AAFCO minimums (Table I). Even the diet with the highest graded concentration of supplemented Trp (15Trp) fell short of the NRC-recommended allowance of Trp on a per-kilogram BW basis (Table II). Also, because the behavioral evaluation demonstrated the dogs' short-term response to an acute stressor, e.g., individual in the kennel for ~90 s, perhaps no level of Trp supplementation — even one that reaches or exceeds the 0.061: 1 Trp: LNAA ratio — would yield an effect. Future studies should investigate the dogs' behavioral patterns over longer periods of the day, in response to a longer-lasting situation/stressor, and/or in response to different situations, such as a social interaction test between dogs.

Every dog, in all diet and approaching individual groups and at every observation point throughout the study, was assigned a 1 for the aggression behavioral parameter, which suggests a complete absence of aggressive behavior in response to an approaching individual. The lack of statistical variation among the aggression scores indicates the dogs' degree of socialization, as well as their genetics, and implies that aggression-related behavioral parameters are negligible when investigating Trp-related effects on the behavior of a healthy, socialized cohort of mixed-breed hounds in response to an approaching individual. The results of this study combined with those of DeNapoli et al (17) could help confirm that the mitigation of aggression induced by dietary Trp supplementation may only be discernible in dogs previously diagnosed with, or predisposed to, aggressive behaviors.

Reisner et al (12) also found that nonaggressive (control) dogs had significantly higher concentrations of 5-Hydroxyindoleacetic acid (5-HIAA) than dominant-aggressive dogs. Indeed, 5-HIAA is a major serotonin metabolite found in cerebrospinal fluid and is an index of brain serotonin metabolism. After controlling for breed, gender, and age within the aggressive dog subgroup, there was still a difference in 5-HIAA between the aggressive and control dogs, which suggests that such differences are not related to any of these factors. Future studies should evaluate the 5-HIAA concentrations of dogs fed different intakes of Trp: LNAA.

The differences observed between the UAI and FAI for a number of behavioral parameters, e.g., activity, $P = 0.0197$; distance, $P = 0.0358$; confidence, $P < 0.0001$; and ear position, $P < 0.0001$, support the efficacy of the particular behavioral ethogram used in this study (Supplementary Table I). The method of evaluating behavioral changes in subjects in response to the approach of a familiar or unfamiliar individual was initially developed to assess attachment and separation anxiety between young children and their caregivers (Ainsworth's Strange Situation Procedure; 22,23), although it has since been adjusted to evaluate attachment behavior in dogs (24,25,26). The attachment theory suggests that, when an individual is with their attached person, they display increased confidence and exploratory behavior and desire to be close to the other individual (27,28). Certain aspects of this theory have been applied to, and are evident in, the relationship between dogs and familiar humans, even with dogs in a laboratory setting (25). These aspects were the basis for the investigation of UAI and FAI in the current study.

Research has shown that when dogs are approached by a familiar individual, such as their owner, as opposed to an unfamiliar individual, the presence of the familiar individual elicited significantly more exploratory activity (24,25,26), active play (23,26), and physical contact (24,26). In the present study, the higher scores in activity (Figure 1) and distance (higher distance scores mean less proximity to evaluator) with the familiar individual are similar to the increased exploration, physical contact, and active play observed in previous studies. These higher scores may indicate increased enthusiasm or excitement when the dogs are with someone they recognized as being familiar.

The closer proximity (higher distance score) and the increased confidence score with the approach of the familiar individual likely demonstrate the dogs' high degree of comfort when in the presence of a familiar individual (Figure 2). The increased fear-related behavior, decreased activity, and greater distance from the unfamiliar person may suggest a cautious approach when in the presence of an unfamiliar individual. These combined behaviors when presented with an unfamiliar individual may indicate a human-oriented neophobic response on the part of the dogs. The dogs may be using behavioral coping mechanisms in response to a fearful reaction to the approach of an unfamiliar person. These observations align with what was seen by Rehn et al (25) as their research dogs showed a significant preference to greet, be nearer to, and be in physical contact with the familiar individual than with a stranger. As well, the higher scores for ear position (more engaged ears; Figure 3) observed with the approach of the UAI aligns with the increased alertness observed by Palmer and Custance (24) with dogs in response to the entrance of an unfamiliar individual, which suggests an increase in wariness or circumspection when approached by someone unfamiliar to them.

While observable differences between level of activity in response to the approach of a familiar or unfamiliar individual lend insight into how inactivity may play a role in dogs reacting to a novel interaction, an opportunity for additional and potentially meaningful data collection was missed in this study. For subsequent studies, discrete recordings of activity level should be taken so that activity in response to the approach of an individual could be differentiated from activity elicited after the individual has departed, e.g., the rate of recovery to baseline activity levels. This may then be used to assess a dog's human-oriented fearfulness, although this method of behavior evaluation could be used for all observed parameters. The rate of recovery to baseline behavior may prove to be more modifiable by Trp supplementation than the behavioral responses to the initial stressor or situation.

In the future, the use of tools to quantify physiological indicators is warranted as a way of supporting the accuracy of behavioral ethograms. While some devices, such as heart rate monitors, are being used in similar studies to support the observable behavioral changes (25), devices such as ActiCal monitors (Bio-Lynx Scientific Equipment, Montreal, Quebec), internal telemetry instruments (Data Sciences International, New Brighton, Minnesota, USA), or cortisol/glucose monitors, may allow changes in behavior to be more precisely categorized. Also, the genotype and phenotype of the dogs

studied, as well as the interaction between the genotype and the environment, should be considered to identify aggressive dog breeds or those with behavior problems and facilitate their management throughout their lifetime. Finally, in an attempt to demonstrate a more significant behavior or behavior-by-treatment interaction effect, future studies should aim to either increase the sample size of dogs evaluated or improve the ways by which dogs that are phenotypically and genetically similar are allocated into treatment groups. For example, it would be advantageous to record baseline behavioral measurements that could be used as an additional parameter when allocating dogs to treatment groups. As well, an experimental design could be implemented, e.g., cross-over or Latin square design, in which each dog is used as its own control. While this may reduce statistical variation it would greatly extend the trial period.

In conclusion, within this cohort of healthy, socialized, mixed-breed hounds, none of the Trp-supplemented diets elicited a consistent or significant enough observable response to conclude that the Trp concentrations used in the present study resulted in an improvement in behavior associated with approaching individuals when compared with a nutritionally complete and balanced control diet that met AAFCO guidelines. The results did, however, confirm differences in a number of behavioral parameters when dogs were approached by familiar or unfamiliar individuals, which supports the effectiveness of the behavioral ethogram method used in laboratory dogs. Future studies should further increase the dietary concentration of Trp to reach the optimal Trp: LNAA ratio of 0.061:1 with the aim of improving aspects of behavior related to the approach of a familiar or unfamiliar individual in healthy, socialized or unsocialized dogs.

Supplementary Table I. Behavioral ethogram for assessing dogs consuming a complete and balanced dry diet containing graded concentrations of supplemental tryptophan (Trp) at 0% (control), 0.05%, 0.1%, and 0.15%.

	Score assigned									
Parameter	1	2	3	4	5	6	7	8	9	10
Pen position (pre-entry)										
Activity level										
Distance from individual										
Response to approach										
Confidence										
Posture										
Tail position										
Ear position										
Aggression										

Supplementary Table II. Descriptions of behavioral parameters and criteria for evaluating dogs consuming a complete and balanced dry diet containing graded concentrations of supplemental tryptophan (Trp) at 0% (control), 0.05%, 0.1%, and 0.15%.

Behavioral parameter	Description of behavior
Pen position (evaluation of pre-kennel-entry period)	The dog's position relative to the kennel gate (at the front of the pen) in response to an individual (familiar/unfamiliar) approaching its kennel gate. 1 signifies a dog located as far at the back of the pen as possible and 10 signifies a dog jumping at the kennel gate.
Activity level (evaluation of post-kennel-entry period)	The dog's level of activity or excitability in response to an individual (familiar/unfamiliar) entering its kennel. 1 signifies a dog remaining completely inactive and 10 signifies a dog that is uncontrollably/overly active, e.g., excessive pacing/circling.
Distance from approaching individual (evaluation of post-kennel-entry period)	The dog's position relative to an individual (familiar/unfamiliar) entering its kennel. 1 signifies a dog that is the maximal distance from the individual and 10 signifies a dog that is as close to the individual as possible, e.g., physical contact with or jumping on the individual.
Response to approach (evaluation of post-kennel-entry period)	The apparent attitude of the dog in response to an individual (familiar/unfamiliar) entering its kennel. 1 signifies a dog demonstrating avoidance-related behaviors, e.g., unresponsive upon entry, ears and tail low, lack of eye contact, refusal of interaction, and 10 signifies a dog demonstrating affiliative and affectionate behavior, e.g., actively wagging tail, relaxed/open mouth, vigorous gait, may vocalize, ears forward, seeking interaction.
Confidence (evaluation of post-kennel-entry period)	The degree of confidence-related behavior the dog directs towards an individual (familiar/unfamiliar) entering its kennel. 1 signifies a dog demonstrating human-oriented fearfulness, e.g., cowering, low posture, tail tucked or depressed, maintaining eye contact but avoiding close proximity, and 10 signifies highly confident behavior, e.g., front-sided and direct approach, resting tail, ears forward, close visual, olfactory, and/or gentle oral inspection.
Posture (evaluation of post-kennel-entry period)	The dog's posture or stance in response to an individual (familiar/unfamiliar) entering its kennel. 1 signifies a dog that is lying down on its side, 5 signifies a dog crawling sternally, and 10 signifies a dog actively walking or moving.
Tail position (evaluation of post-kennel-entry period)	The position of the dog's tail in response to an individual (familiar/unfamiliar) entering its kennel. 1 signifies the dog's tail is tightly tucked between its legs, 5 signifies a resting tail, and 10 signifies a tail that is held high and actively wagging.
Ear position (evaluation of post-kennel-entry period)	The position of the dog's ears in response to an individual (familiar/unfamiliar) entering its kennel. 1 signifies ears that are completely laid back and 10 signifies ears that are fully erect and facing forward.
Aggression (evaluation of post-kennel-entry period)	The degree of aggressive behavior a dog directs towards an individual (familiar/unfamiliar) entering its kennel. 1 signifies a complete absence of aggressive behavior and 10 signifies extreme aggression, e.g., hackles raised, lips curled, growling, stiffened posture, lunging, snapping, and attempting to bite.

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