Short-term determination and long-term evaluation of the dietary methionine requirement in adult dogs

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Abstract

Methionine, an essential sulphur-containing amino acid (SAA), plays an integral role in many metabolic processes. Evidence for the methionine requirements of adult dogs is limited, and we employed the indicator amino acid oxidation (IAAO) method to estimate dietary methionine requirements in Labrador retrievers (\(n = 21\)). Using semi-purified diets, the mean requirement was 0.55 (95% CI 0.41, 0.71) g/4184 kJ. In a subsequent parallel design study, three groups of adult Labrador retrievers (\(n = 52\)) were fed semi-purified diets with 0.55 g/4184 kJ (test diet 1), 0.71 g/4184 kJ (test diet 2) or 1.37 g/4184 kJ (control diet) of methionine for 32 weeks to assess the long-term consequences of feeding. The total SAA content (2.68 g/4184 kJ) was maintained through dietary supplementation of cystine. Plasma methionine did not decrease in test group and increased significantly on test diet 1 in weeks 8 and 16 compared with control. Reducing dietary methionine did not have a significant effect on whole blood, plasma or urinary taurine or plasma N-terminal pro B-type natriuretic peptide. Significant effects in both test diets were observed for cholesterol, betaine and dimethylglycine. In conclusion, feeding methionine at the IAAO-estimated mean was sufficient to maintain plasma methionine over 32 weeks when total SAA was maintained. However, choline oxidation may have increased to support plasma methionine and have additional consequences for lipid metabolism. While the IAAO can be employed to assess essential amino acid requirements, such as methionine in the dog using semi-purified diets, further work is required to establish safe levels for commercial diet formats.

Key words: Methionine: Indicator amino acid oxidation: Sulphur amino acids: One-carbon metabolism

Methionine is an essential sulphur-containing amino acid (SAA), a constituent of proteins, a methyl donor and an integral part of one-carbon metabolism. It is also a precursor for cysteine (non-essential amino acid (AA)), and subsequently glutathione and taurine. To support its availability to maintain these diverse roles effectively, methionine is recycled via the transsulphuration pathway and also the remethylation pathway from homocysteine, with a methyl group donated by folate and also, to a lesser extent, choline derivatives\(^{(1)}\). However, homocysteine is also a substrate for glutathione and taurine synthesis and can diminish the methionine pool over time\(^{(2)}\).

One of the challenges in determining dietary methionine requirements is that under certain circumstances other non-essential nutrients, including cysteine and taurine, are able to promote methionine sparing or alternatively substitute for methionine in some specific functions\(^{(3–10)}\). Methionine dietary requirements are therefore dependent to some extent on availability of other SAA-containing substances such as cysteine, taurine and glutathione, as well as the availability of folate and choline for recycling and the demand for methyl groups, protein synthesis and methionine oxidation for energy metabolism. The extent to which cysteine can be substituted in diets to replace methionine as an SAA donor is debated\(^{(11)}\); however, in a mouse growth study\(^{(12)}\), normal growth was achieved with cysteine supplementation in a 50:50 ratio to methionine, with a reduction in total SAA requirement of 50 % compared with methionine as a sole donor. This study indicates that growth with methionine as sole SAA source is an inefficient process and that the absolute requirement for methionine is dependent upon the availability of other non-essential nutrients capable of complementing methionine for some roles.

In dogs, the demand for other nutrients such as taurine, cysteine, folate and choline can also change and may impact on methionine availability as well as have significant impact on dog health. For example, the sometimes fatal condition of dilated cardiomyopathy (DCM) as a result of insufficient taurine has been identified in dogs fed diets formulated using some natural ingredients (e.g. containing both lamb and rice\(^{(13–16)}\)). While

Abbreviations: AA, amino acid; BW, body weight; CL, confidence limit; DCM, dilated cardiomyopathy; DMG, dimethylglycine; IAAO, indicator amino acid oxidation; NRC, National Research Council; NT proBNP, N-terminal pro B-type natriuretic peptide; Phe, phenylalanine; RCO₂, carbon dioxide production rate; RF, retention factor; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SAA, S-containing amino acid.

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taurine can be synthesised by dogs, these diets were shown to reduce taurine availability, resulting in an increased requirement for taurine synthesis (14-16). SAA supplementation above that recommended for most other diets can support dogs in these situations and has been demonstrated to resolve cases of DCM (17,18). As the sole essential AA of the SAA group, the importance of establishing a safe and effective methionine requirement is important. The National Research Council (NRC) recommendations (19) are based on a number of studies assessing total SAA requirements with methionine supplementation of low-protein diets and one study identifying taurine deficiency as an adverse response to long-term feeding (4 years) of a protein-restricted, high-fat diet. The NRC (19) methionine minimal requirements (fed at 543-92 kJ/kg body weight (BW)) (20) for diet manufacturing are 0.65 g/4184 kJ, while total SAA with cysteine is approximately double this value at 1.30 g/4184 kJ.

The indicator AA oxidation (IAAO) method enables an estimation of the absolute requirement for essential AA. It has been developed as a reliable and minimally invasive method of determining AA requirements in humans and livestock (20-23). The technique is based upon the following principles: AA cannot be stored and must be either incorporated into protein, used to generate AA derivatives or be oxidised. When an essential AA is deficient for protein synthesis, then all other AA are utilised for other functions. For phenylalanine (Phe), in the presence of excess tyrosine, excess can only be oxidised. Using a stable isotopic (1-13C) Phe as a tracer, it is possible to track the extent of 13CO2 production (Phe oxidation) when individuals are fed diets containing a range of an AA. As the limiting AA increases in diets, it allows more incorporation of Phe into protein and a concomitant decrease in Phe oxidation/13CO2 production. Once the requirement is met for the limiting AA, there is no further decrease in the rate of Phe oxidation (20). The ‘breakpoint’ or inflection point where the 13CO2 production rate changes from decreasing to level is considered to be the minimum requirement level for the test AA.

A benefit of the IAAO method in estimating essential AA requirements is that it provides an estimate following only a short-term exposure to diets, so individuals are not put at risk from insufficient nutrient intake. However, the method measures the requirement of an essential AA based on the amount no longer limiting protein synthesis. This approach may account for other roles and regulatory processes that dominate availability for protein synthesis but cannot account for those requirements that are not sufficiently important relative to protein synthesis. Subsequently, the longer term consequences of providing methionine at a level sufficient to meet protein synthesis requirements in the short term are not accounted for and can only be assessed through a longer term feeding study. Relevant primary measures of insufficient dietary methionine would be concentrations of plasma methionine, plasma choline metabolites, plasma S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) and taurine status (whole blood, plasma and urinary taurine). Additional health measures include the cardiac stress marker N-terminal pro B-type natriuretic peptide (NT proBNP) and standard clinical health parameters, such as complete blood count and serum biochemistry.

The hypothesis tested here was that the mean amount of dietary methionine determined by IAAO, required to meet protein synthesis requirement of adult dogs, would be sufficient to maintain plasma methionine in a long-term feeding study. To test this hypothesis, an IAAO study was undertaken to estimate the mean requirement and also the upper 95% confidence limit (CL) for methionine in adult Labrador retrievers, a common medium size breed within the UK. Semi-purified diets were then manufactured to achieve these values and were fed to adult dogs over a period of 32 weeks. As the aim was to determine the essential requirement of methionine, rather than total SAA, these diets were supplemented with dietary cystine to maintain total dietary SAA.

Materials and methods

The studies described here were reviewed and approved by the WALTHAM© Animal Welfare and Ethical Review Body, and the work was carried out under the authority of the Animals (Scientific Procedures) Act 1986.

Indicator amino acid oxidation determination of the requirement for methionine

Animals. Twelve male and nine female (all neutered) adult Labrador retrievers (n = 21, average age 4-9 years, range 3-8 years) were used in this study. The number of dogs was determined using simulation analyses based on variance estimates of F13C02 from an unpublished IAAO study (Gray et al.) to provide a 95% CI for the breakpoint concentration of methionine within 25% of the mean with 90% power. Dogs were housed in purpose-built, environmentally enriched housing at the WALTHAM Centre for Pet Nutrition. They interacted with other dogs within their sampling group and other, non-study dogs, but interaction with other study dogs was avoided to prevent any isotopic contamination. On IAAO sampling days, dogs were isolated from other animals to prevent coprophagia. Dogs were habituated to all trial procedures before the start of the study. The trial took place between January and June.

Diets. A semi-purified base diet was used to have consistency in the nutritional composition, minimising complex matrix effects while manipulating the methionine content. Nine diets (manufactured by ssniff Spezialdiäten GmbH) were nutritionally complete apart from methionine and formulated for dogs with an energy requirements of 397-48 kJ/kg (17,18), iso-energetic and iso-nitrogenous (see online Supplementary Tables S1 and S2 for formulation and nutritional profile analysis). The nine diets differed in methionine content: 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1 and 2.4 g/4184 kJ (analysed values 0, 0.31, 0.60, 0.97, 1.30, 1.61, 1.83, 2.15 and 2.47 g/4184 kJ, respectively). These values were selected to have four diets above and four diets below the methionine inclusion level of 1.16 g/4184 kJ (the NRC minimum recommended allowance adjusted to a maintenance energy requirement of 397-48 kJ/kg (19)). To provide an acceptable consistency for consumption, the powdered diet was hydrated (15%, w/w) immediately before offering to the dog.
All diets were offered in amounts to maintain individual energy requirements, with BW and body condition score assessed weekly.

**Study design.** Each dog received each test diet in a randomised order. Dogs had at least 5 d washout between each test diet assessment, where they were offered Royal Canin Medium Adult (single batch, nutritionally complete) at 100% maintenance energy requirement (see online Supplementary Table S3 for proximate analysis). For each test diet, dogs received the test diet for 2 d (four meals/d) prior to the assay day (day 3). On the 3rd day, dogs were fed to their maintenance energy requirement (based on intake in the week prior) distributed across eight equal aliquots (given at hourly intervals). Isotope (described below) was added from meal 4 onwards (Fig. 1).

Two groups of dogs were fed the incorrect diet on one study day, and so for the diet containing 1.2 g/4184 kJ only fifteen dogs' data were analysed.

**Isotope tracer addition.** The first three meals were used to obtain a baseline measure of breath $^{13}$CO$_2$ enrichment (i.e. no isotope added). Breath samples were taken immediately before feeding meal 3 and meal 4. In meal 4, a priming dose of 3 mg/kg BW $^{13}$C-Phe (Cambridge Isotope Laboratories) was added when the meal was hydrated. Further doses (2 mg/kg BW) were given in meals 5, 6, 7 and 8. Subsequent to meal 3, breath samples were taken at 30-min intervals, just prior to, and 30 min after, each meal (Fig. 1).

**Sample collection and analysis.** Breath samples were collected using a veterinary respiratory mask connected to a breath collection bag (Quintron, QT00830-P). Approximately 12 ml of breath was syringed from the bags and injected into a vacutainer, two vacutainers were collected for each breath sample. The samples were then run on a Continuous Flow Isotope Ratio Mass Spectrometer (CF-IRMS AP2003, Analytical Precision) to determine the delta $^{13}$C.

**Carbon dioxide production rate and retention factor calculation.** The CO$_2$ production rate (RCO$_2$) and bicarbonate retention factor (RF) were calculated using the following equations, where $D$ represents the tracer dose administered, AUC represents AUC (sodium bicarbonate analysis) and $A$ represents abundance of $^{13}$CO$_2$ (parts per million) in expired breath samples:

$$\text{RCO}_2 = \frac{D}{AUC} \times \text{RF}$$

$$\text{RF} = \frac{A \times \text{RCO}_2}{D}$$

**F$^{13}$CO$_2$ calculation.** The rate of appearance of $^{13}$CO$_2$ in the breath ($F^{13}$CO$_2$ ($\mu$mol/kg per h)) was calculated using the following equation:

$$F^{13}\text{CO}_2 = \left(\frac{\text{RCO}_2 \times E\text{CO}_2 \times 44 \cdot 6 \cdot 60}{(BW \times 0 \cdot 82 \cdot 100)}\right)$$

where $E\text{CO}_2$ is the $^{13}$CO$_2$ enrichment in expired breath at isotopic steady state (APE), and BW is the body weight (kg) of the dogs. The constants 44.6 and 60 convert FCO$_2$ into $\mu$mol/h. The factor 100 changes APE to a fraction. The factor 0.82 was used to account for the retention of $^{13}$CO$_2$ in the bicarbonate pool of the body in the fed state (RF).

**Calculation of the breakpoint (mean methionine requirement).** $F^{13}$CO$_2$ was analysed using a mixed-effects breakpoint regression model$^{25}$. Using a mixed-effects model accounts for the correlation between repeated measures within each dog, thus random effects of dog were fitted, with a random $y$-intercept and random slope variances estimated. The breakpoint analysis modelled two regression slopes, one before the breakpoint and one after the breakpoint, where the slope after
the breakpoint was forced to have a slope of zero. The model is defined by:

\[ y_{id} = \beta_0 + \beta_1 \times (BP - x_{id}) \times I(x_{id} < BP) + b_0 + b_1 x_{id} + \varepsilon_{id}, \]

where \( i = 1 \ldots \text{no. of dogs}, d = 1 \ldots \text{no. of diets}, BP \) is the breakpoint and so the estimated mean methionine requirement, \( x_{id} \) is the \( d \)th diet on the \( i \)th dog, \( I(x_{id} < BP) = 1 \) if \( x_{id} < BP \) or 0 otherwise, \( I(x_{id} \geq BP) = 0 \) if \( x_{id} < BP \) or 1 otherwise, \( \beta_1 = F^\text{13CO}_2 \text{ value at the breakpoint}, -\beta_1 \) is the slope when \( x_{id} < BP, b_0 \) is the random intercept of the \( i \)th dog (normally distributed), \( b_1 \) is the random slope of \( i \)th dog when \( x_{id} < BP \) (normally distributed) and \( \varepsilon_{id} \) is the independently normally distributed random errors.

The model was fitted by allowing the breakpoint to vary from minimum +0.5 to the maximum –0.5 methionine contents offered, with 1000 breakpoints fitted in total. The Akaike information criterion of the fitted models conditional upon the breakpoint was then minimised. This maximised the profile log likelihood for the breakpoint, giving the best fitting model and associated breakpoint. We calculated 95 % CI for the breakpoint using the profile log likelihood, with the lower and upper limits set at the models with an Akaike information criterion equal to the minimised + \( \chi^2 \).

The breakpoint analyses were performed in R version 3.0.2 using the lme4 library.

Evaluation of the effects of long-term feeding of diets providing methionine at the predicted mean and upper 95 % confidence limit requirement values

**Animals.** Adult Labrador retrievers (\( n = 52 \)) were used in this study (average age 5.2 years, range 2.3–8.2 years at start of study). One dog was removed from the study at week 8 (\( n = 51 \)) and another at week 16 (\( n = 50 \)) for reasons unrelated to the study. Data from dogs removed from the study were included in the analysis up to the point they were removed from the study. Dogs were housed and exercised with other dogs in their dietary group throughout the whole trial. Dogs were habituated to all trial procedures in accordance with WALTHAM welfare and ethical principles. The trial took place between January and October.

**Diets.** To remain consistent with the IAAO study, all diets were semi-purified (ssniff Spezialdiäten GmbH) and produced in a pelleted format (2.5-cm), differing in methionine and cystine content to maintain total SAA (see online Supplementary Tables S4 and S5 for formulation and nutritional profile analyses). control diet (control diet) contained 1.37 g/4184 kJ methionine; the breakpoint diet (test diet 1) 0.55 g/4184 kJ methionine and the upper 95 % CL diet (test diet 2) contained 0.71 g/4184 kJ methionine. All diets were iso-energetic and iso-nitrogenous (via alanine) and nutritionally complete except for the reduced level of methionine in test diets (total SAA content being maintained by the addition of dietary cystine). Diets were fed at 100 % of individual maintenance energy requirement, estimated from 28 d of pre-feeding the control diet to maintain a stable BW and body condition score using the SHAPE scoring system. Food was provided in one meal (80 % ration) at 08.30 hours and the remaining 20 % provided as treats throughout the day (all fed by 16.00 hours).

**Diet analysis and protein digestibility.** All diets underwent full nutritional analysis (online Supplementary Table S5 (Eurofins)). The pelleted diets used in the longitudinal study were used to determine protein digestibility. A mixed breed panel (\( n = 8 \)) was fed study diets for 9 d, faeces collections were made on days 7–9 and samples were analysed for protein digestibility (Mars Petcare Europe Central Laboratory).

**Study design.** The primary objective of the study was to determine whether a reduction in dietary methionine intake would result in a physiologically meaningful change in plasma methionine concentrations between the test and control diets. Canine plasma methionine can differ by at least 20 % between dogs fed nutritionally complete diets. Sample size analyses by simulation were performed using methionine data derived from an unpublished results (Gray et al.) using a standard diet to inform estimates of the within and between dog variability. The method estimated that seventeen dogs per group were needed to detect a 20 % change in methionine with at least 90 % power using a test level of 5 %.

All dogs were fed the control diet (1.37 g/4184 kJ) for 4 weeks at the beginning of the study, all dogs then had a baseline sample taken before being randomised to one of three dietary groups balanced for age and sex. Group 1 remained on the control diet. Group 2 (IAAO Breakpoint) transitioned to test diet 1 (0.55 g/4184 kJ) and Group 3 (upper 95 % IAAO CL) transitioned to test diet 2 (0.71 g/4184 kJ) for 32 weeks. Fasted blood and urine samples were taken every 8 weeks (weeks 8, 16, 24 and 32). To minimise scavenging of bile/SAA derivatives, all dogs were monitored 24 h/d for 1 week prior to blood sampling to ensure all faeces samples were collected immediately and the dogs did not practice coprophagia. BW and body condition score were assessed weekly.

**Blood sample preparation.** Whole-blood samples (EDTA) were centrifuged and plasma collected in aliquots and frozen at –80°C until analysis, unless otherwise stated.

**Plasma amino acid analysis.** Sample processing buffer (5 % SSA + 500 μmol/l norleucine + n-glucosaminic acid) was added 1:1 to plasma, mixed using a Vortex Whirlimixer and allowed to incubate at room temperature for 20 min. Sample was then centrifuged at 7000RCF, for 5 min at 4°C. Supernatant was then removed and filtered with a Whatman Mini-Uniprep Syringeless 0.2 μm filter, aliquoted and frozen at –80°C for later analysis. Samples were analysed in duplicate on the Biochrom 30+ analyser (Biochrom).

**Plasma choline, betaine and dimethylglycine analysis.** Plasma (50 μl) was combined with 20 μl of internal standard working solution (2.0 mg/l D9 choline chloride and 2.5 mg/l D11 betaine), to standardise the results from the sample, and 200 μl of acetonitrile for protein precipitation. The resulting
solution was mixed with a Vortex Whirlimixer before being  centrifuged for 10 min at 7000RCF. The supernatant was filtered and 50 μl transferred to a culture tube, where 2.45 ml acetonitrile:  water solution (85:15) was added and the resulting 50x diluted solution was vortexed and filtered through a 0.2 μm syringe filter.  From this, 5 μl was injected in duplicate. The analysis was carried out by a coupled chromatograph and MS (Agilent 1290 UPLC and Agilent 6460c Mass Spectrometer) at 30°C for a total of 6 min (Talbot & Coffey, unpublished results).

Plasma homocysteine analyses. Plasma (50 μl) combined with 20 μl of internal standard working solution (containing 20 μM DL-homocysteine-3, 3, 3″, 3‴, 4, 4′, 4″-D) and 20 μl of dithiothreitol (500 μM) to reduce the homocysteine to homocysteine so all homocysteine present was measured. The sample was then mixed using a Vortex Whirlimixer and left to stand for 15 min before the addition of acetonitrile (200 μl). It was vortexed, left to settle and vortexed again, followed by being centrifuged at 7000RCF for 10 min. The supernatant was filtered through a 0.2 μm PVDF filter, by syringe filter into a HPLC autosampler vial and analysis was carried out with a coupled chromatograph and MS (Agilent 1290 UPLC and Agilent 6460c Mass Spectrometer) at 30°C for a total of 6 min (Talbot & Coffey, unpublished results) with each sample being injected into the instrument twice. This process was carried out in duplicate to get a number of replicates to give the total amount of homocysteine within each sample.

Plasma S-adenosylmethionine and S-adenosylhomocysteine. Plasma (300 μl) was acidified at point of collection with 30 μl 1M acetic acid to prevent SAM degradation and vortexed for 5 s, plasma was then snap-frozen and placed in storage at −80°C until analysis. SAM and SAH analysis (Center of Metabolomics at the Baylor Scott & White Research Institute) was conducted for control diet and test diet 1 at 0, 16 and 32 weeks of the study.

Whole-blood taurine analysis. Whole blood (200 μl) was aliquotted into three Eppendorf tubes and frozen at −20°C. To lyse erythrocytes, samples were subjected to two freeze/thaw cycles. After the final thaw, sterile water was added to a dilution of 1:4 to lyse the cells further. Sampling processing buffer (5 % SSA, 500 μmol/l norleucine and β-glucosaminic acid made up in lithium loading buffer) was added at a 1:2 dilution in order for analysis to be normalised, positively charge the AA and also deproteinise the sample. The sample was mixed using a Vortex and incubated at room temperature for 20 min, before being centrifuged for 5 min at 4°C at 7000RCF. Supernatant was removed and transferred to a Whatman Mini-Uniprep Syringeless 0.2 μm filter vial to filter the sample. The final sample was at a 1:8 dilution and was injected into the instrument in duplicate. The analysis was carried out using a Biochrom 30+ Analyser (Biochrom).

Urinary taurine analysis. Urine samples were snap-frozen after collection using dry ice and transferred to a −80°C freezer until ready for analysis. Samples were thawed on the day of analysis and diluted with sample diluent (5 % SSA (Sigma) and loading buffer (Biochrom)), one part urine to five parts sample diluent. Sample processing buffer was added at a 1:2 dilution, mixed with a Vortex mixer and left at room temperature for 20 min. The sample was centrifuged for 5 min at 4°C and 7000RCF. Supernatant was removed and filtered using a Whatman Mini-Uniprep Syringeless 0.2 μm filter vial. Samples with a final dilution of 1:12 were then run on the Biochrom 30+ Analyser (Biochrom).

Urinary creatinine. Urine samples were stored at 4°C after collection until analysis, at which point the samples were centrifuged at 2000RCF for 5 min at 4°C. Urine was then analysed on an AU480 Analyser (Beckman Coulter).

Plasma biochemistry and creatine kinase. Whole-blood samples (EDTA) were centrifuged for 10 min at 4°C at 2000RCF. The plasma was removed and transferred into 2.5 ml sample cups. Samples were then run on the AU480 Chemistry Analyser (Beckman Coulter), analysed parameters include total protein, albumin, phosphate, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, AST, cholesterol, urea, creatinine, TAG, glucose and creatine kinase. Further parameters measured were lipidemia/turbidity, icterus and haemolysis as an internal test to ensure no photometric interference was present within the plasma.

Plasma N-terminal pro B-type natriuretic peptide. Plasma samples were packed frozen on dry ice and shipped overnight to a reference laboratory for analysis (IDEXX Laboratories).

Statistics. The primary response variable was plasma methionine. Secondary supportive response variables were plasma choline, betaine, dimethylglycine (DMG), homocysteine, SAM and SAH. Additional recorded measures included whole-blood taurine, plasma taurine, urinary taurine, clinical health measures (plasma NT proBNP (IDEXX) and biochemistry) and daily energy intake (calculated from the amount of diet offered minus left after meal and energy density of the diet).

For each response measured, linear mixed model analyses (restricted maximum likelihood) were used to allow for repeated measures on each dog over time. Specifically, where there were technical repeats for a measure at each time point, random effects of week nested in dog were used, but where only single measurements were available at each time point a random effect of dog was used. Categorical fixed effects of diet, week and their interaction were used.

Distributional assumptions were checked to ensure robustness of the statistical models by performing residual checks (e.g. for randomness and constant variance). Residuals were found to have increasing variability for a number of measures (homocysteine, choline and betaine); therefore, these measures were log10 transformed prior to analyses.

As plasma methionine was the only primary measure, an overall test level of 5 % was used. Secondary measures were also tested against a test level of 5 % as they were supportive. Planned contrasts investigated the changes from week 0 (baseline) to weeks 8, 16, 24 and 32 within each diet and the differences between each diet at each week. Family wise adjustments were made for the number of comparisons to maintain the overall test level at 5 % for each measure. Accordingly, means, differences in...
means or fold changes of means (where log10 transformation was necessary) are reported with 95 % family wise CI.

Analyses were performed in R version 3.2.4 using libraries nlme for linear mixed effects models, multcomp for simultaneous inference of planned contrasts and ggplot2 for figures.

Results

Indicator amino acid oxidation determination of methionine requirements

All dogs maintained an ideal body condition score (BCS) throughout the trial using the SHAPE system. BW average was 27.3 kg (range 22.8–35.4 kg) and average intake was 451.9 kJ/kg0.75 (range 401.7–548.1 kJ/kg0.75) based on weekly readings. On two occasions, sampling was stopped on the day due to intake irregularities (non-consumption and vomiting). These samples were not used in the study and the occasions repeated. The calculated mean minimum requirement for methionine (Fig. 2) in adult Labrador retriever dogs was estimated to be 0.55 (95 % CI 0.41, 0.71) g/4184 kJ.

Evaluation of the effects of long-term feeding of diets providing methionine at the estimated mean and upper 95 % confidence limit requirement values

Diets digestibility. Faecal crude protein apparent digestibility was determined to be 96 %. Full dietary analysis can be found in the online Supplementary material (online Supplementary Table S2).

Body weight and daily intake. A significant increase in BW (kg) over the duration of the study was noted for each diet group when compared with baseline BW (P < 0.05), specifically for test diet 1 at weeks 14, 15, 17 and 18 (by 0.86–1.1 kg), for test diet 2 at week 6 (by 0.79 kg) and for the control diet at weeks 3–15 and week 23 (by 0.71–0.97 kg). This response does not appear to be influenced by the methionine content of the diet, as no significant differences were noted between dietary groups at any time point. To ensure an ideal body condition score was maintained, the amount of diet offered was adjusted and subsequently the average daily intake (kJ/kg0.75) significantly reduced over the study period (online Supplementary Fig. S1(a)) for all dietary groups (P < 0.001). No significant differences in energy intake were found between diets at any week. Daily average methionine and average total SAA intake (methionine and cystine) (g/kg0.75) also reduced significantly over time for all dietary groups (P < 0.001), no difference between dietary groups was noted for total SAA; however, methionine intake did vary significantly due to the diet formulation and study design (online Supplementary Fig. S1(b) and (c)).

Reduced dietary methionine did not reduce plasma methionine but altered some intermediates in methionine pathways. Dogs in all groups remained healthy throughout the trial. No significant decreases in plasma methionine were observed at any time point for test diets compared with control (Fig. 3(a)). Indeed, plasma methionine was significantly increased at weeks 8 and 16 for test diet 1 compared with the control diet (30 %, range 7–58 %, P = 0.001; 24 %, range 2–51 %, P = 0.022, respectively). Plasma homocysteine (Fig. 3(b)) was significantly

Fig. 2. Effect of methionine intake on production of 13CO2 from phenylalanine oxidation ([F13CO2]). All observations (n 189) and all dogs (n 21) are shown. Symbols represent individual dogs, the black solid line is the fitted breakpoint regression for the minimised Akaike information criterion (AIC) model, the dotted lines are the models at the minimised AIC and the dashed vertical lines represent the 95 % confidence limits for the breakpoint. The breakpoint of the two-phase mixed-effect model represents a methionine requirement of 0.547 g/1000 kcal (4184 kJ).
decreased at weeks 8, 16, 24 and 32 compared with baseline levels (week 0) in both test diet groups ($P < 0.005$) and not in the control diet group (range 75–83 % for test diet 1, $P < 0.005$; range 70–79 % for test diet 2, $P \leq 0.001$). However, no significant differences between diets were noted at any time point. Analysis of individual contrasts revealed that at 8 weeks, both plasma SAM and SAH (online Supplementary Fig. S2(a) and (b), respectively) reduced significantly in both test diet 1 and the control diet groups from baseline, and also the SAM/SAH increased in the test diet 1 group at week 8 compared with baseline (Fig. 3(c)). The only significant difference between groups was observed at 32 weeks, when plasma SAM was greater in the test diet 1 group by 11.77 (95 % CI 0.97, 22.58) nmol/l ($P = 0.025$) (online Supplementary Fig. 2).

Reduced dietary methionine resulted in increased choline oxidation intermediates. Plasma choline levels (Fig. 4(a)) analysis revealed the test diet 2 group was significantly raised from baseline (week 0) levels at week 8 by 21 (95 % CI 2, 43) % ($P = 0.014$), whereas the test diet 1 group was significantly reduced from baseline levels at week 32 by 20 (95 % CI 5, 33) % ($P = 0.002$). Analysis between dietary groups revealed that the test diet 1 group was significantly less than the control diet group at 24 and 32 weeks (by 22 (95 % CI 5, 36) %, $P = 0.002$, and 20 (95 % CI 2, 34) %, $P = 0.016$, respectively) and test diet 2 group at 32 weeks by 24 (95 % CI 2, 51) % ($P = 0.018$).

Compared with baseline, plasma betaine levels (Fig. 4(b)) were significantly elevated by 2.3–5.3 times ($P < 0.001$) at all
sampling points compared with baseline for both test diet 1 and test diet 2 groups. No change from baseline values was noted in the control diet group. Analysis between dietary groups following transition to the test diets revealed plasma betaine levels were higher for both test diet groups compared with the control diet group at all time points by 2.7-6.1 times (\( P < 0.001 \)), in addition plasma betaine levels were also significantly elevated in the test diet 1 group compared with the test diet 2 group at all time points by 53-88 % (\( P \leq 0.010 \)).

Plasma DMG levels (Fig. 4(c)) were significantly increased from baseline levels at weeks 8, 16, 24 and 32 for the test diet 1 group and weeks 8, 24 and 32 for the test diet 2 group (\( P \leq 0.012 \)). No change in DMG levels from baseline was noted in the control group. Analyses between dietary groups revealed that DMG levels were significantly higher in the test diet 1 group compared with the control group at all sampling occasions subsequent to diet change by 2.24-2.74 times (\( P < 0.001 \)). In addition, DMG levels in the test diet 1 group were also significantly elevated above levels in the test diet 2 group at week 16 by 19 (95 % CI 1, 40) % (\( P = 0.023 \)). No difference between the test diet 2 group was noted compared with the control diet group. Total urinary taurine levels (online Supplementary Fig. 3) and urinary taurine:creatinine (Fig. 5(c)) also decreased over the duration of the study in all dietary groups, specifically a significant reduction in urinary taurine was noted at week 32 compared with baseline in all diets and additionally at weeks 16 and 24 for the test diet 1 group and a significant reduction in urinary taurine:creatinine at weeks 24 and 32 for test diet 1. However, no differences were found between diet groups. Plasma NT ProBNP levels were monitored as a marker of cardiac stress, and no significant effects of diets or time were noted (Fig. 5(d)).

Reduced dietary methionine did not influence taurine status or plasma N-terminal pro B-type natriuretic peptide levels. Plasma taurine (Fig. 5(a)) and whole-blood taurine levels (Fig. 5(b)) had significant decreases overtime in all dietary groups. In addition, plasma taurine levels in the test diet 1 group were significantly elevated above levels in the control group at week 16 by 19 (95 % CI 1, 40) % (\( P = 0.023 \)). No difference between the test diet 2 group was noted compared with the control diet group. Total urinary taurine levels (online Supplementary Fig. 3) and urinary taurine:creatinine (Fig. 5(c)) also decreased over the duration of the study in all dietary groups, specifically a significant reduction in urinary taurine was noted at week 32 compared with baseline in all diets and additionally at weeks 16 and 24 for the test diet 1 group and a significant reduction in urinary taurine:creatinine at weeks 24 and 32 for test diet 1. However, no differences were found between diet groups. Plasma NT ProBNP levels were monitored as a marker of cardiac stress, and no significant effects of diets or time were noted (Fig. 5(d)).

Little effect of dietary methionine:cystine was detected in a range of standard analyses. Comparing amino acid metabolite plasma pools at each time point, four differed between diets at
Fig. 6. Effect of reduced methionine on plasma (a) cholesterol (mmol/l) and (b) TAG (mmol/l) levels in dogs over a 32-week period. Values are observations and means with their 95% confidence intervals represented by vertical bars. ●: Control diet (n=17); ▲: test diet 1 (n=16); ▼: test diet 2 (n=17). * Significant difference between the control diet and the respective test diet within a time point.

Methionine requirement of adult dogs

Discussion

The objective of these studies was to determine the methionine requirement of adult Labrador retrievers using a short-term assay and to evaluate the safety of feeding at this revised level in a longer-term study. The hypothesis was that the mean level estimated using IAAO would result in no significant reduction in plasma methionine in animal fed this over a 32-week period. In addition, to evaluate the safety of these reduced methionine diets, a broad range of health and metabolic parameters were also measured. Both studies used semi-purified diets to ensure precise amounts of amino acids were delivered, both in the amount supplemented and in their availability, with minimal loss due to matrix effects, with the inclusion of all other nutrients standardised. The dietary methionine minimum requirement determined using the IAAO methodology was approximately 30% lower than the published NRC minimum for adult dogs and was subsequently shown to be sufficient to maintain plasma methionine over a period of 32 weeks when total S amino acid levels were maintained with dietary cystine.

The IAAO study estimated a mean dietary requirement of 0.55 (95% CI 0.41–0.71) g/4184 kJ, compared with the NRC values of 0.65 g/4184 kJ and 0.83 g/4184 kJ for minimum requirements and recommended allowance, respectively. Contextualising this against individual animal requirements, the NRC minimum requirement for methionine can be expressed as 0.085 g/kg BW<sup>0.75</sup> per d. The mean value we estimated was determined as approximately 0.059 g methionine/kg BW<sup>0.75</sup> per d (approximately 70% of the NRC minimum), with the upper 95% CI, was 0.076 g methionine/kg BW<sup>0.75</sup> per d (90% of the declared NRC minimum). The lower values reported here reflect the differences in methodology and objectives between this short-term study and previous studies used to inform the NRC guidelines. For example, previous studies investigated responses to methionine supplementation in low-protein diets<sup>(33–40)</sup> and estimated total SAA as approximately 0.5–1 g/4184 kJ. One long-term study<sup>(41)</sup> found no adverse effects when beagles were fed a diet containing total SAA of 1.3 g/4184 kJ for 4 years. However, one dog developed DCM as a result of taurine deficiency when fed a high-fat, protein-restricted diet with a total SAA of 1.2 g/4184 kJ. The study concluded that the Association of American Feed Controls Officials requirement for methionine was insufficient to maintain taurine on protein-restricted diets. Our study estimated the requirements of methionine (delivered as a free amino acid) in adult dogs based on availability of Phe for protein synthesis rather than detecting health consequences over a long-term feeding period. While the IAAO approach has been deployed as a reliable and minimally invasive method of determining amino acid requirements in humans and livestock<sup>(20–23)</sup>, the mean value alone cannot provide the basis for a minimal requirement, as it only secures 50% of the population. However, data from this acute study were used to inform the methionine inclusion studied in the long-term feeding trial to provide additional certainty around the adequacy of the revised feeding levels. In addition, the observed variability was greater than that had been expected based on unpublished in-house pilot studies, potentially resulting from a combination of biological and technical variability. Sources of biologically related variability between dogs could arise from the differential distribution of methionine between protein anabolism and transmethylation and the distribution of homocysteine between remethylation and transsulphuration<sup>(37)</sup>. The challenges of determining methionine requirements when cysteine can substitute for some activities
have been previously discussed\(^3\). However, further method development and validation is required, including repeated measures within dog to provide insights into if, and how, the IAAO method could be improved.

The accurate determination of methionine requirements is challenging for a number of reasons. Firstly, other amino acids or their derivatives can be used as SAA donors for some activities (e.g. cysteine for taurine and cystathionine synthesis\(^7\,11\)). In addition, the replacement of one dietary amino acid for another does not follow a simple stoichiometric relationship. Evidence indicates that cysteine can reduce total SAA requirements by 50 %, compared with situations where methionine is the sole SAA\(^1\,12\). This indicates that cysteine is a preferred precursor for some metabolic functions and there is a regulatory cost of having methionine as a sole SAA. Secondly, methionine is able to be recycled and salvaged from different pathways, with some dependency on/crosstalk between resources, such as folate or choline\(^3\,9\,10\). Thirdly, the physiological demands for products of SAA may alter with age, breed, immune needs, physical activity level and diet composition\(^4\,2\,3\,3\). For example, reports indicate that components found in some commercial diets may result in an increased requirement for dietary SAA to reduce the risk of DCM\(^14\,4\). This latter point is clearly an issue for assessing meaningful values in nutritional studies, where anti-nutritive components and lipid composition may create much higher demands for total SAA, consequently influencing methionine requirements. The semi-purified diet used here provides a baseline to allow the effect of dietary factors on methionine requirements to be characterised more effectively, but future work in more complex dietary matrices that more closely represent commercially available canine diets would be important to further validate the present findings.

The mean and upper 95 % interval values sufficiently differed to the current NRC recommended dietary requirements to warrant further investigation in a longer term (32 weeks) study. To ensure that the essential requirement for methionine was being tested, rather than a change in the total SAA requirement, diets were supplemented with cystine to maintain the total dietary SAA content. It should be noted that this was a difference to the IAAO study, where diets varied in total SAA due to cystine being a fixed concentration. The hypothesis tested here was that the IAAO-determined mean value would be sufficient to maintain plasma methionine throughout a 32-week feeding period, determined as no significant reduction in the plasma methionine pool compared with the control diet. In fact, the methionine pool was higher than the control diet at 8 and 16 weeks for test diet 1. We also hypothesised that a reduction in dietary methionine intake may result in a reduced plasma SAM:SAH and an increased homocysteine concentrations. These effects were not observed, perhaps as a consequence of methionine sparing of cystine. However, no indication of a negative consequence overall health or on the plasma pools of metabolites in this pathway was observed.

The most biologically significant effects observed were the increase in plasma pools of betaine and DMG, in both test diet groups, more so in the lower methionine group. In humans, plasma DMG has been demonstrated to be a good indicator of betaine being utilised as a methyl donor when folate was low/deficient\(^4\,5\,6\), though plasma betaine was not. However, both plasma betaine and DMG have been reported to increase in response to dietary methionine restriction and are considered to be good plasma biomarkers of methionine restriction\(^7\). The elevated amounts of these products of choline oxidation when dietary methionine is lower may therefore be used to suggest that increased methylation of homocysteine replenished methionine via betaine-homocysteine methyltransferase. This interpretation of the data suggests that the estimate derived from protein synthesis requirements in the short-term study was insufficient to cover all methionine needs in the longer term. We speculate that the relative increase in plasma methionine on the lowest methionine diet (test diet 1) is a response to activation of choline oxidation to deliver methyl groups to increase flux through the methionine cycle. The intermediate levels of betaine and DMG for the test diet 2 group are consistent with this view.

Regarding other measures of note, taurine is able to be synthesised by dogs, but when fed certain diets with low inclusion levels of total SAA the rate of synthesis can be insufficient to meet the needs of some dogs, which in the long-term can result in DCM\(^14\,4\). Taurine status is therefore an important marker of SAA metabolism in canine. We maintained total SAA with dietary cystine and, as expected, saw no significant effect of reduced dietary methionine on taurine status (whole blood, plasma and urinary). Both cholesterol and TAG reduced in test diet 1 dogs, which may reflect a change in lipid metabolism related to diversion of choline from lipid synthesis to methionine synthesis or altered bile acid production. Alterations in lipid metabolism have also been reported as consequences of methionine restriction\(^8\).

Based on the findings presented here, future work will be divided into three main work streams. Firstly, metabolomics will be used to address some of the remaining questions about the response to the lower methionine diet, as well as to long-term consumption of semi-purified diets. Secondly, improvement of the IAAO experimental protocol to enhance accuracy and alignment with the principles of the NC3R. Thirdly, transitioning these findings into commercially relevant diet formulations and characterising how other dietary components, such as cysteine and choline, or anti-nutritive factors impact estimated methionine requirements using IAAO.

### Considerations

A concern from the calculated breakpoint is the 30 % range of the CI limits, exceeding the estimate from the powering. The extent of this variability may be indicative of wider ranges requirements for methionine between dogs, variability in requirements (e.g. seasonal hair growth) or a high variability in the methodologies employed. Potential technical causes include insufficient priming dose and variability in breath collection success. While the use of the same purified diet across both studies enabled a direct comparison to the IAAO data, extrapolation of the results to commercial diet formats is not recommended due to confounders related to protein digestibility and the impact of other dietary factors, such as fats on bile acid requirements or...
interpretation of data, conceived and designed study 2, interpreted the data and co-wrote the manuscript. There are no conflicts of interest to declare.

Supplementary material
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