



Original Research Article

Increased dietary intake of tyrosine upregulates melanin deposition in the hair of adult black-coated dogs



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ABSTRACT

The principle determinant of melanin derived hair colour and patterning in mammals is genetic, but environmental factors are now thought to play a role. It has been shown that the concentration of melanins in cat hair is influenced by the amino acid composition of their diets. Also, puppies were found to require tyrosine (Tyr) intake significantly greater than that recommended for normal growth and development in order to optimize melanin expression in their coats. Much of the work to date has been conducted in growing animals. Less is known about the relationship between nutrition and hair melanin deposition in healthy adult animals. In this study, we fed 2 groups of adult black Labrador retrievers (12 dogs/group) different concentrations of Phe + Tyr (5.6 vs. 3.5 g/Mcal) for 24 weeks and used spectrophotometric measurements every 8 weeks to detect any associated changes in the dogs' hair colour. The higher intake dogs showed reduced dilution of their black coat pigment compared with the lower intake dogs. Specifically, following 16 weeks at the higher intake, the dogs showed less yellow pigmentation to their coats ($P = 0.0032$), and after 24 weeks at the higher intake, the dogs showed less red ($P < 0.0001$) and yellow ($P < 0.0001$), as well as greater overall dark pigmentation ($P < 0.0001$). In conclusion, we have demonstrated for the first time that colour expression in the hair-coat of adult dogs is dependent on dietary intake of Tyr, and that the requirement appears to be in excess of the minimum level recommended to maintain health.

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1. Introduction

Hair colouration in mammals depends on the deposition of melanins in the hair shaft as it develops within the follicle (Ortonne and Prota, 1993). Follicular melanocytes produce black eumelanin and brown pheomelanin at differential concentrations, a process principally controlled by genetic factors, resulting in the myriad of colours and patterns seen in the natural world (Ozeki et al., 1995).

Ito (1993) showed that the relative proportion of eumelanin and pheomelanin was controlled by activity of the enzyme tyrosinase. Lower levels of activity favour production of pheomelanin, while higher levels favour eumelanin. What is more, tyrosinase activity is stimulated by tyrosine (Tyr) concentration, with an increase leading to greater eumelanin production, as shown by Slominski (1989). Tyrosine can come directly from the diet, or from the hydroxylation of phenylalanine (Schallreuter et al., 2008). Therefore, both amino acids are relevant in terms of dietary intake.

Generating an entirely black hair coat, as is seen in a number of canine breeds, requires virtually exclusive deposition of eumelanin. It is commonly observed however, that such an ostensibly black coat can be affected by an 'off-black' dilution, often resulting in a red hue to the hair (Yu et al., 2001). The reasons for this are not completely understood, although environmental factors undoubtedly contribute. For example, light, particularly sunlight, moulting, seasonal changes and even some cleaning products are associated

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with colour dilution. Probably the most recognised non-genetic influence on coat colour, however, is nutrition.

A number of publications have highlighted the nutritional connection, with much of the work focused on feline and canine models. For example, in cats, a telling discovery was made that the intake of the aromatic amino acids Tyr and phenylalanine (Phe) required for normal, healthy growth and development was insufficient to support maximal expression of melanins in the hair (Morris et al., 2002; Anderson et al., 2002). These studies have illustrated the importance of adequate Phe and Tyr intake for the biosynthetic processes responsible for melanin synthesis. The group also demonstrated a correlation between blood Tyr concentration and the amount of melanin found in the hair of cats. Similar work conducted in puppies showed a comparable phenomenon was present here also; Watson et al. (2015) demonstrated that, in order to enrich black pigmentation in growing Labrador retrievers and Newfoundlands, it was necessary to increase the Phe and Tyr concentration from the previously predicted requirement >3 months of 2.5 to 5.8 g/MCal. The pigmentation could be further increased when Phe + Tyr intake was raised to 7 g/MCal.

What has not been investigated to date, however, is the influence of nutrition on the melanin deposition, and associated pigmentation, in the hair of adult dogs. The current FEDIAF/NRC recommendation for Phe + Tyr in adult dogs is 2.23 g/Mcal. Here we demonstrate the value of ensuring that minimum combined concentrations of Phe + Tyr, defined here as 5.6 g/Mcal, is included in the diet to augment pigmentation in black dogs.

2. Materials and methods

2.1. Animals

Twenty four sterilized adult black Labrador retrievers were divided into 2 groups of 12, matched as far as possible for age and sex. The number of dogs used was defined by reference to a previous study using similar study parameters (Watson et al., 2017). Group A consisted of 5 males, and group B consisted of 6 males. Age range for group A was 1.1 to 8.3 years, mean 4.2; age range for group B was 1.1 to 8.2 years, mean 3.7. In addition to these criteria, the dogs' coats were categorized as straight or wavy. As the physical form of the hair could influence the subjective scoring, the groups were balanced 6 straight and 6 wavy hair per group. As such, the study design was double blinded parallel study design with stratified randomization for sex, age and straight vs. curly hair. Dogs on this study were housed in purpose-built, environmentally-enriched housing at the Pet Health Nutrition Center, Lewisburg, OH, USA, for the duration of the study. All kennelling, care and procedures were in keeping with the requirements of the Animals (Scientific Procedures) Act 1986. The study was approved by both the Royal Canin Ethical Committee and the Institutional Animal Care and Use Committees. The study was performed blinded: animals groups were selected according to predefined criteria and then assigned randomly to the supplemented diet (diet A) or baseline diet (diet B). All experimental and dog care staff as well as the subjective assessors were then blinded to which animals were fed.

2.2. Feeding study

Summary analytical results of the diets fed to the 2 groups can be found in Table 1. Control of protein composition in the 2 diets was achieved by alteration of poultry content. The quantity was reduced by 1% of total composition in diet A to allow for Tyr (2.1 g/Mcal) to be added. Dogs were fed twice daily according to

Table 1

Dietary analysis information for study diets (quantities are in g/Mcal unless otherwise stated).

Dietary constituent	Diet A	Diet B
Aspartic acid	2.75	2.79
Threonine	1.43	1.45
Serine	1.87	1.89
Glutamic acid	5.63	6.30
Hydroxyproline	0.77	0.86
Proline	2.90	3.06
Glycine	2.93	3.08
Alanine	2.42	2.51
Cystine	0.75	0.64
Valine	1.78	1.87
Methionine	0.81	1.15
Isoleucine	1.34	1.37
Leucine	2.95	3.08
Tyrosine	3.96	1.84
Phenylalanine	1.60	1.61
Histidine	0.75	0.79
Hydroxylysine	0.09	0.07
Lysine	2.64	2.55
Arginine	2.20	2.29
Copper, mg/kg	11	11
Ash, wt%	5.9	5.8
Crude fibre, wt%	2	2.3
Fat, wt%	15.1	14.8
Protein, wt%	18.1	18.5
Total dietary fibre, wt%	7	6.3

rations based on standard feed intake guidelines (110 kcal/kg^{0.75}). Intake was recalculated weekly based on current weight combined with weight change in the previous week. Group A were fed exclusively the test diet with supplemented Phe + Tyr; group B were fed exclusively the control diet. Both groups were fed their respective diets for 6 months (24 weeks). Information regarding the consumption of the daily rations was captured by the dog care staff at each colour assessment point (Appendix A). All dogs were allowed fresh water *ad libitum* throughout the study.

2.3. Assessments of coat colour

Colour assessment of the dogs was undertaken with a hand held spectrophotometer (Spectro-guide 45/0, gloss, BYK Gardner, Brant Industries, Germany) according to the CIE Lab colour measurement system (Hunter Associates Laboratory, Inc. Reston, VA, USA). Under this system, colour is quantified using values for a* (red to green axis, 100 to -100) and b* (yellow to blue axis 100 to -100). Light to dark is determined as L* on a separate numerical scale (from black = 0 to white = 100). Measurements were taken at the start of the study and then following 2, 4 and 6 months. Five measurements were performed at 4 distinct body sites at each time point (site 1, left shoulder; site 2, right shoulder; site 3, withers; site 4, rump). In parallel, 5 assessors were requested to complete a subjective study questionnaire at each sampling time-point (for questionnaire details see Appendix A). The questionnaire was primarily designed to determine the opinion of the assessor regarding coat colour/pigmentation during the study. Each assessor viewed and scored a dog under standardised lighting conditions, without sharing opinions with fellow assessors. An arbitrary scoring scale of 1 to 10 was used to generate the subjective measures, 1 representing 'very poor' and 10 'perfect'.

2.4. Blood Analysis

Plasma amino acid analyses were performed to coincide with coat colour spectrophotometry. Blood samples (2 mL) were taken

after overnight fasting first thing in the morning and collected into heparinized tubes and spun at $2,000 \times g$ for 15 min at 4°C . The plasma was carefully removed from the pellet, leaving a small amount behind to reduce risk of contamination. For deproteination, 200 μL homogenized plasma was pipetted into pre-labelled 1.5 μL Eppendorf centrifuge vial, followed by addition of 6% sulfosalicylic acid (containing 200 nmol/mL norleucine) in a 1:1 (vol/vol) ratio. The closed vial was immediately vortexed for 3×10 s to ensure complete mixing. After 15 min, the sample was centrifuged at $16,000 \times g$ for 15 min. Supernatant (200 μL) was pipetted into a 400- μL glass insert (National C4011-631) in a 2-mL auto sampler vial (Fisher 03-391-16). The vials were then kept in the refrigerated auto sampler for sample loading. A Biochrom 30 amino acid analyser with biological fluid program was used for analysis. Separation was done by ion exchange column and quantification by post column ninhydrin colour metric method. A 25- μL supernatant was loaded through the auto sampler. Calibration standards (A6407-acids and neutral, and A6282-basics) and internal standard (norleucine) were purchased from Sigma. Variances of major amino acids between duplicates were $<5\%$.

For plasma copper and iron analysis, 0.5 mL of sample was placed in a test tube to which 4 mL of a protein precipitating internal standard solution (trichloroacetic acid, hydroxylamine sulphate, HCl, with 0.001% [wt/vol] yttrium) was added. After mixing the sample was centrifuged ($20,000 \times g$ at 4°C for 15 min) to produce a clear protein-free supernatant. The copper and iron concentration of the supernatant was analysed by inductively coupled plasma–optical emission spectroscopy using a Thermo-Fisher iCAP 6500 Radial ICP-OES.

2.5. Statistics

Statistical analyses were performed with the JMP version 12 and the SAS version 9.3 software (SAS Institute Inc., Cary, NC, USA). Linear mixed models were used to assess the influence of diet and time with the respected interaction on hair colour evolution (L^* , a^* , b^*). As data of each site of measurement were nested within naturally occurring hierarchies (sites within dog), site of measurement variable nested within dog written as site (dog) was defined as a random term. Attention was particularly paid on normality and homoscedasticity of residuals. Rank transformation of data was performed if required. Moreover, the studentized maximum modulus adjustment (Dunnnett, 1980) for post hoc analysis was used as appropriate, in the presence of heteroscedasticity. Plasma Amino acid and questionnaire coat scores were analysed using repeated measures one way ANOVA with post-hoc Tukey's Test. Significance was set at $P < 0.05$.

3. Results

3.1. Animals and diets

All dogs ate their full food rations throughout the study. No significant health issues which could impact the study were reported for either group. Average body weights at the start of the study were 25.6 kg (SD \pm 4.1) for group A and 24.5 kg (SD \pm 3.7) for group B. These did not change significantly during the study and there was no variation between the 2 groups. Dietary analysis showed that, with the exception of Tyr, most amino acids were slightly reduced in diet A due to the reduced quantity of poultry included. Exceptions included lysine (+hydroxylysine) and cysteine, which were also slightly increased in diet A. Methionine and glutamate were the 2 amino acids most noticeably elevated in diet B versus diet A.

3.2. Spectrophotometry

The 2 groups of dogs showed no significant difference between any of the 3 measurement parameters, L^* , a^* and b^* , at the start of the study. Following 16 weeks, the Labradors fed the Tyr supplemented diet A showed a significant reduction in the b^* (yellow-blue) parameter ($P = 0.0032$) relative to those fed diet B (Fig. 1). At this time-point, there was no difference detected for the a^* red-green colour axis nor in the L^* light–dark axis. Following 24 weeks of feeding diet A, there was a significant reduction in both the a^* (reduction in red pigment; $P < 0.0001$) and b^* (reduction in yellow pigment; $P < 0.0001$) axes relative to dogs fed diet B. At this time point there was also a significant reduction in the L^* parameter in group A versus B, indicating a darker pigmentation of the hair coat with the higher Tyr diet ($P < 0.0001$, Fig. 1C). A such diet interacted highly significantly with time-point for L^* , a^* and b^* , but the kinetics of diet effect through time were different for b^* (earliest sensitivity to diet effect at week 16) and a^* and L^* (diet effect visible from week 24).

3.3. Blood analyses

Analysis of plasma amino acid concentrations indicated no differences between the 2 groups at the start of the study. A significant increase in the level of circulating Tyr was then observed following 8 weeks of feeding the supplemented diet A ($P < 0.01$, Fig. 2A). The difference was maintained for the 16- and 24-week sample points, although the initial increase remained stable through these time-points. When the unsupplemented diet B was fed there was a significant ($P < 0.01$) reduction in plasma Tyr concentration following 8 weeks, and once again this change was maintained at a similar level for the duration of the study. Plasma Phe concentration followed an opposite pattern to Tyr, the level decreasing over the first 8 weeks and then stabilising for dogs on diet A and doing the same for the diet B group of dogs. Cysteine and Met were analysed in the same way (Fig. 2B). There were no significant changes in plasma concentration detected for Cys at any of the time points. Plasma Met however was found to be increased in group B, probably explained by the fact that Met was also one of the amino acids most noticeably elevated in diet B versus diet A. Plasma concentrations of copper and iron were also determined throughout the study and were not found to vary between the groups at any time point nor change significantly during the feeding period.

3.4. Subjective assessment of hair colour

Responses to the questionnaire indicated that the diets as fed were well received by the animals with no issues or refusals reported during the study. Responses to the subjective coat colour assessment (Fig. 3) indicated no significant difference in scores between group A and group B dogs at the beginning of the study ($P > 0.5$). Subsequent to this, for group A there was an increase at lower significance for scores at 8 weeks (8 wk $>$ 0 wk; $P < 0.05$), but a more significant difference was demonstrated by 16 weeks, which was then sustained until the end of the study at 24 weeks ($P < 0.01$). Moreover, there was a significant further increase in scores between 8 weeks and those at 16 and 24 weeks ($P < 0.01$). In group B, there was a difference discerned between baseline and 16 weeks ($P < 0.01$), but this was not sustained up to 24 weeks scoring. There was no evidence of scores changing between the other time-points for group B.

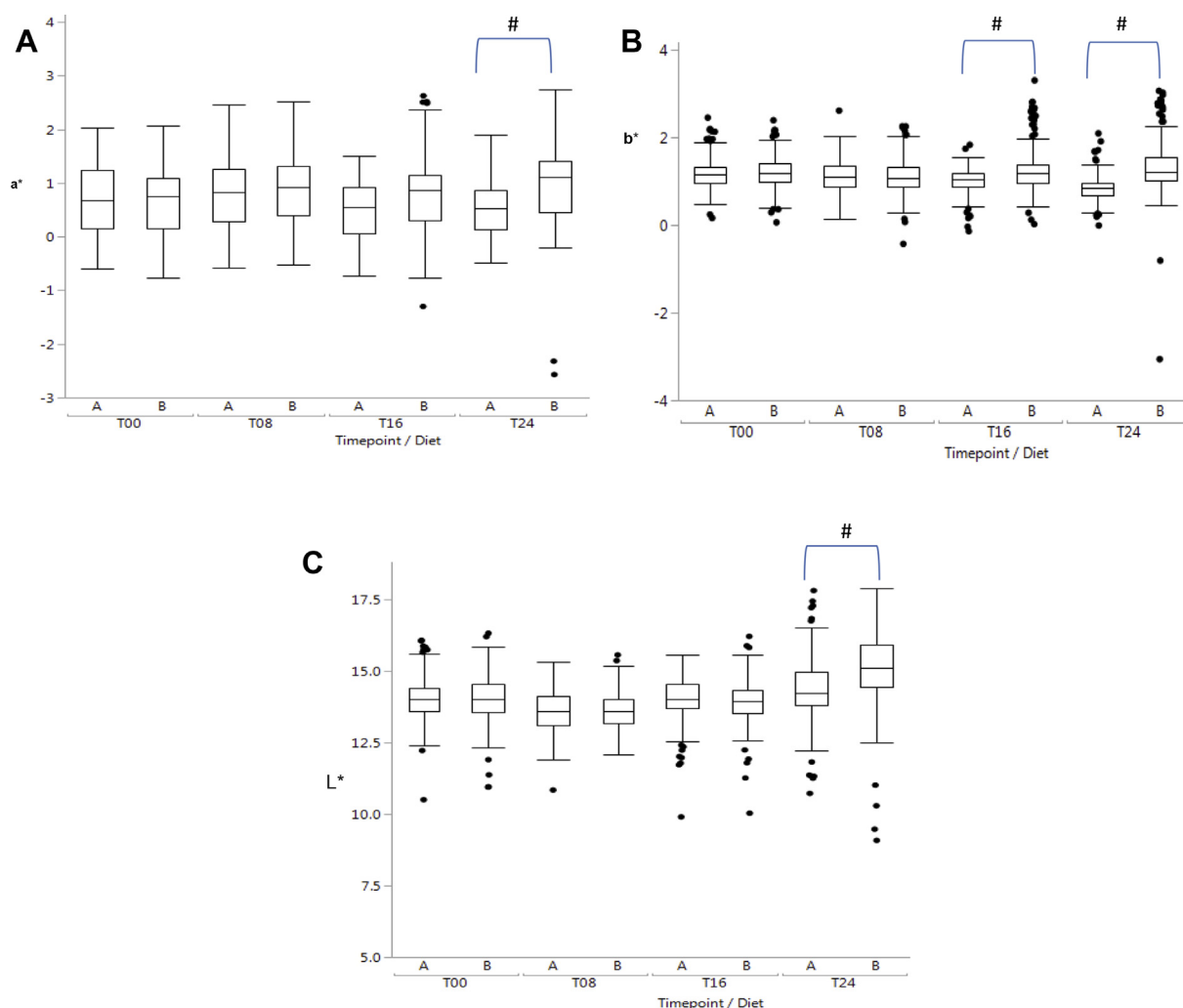


Fig. 1. Box and Whisker plots for Spectrophotometer readings of canine coat colour during the 24-week feeding study. (A) a^* denotes colour expression along red–green axis; $A < B$ at 24 weeks, $P = 0.001$. (B) b^* denotes colour expression along the yellow–blue axis; $A < B$ at 16 weeks, $P = 0.0032$ and 24 weeks, $P < 0.0001$. (C) L^* denotes white to black (light to dark) axis; $A < B$ at 24 weeks, $P < 0.0001$. For x axis, A denotes tyrosine the supplemented diet group; B denotes the baseline non-supplemented diet group; T24 denotes the time point 24 weeks of feeding. # denotes significantly difference between A and B groups at corresponding timepoint.

4. Discussion

Although the primary influence on hair colouration in animals is genetic (Robinson, 1991), a number of exogenous factors are also known to exert an effect (Busch-Kschiewan et al., 2004). The evidence for a nutritional impact has been accumulating over recent times; with intake of the amino acids tyrosine, phenylalanine and cysteine, as well as copper all having been implicated in studies (Anderson et al., 2002; Watson et al., 2015, 2017). The black coat of certain animals is known to discolour, often showing patches of brown or reddening (Yu et al., 2001). Apart from being an aesthetic concern for breeders and owners, research into the phenomenon has suggested that it may have implications for differentiating between adequate versus optimal nutritional requirements. For example Anderson et al. (2002) for kittens and Watson et al. (2015) for puppies both highlighted that nutritional requirements for growth appear insufficient to also meet the demands of regular melanin synthesis.

4.1. Tyrosine requirements and hair pigmentation

Determining whether a similar hierarchy of nutritional requirements exists for adult dogs was an intention of the study

described here. The lower concentration of the Phe + Tyr fed here (3.5 g/Mcal) was already 52% higher than the FEDIAF recommended minimum intake for adults, which was compared to a diet set at 5.6 g/Mcal Phe + Tyr, nearly 2.5× the minimum. It was clearly illustrated that there was an additional enrichment of black pigmentation in the hair of the dogs fed the supplemented diet, most likely due to eumelanin deposition. The duration of feeding required to observe these changes is not surprising; as it is not possible to introduce new melanin pigment into already formed hair follicle, extensive regrowth across the entire body is required in order for differences to become detectable via spectrophotometry. It has been shown that regrowth of a hair to full length in adult Labradors takes around 14 weeks (Diaz et al., 2004). Given that an estimated 80% of hairs are in the telogenic resting phase at any time, 16 weeks would be the minimum time required to see a significant number of hairs emerge with altered melanin deposition (Al-Bagdadi et al., 1977). Thus, it is also not surprising that the more pronounced effects on coat pigmentation were not observed until 24 weeks of feeding the supplemented diet. In a previous similar study in growing dogs a change in pigmentation was first detected after 4 months (Watson et al., 2015). In contrast, no further change was seen at 6 months, probably due to the different study

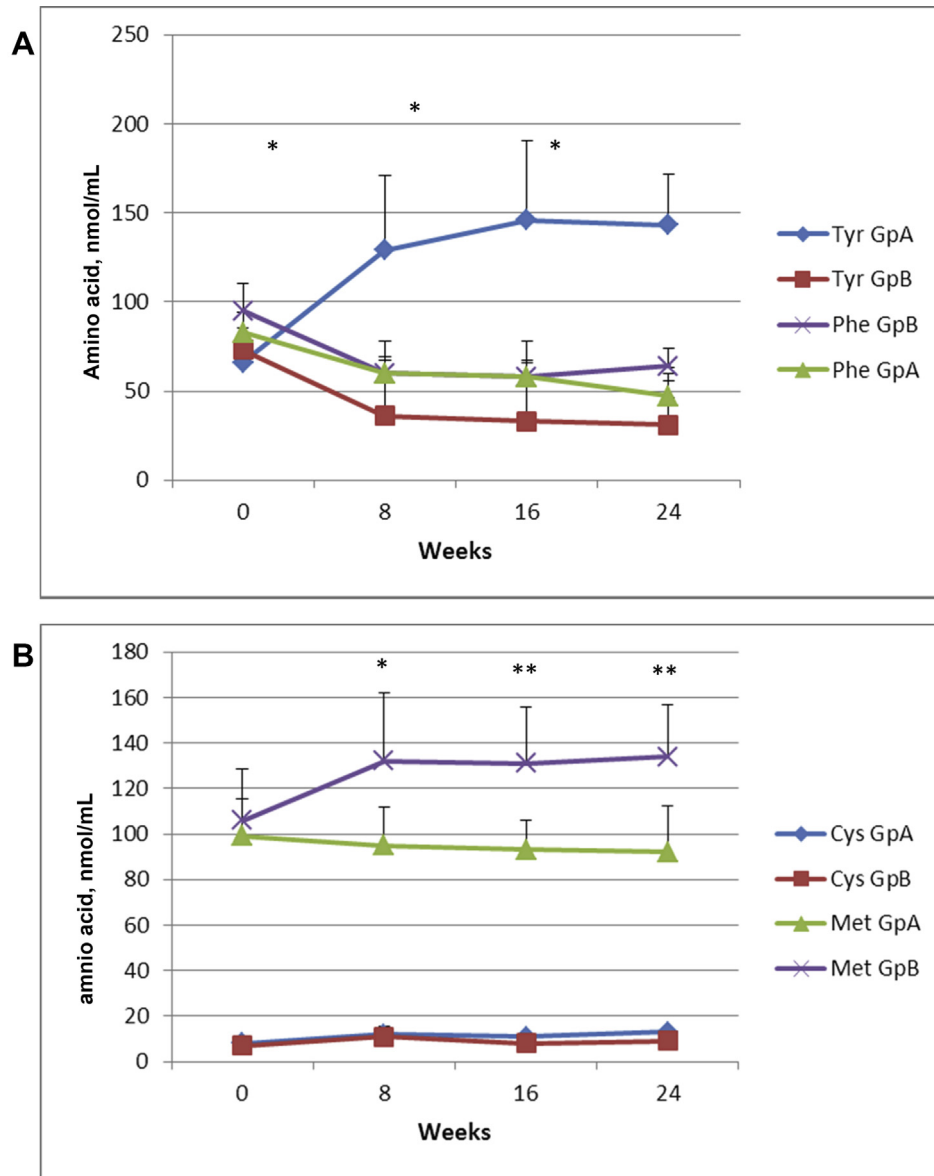


Fig. 2. (A) Changes in plasma concentrations of tyrosine and phenylalanine during the 24-week feeding study. GpA, tyrosine supplemented. Means \pm SD. * Tyr GpA > GpB, $P < 0.0001$. (B) Changes in plasma concentrations of cystine and methionine during the 24-week feeding study. GpA, tyrosine supplemented. Means \pm SD. * Met GpA < GpB, $P < 0.001$; ** Met GpA < GpB, $P < 0.0001$.

design involved. The presence of a number of outliers illustrated on the box and whisker plots reflects the fact that not all animals reacted uniformly to the dietary regimes, probably due to the natural variation and genetic components which can come into play in such studies. Multiple body sites were targeted for the colour measurements in order to minimise the influence of localised variation in the hair. The results indicate that there was some differential reactivity to the regimes within the diet groups.

4.2. Plasma amino acid changes

In this study a significant increase in the plasma concentration of Tyr was observed at the first sampling time-point of 8 weeks for diet group A, but did not change significantly again up to 24 weeks. In the same group Phe dropped between the start of the study and 8 weeks, after which no further decline was seen. All dogs were being fed a maintenance diet product prior to the start of the study, the amino acid composition of which, although within feeding

guidelines, will have been somewhat different. As a consequence a number of plasma amino acid concentrations will have changed upon diet switching. It was clear that the Tyr concentrations readily re-equilibrate to their new level in response to the dietary change. In the case of plasma Tyr for dogs fed diet A this should have enabled a greater supply to the melanin biosynthetic pathway of melanocytes. Phenylalanine was not supplemented in diet A, and plasma concentrations responded similarly in the 2 groups. Therefore, it would not appear that providing additional Tyr had any sparing effect on Phe. Measurements made for other unrelated amino acids, showed no significant differences between the two groups, apart from Met. Cysteine is another amino acid which plays an important role in melanin biosynthesis, specifically by facilitating the generation of cysteinyl-DOPA in the pathway to pheomelanin production. There is evidence that greater availability of Cys can favour red/yellow pigmentation over black (Ito, 1993). Despite the moderately increased Cys found in diet A, this did not appear to translate to plasma concentration and so is unlikely to

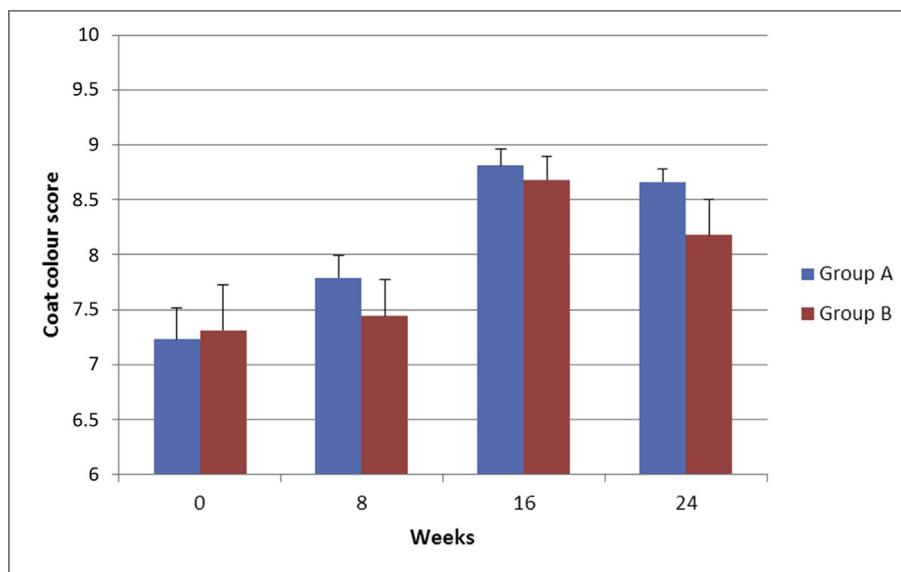


Fig. 3. Subjective coat colour assessment for dogs during the 24-week feeding study. Coat colour scores are in arbitrary units based on an original scale of 1 to 10. Group A, tyrosine supplemented. Group A 8 wk > 0 wk, $P < 0.05$; Group A 16 wk > 8 wk, $P < 0.01$; Group A 24 wk > 8 wk, $P < 0.01$; Group B 16 wk > 8 wk, $P < 0.01$. Means \pm SEM.

have had a bearing here. Plasma concentration of copper, a cofactor for tyrosine decarboxylase, the rate limiting enzyme for melanin synthesis, was the same in diet A and diet B (0.47 mg/kg). As such, the pigmentation differences observed are most likely attributable to the increased availability of Tyr, presumably exerted by stimulating production and secretion of melanins into the nascent hair.

4.3. Subjective assessment of hair colour

The CIELab based spectrophotometric measures have been used as an alternative to chemical methods for assessing hair eumelanin and pheomelanin content in humans (Shekar et al., 2008). It was shown that dimension a^* of spectrocolourimetric index is a good approximation of pheomelanin concentration in hair, as it measures a continuum of the red-green spectra. A lower a^* value therefore indicates reduced hair pheomelanin concentration, manifesting as a less reddened hair shaft. Subjective scoring of the coat colour of the animals suggested that the pigmentation changes detected experimentally could be discerned by the naked eye, which has been demonstrated previously (Shekar et al., 2008). A discernible difference was first suggested as early as 8 weeks in group A, though this became more obvious at 16 weeks and beyond, corresponding to the times at which colour change was detected spectrophotometrically for group A. The fact that a difference was also reported for the group B dogs after 16 weeks suggests there was perhaps a slight 'oversensitivity' in the scoring during the study, potentially caused by evaluators anticipating a change in coat colour. However, the significance and consistency of the increased scores for group A supports the overall effect being real. Visually determined differences in white coated dogs correlated to CIELab measurements in a previous study by Busch-Kschiewan et al. (2004), suggesting that spectrophotometer measurements in combination subjective scoring is a reliable way of determining colour changes in canine hair.

5. Conclusion

It has been previously shown in growing animals that there appears to be a distinction between the concentration of

Tyrosine required to for healthy growth and development and that required to maintain full hair pigmentation. Evidence is provided here that a similar phenomenon exists in adult dogs, whereby a tyrosine intake $2.5\times$ the recommended minimum was able to significantly reduce off-black colouration in healthy Labrador retrievers.

Appendix A

Dog assessment questionnaire:

1. Have you had any problems associated with the study diet, for example refusals? If so, how often?
2. Overall how satisfied are you/is the dog with the study diet? (1 – completely dissatisfied; 10 – completely satisfied)
3. On the scale below how would you score the dog's current coat colour (1 – Very poor; 10 – Perfect)

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