Glycine supplementation can partially restore oxidative stress associated glutathione deficiency in ageing cats

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Short title: Glycine supplementation in ageing cats

Abbreviations: 8-iso-$PGF_{2\alpha}$ (8-iso-prostaglandin $F_{2\alpha}$), 8-OHdG (8-hydroxy-2' -deoxyguanosine), c–GC (c-glutamylcysteine), AA (amino acid), AAFCO (Association of American Feed Control Officials, APP (acute phase proteins), BCS (body condition score), CI (confidence intervals), CYS (cysteine), F_2-IsoPs (F_2-isoprostanes), GLY (glycine), GSH (reduced glutathione), GSR (glutathione reductase), GSSG (oxidised glutathione), IS (internal standard, MFI (median florescence intensity), $PGF_{2\alpha}$ (prostaglandin $F_{2\alpha}$), ROS (reactive oxygen species), RBC (red blood cell), RT (room temperature), SAA (serum amyloid A), WB (whole blood), WBCs (white blood cell)

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Abstract

Intracellular levels of glutathione, the major mammalian antioxidant, are reported to decline with age in several species. To understand whether ageing affects circulating glutathione levels in cats, blood was sampled from two age groups, < 3 years and > 9 years. Further, to determine whether dietary supplementation with glutathione precursor glycine (GLY) affects glutathione concentrations in senior cats (> 9 years), a series of free GLY inclusion level dry diets were fed. Subsequently, a 16-week GLY feeding study was conducted in senior cats (age), measuring glutathione, and markers of oxidative stress. Whole blood (WB) and red blood cell (RBC) total, oxidised and reduced glutathione levels were significantly decreased in senior cats, compared to their younger counterparts ($P \leq 0.02$). The inclusion level study identified 1.5% free GLY for the subsequent dry diet feeding study. Significant increases in RBC total and reduced glutathione were observed between senior cats fed supplemented and control diets at 4 weeks (*P* £ 0.03; maximum difference of 1.23 mM). Oxidative stress markers were also significantly different between groups at 8 ($P = 0.004$; difference of 0.68 nG/mL in 8-OHdG) and 12 weeks ($P \leq 0.049$; maximum difference of 0.62 nG/mG Cr in F_2-isoprostane $PGF_{2\alpha}$). Senior cats have lower circulating glutathione levels compared to younger cats. Feeding senior cats a complete and balanced dry diet supplemented with 1.5% free GLY for 12 weeks elevated initial RBC glutathione, and altered markers of oxidative stress. Dietary supplementation with free GLY provides a potential opportunity to restore age-associated reduction in glutathione in cats.

Keywords: antioxidant, feline, senior, amino acid

Introduction

A key hallmark of ageing is increased oxidative stress[1]. This entails a progressive decline in the ability to manage levels of oxidative damage, which results in changes to cellular proteins, lipids, and nucleic acids[2]. DNA and RNA damage, in particular, have been postulated to play a central role in age-related loss of physiological functions, including immunosenescence[3]. Age-related increases in oxidative stress have been associated with chronic inflammation, cardiovascular and neurodegenerative disease, osteoarthritis, and type 2 diabetes in humans[4,5,6] and companion animals, namely cats[7,8] and dogs[7,8,9,10].

The oxidative damage equilibrium is dependent on the neutralisation of generated reactive oxygen species (ROS), a process governed by the capacity of several antioxidant defence systems. The most abundant intracellular antioxidant in mammals is glutathione, a tripeptide of glutamate, cysteine (CYS) and glycine (GLY). Availability of glutathione is critical; it regulates the oxidation state of cells. In turn, the ability to upregulate glutathione synthesis in response to demands is hypothesised to be an important determinant of cell survival[11]. Evidence from several rodent[12-14], dog[15,16] and human[17-21] studies suggest that glutathione levels in organs, red blood cells (RBCs) and plasma decline with age.

Studies in aged humans and rodents have shown that dietary supplementation with the precursors of glutathione, CYS and/or GLY, can restore glutathione synthesis and reduce levels of oxidative stress. Sekhar *et.al.*[21] observed that the significantly lower concentrations of RBC glutathione in elderly human subjects, compared with younger control subjects, could be re-established by dietary GLY (1.33 mM/kg/day) and CYS (0.81 mM/kg/day) supplementation for 14 days. Not only was RBC glutathione significantly increased, but plasma reactive oxygen metabolites, plasma F_2-isoprostanes (F_2-IsoPs) and lipid peroxides were significantly reduced. A recent intervention study by Kumar *et al.*[22] supplementing 100 mg/kg/d GLY and CYS, provided as N-acetylcysteine (100 mg/kg/d), improved glutathione deficiency, oxidative stress, mitochondrial dysfunction, inflammation and physical function. Perturbations to glutathione homeostasis have also been explored in disease models. Linking human immunodeficiency virus (HIV) to glutathione deficiency, Nguyen *et al.*[23] explored supplementation with both glutathione precursors in infected male patients. The authors observed two weeks of oral supplementation of 1.33 mM/kg/day GLY and 0.81 mM/kg/day CYS restored glutathione synthesis, improved mitochondrial fat and

carbohydrate oxidation, insulin sensitivity, body composition, muscle strength, and dyslipidemia.

Supplementation of GLY alone has been investigated in rodent models. In a sucrose-fed rat model, where animals developed significantly lower aortic tissue glutathione and higher aortic tissue oxidised glutathione (GSSG) compared to controls, 1% v/v GLY offered in drinking water for 4 weeks restored glutathione levels in vascular tissue and significantly reduced markers of oxidative damage[24]. Furthermore, GLY administration (1g/kg for 21 days) was reported to attenuate the loss of fat and muscle mass and to reduce skeletal muscle inflammation in a mouse model of cancer cachexia[25].

Oxidative stress and abnormal glutathione metabolism have been implicated in various feline diseases. Cats with chronic kidney disease have been shown to exhibit significantly lower reduced glutathione (GSH) and GSH to GSSG ratio and significantly higher GSSG compared to clinically normal, age-matched cats[26]. Lower concentrations of GSH have also been characterised in the liver tissue of cats with necroinflammatory liver disease and hepatic lipidosis compared with healthy cats[27]. Furthermore, a study of feline immunodeficiency virus (FIV) infection by Webb *et al.*[28] identified significantly increased whole blood (WB) GSH and intracellular GSH concentrations in a reduced CD4+ T cell population. Despite these substantiations, knowledge of how WB or RBC GSH, GSSG, GLY or CYS concentrations vary in cats of different ages is lacking. To elucidate this, we report a cross-sectional study to determine levels of total glutathione, GSH and GSSG in WB and RBCs, as well as plasma free GLY, CYS and methionine concentrations in young adult and senior cats offered a complete and balanced diet. Following the confirmation of reduced glutathione levels in senior cats, we explored the effect of free GLY supplementation in a complete and balanced dry diet, containing excess CYS, via two additional studies. Supplementation of GLY alone was selected as opposed to GLY and CYS since CYS can be toxic[29]. The impact of the intervention was determined by measuring the resulting RBC and white blood cells (WBCs) glutathione concentration, and biomarkers of oxidative stress.

Experimental methods

Animals

Domestic short haired cats (all neutered) were housed at the Pet Health and Nutrition Center (PHNC), Lewisburg, OH, USA and all procedures were approved by the WALTHAM Animal Welfare and Ethical Review Body and the PHNC Institutional Animal Care and Use Committee. Cats were deemed healthy by a veterinarian at the start of the study with no evidence of systemic disease requiring treatment e.g. arthritis, diabetes, thyroid disorder, liver, or renal impairment and had not been vaccinated or prescribed any medication within 2 weeks of blood sampling. Routine housing, husbandry and exercise regimes were maintained throughout the course of the study. Cats were group housed in a free-living environment with indoor/outdoor access during the day (weather permitting) except twice daily for a period of 30 mins, when they were individually housed for feeding. Rooms were fitted with environmental enrichment and all cats had daily social human interactions which included grooming and play with toys for a minimum of 20 mins. Water was provided *ad libitum* at all times. The general health and overall condition of each animal were monitored daily by the animal care staff.

Impact of ageing on glutathione (cross-sectional study)

The first study consisted of 32 healthy adult cats (22 female, 10 male) that took part in a cross-sectional study. Sixteen were classified as the "young" group (12 female, 4 male), with a median age of 2.9 years (range 1.3 to 2.9 years) and 16 as the "senior" group (10 female, 6 male), with a median age of 9.7 years (range 9.2 to 13.2 years). Cats in both age groups were fed a commercially available complete and balanced dry diet (IAMS Multi-Cat, Mars Petcare, USA) meeting the Association of American Feed Control Officials (AAFCO) nutrient profile for adult cats throughout the study. All animals were within 10% of ideal body condition score (BCS) and diets were provided at levels to maintain ideal BCS throughout the study. Ideal BCS was determined using the Size, Health And Physical Evaluation (S.H.A.P.E.) system[30]. Maintenance energy requirements (MER) were calculated using an average energetic intake estimated for each cat based on individual feed intakes required to maintain an ideal BCS. Two, 3 mL blood samples were collected from the medial saphenous vein at an

interval of one month to compare WB and RBC total glutathione, GSH and GSSG and free plasma CYS, GLY and methionine concentration.

Comparing different levels of glycine supplementation (GLY inclusion level study)

To determine the level of GLY required to increase GLY concentrations in blood, 52 senior cats (33 female, 19 male), with a median age of 12.1 years (range 8.1 to 13.6 years), took part in a 16-week study, comprising diet rotations over eight, two-week blocks (Figure 1a). Cats were assigned to one of four groups and alternated between a control dry diet (IAMS Adult Cat Original Chicken, Mars Petcare, USA) or test dry diet supplemented with either 0.5, 1.5 or 6.0% as-fed free GLY. All diets were manufactured from the same batch of ingredients to the same formulation as commercially available diet. For test diets, glycine was added, as a dry powder ingredient, to the dry feed mix prior to extrusion. All diets were analysed for nutrient composition (Supplementary Table 1) (Total amino acid profiling method reference: AOAC 982.30 mod/AOAC 994.12 mod/AOAC 988.15; free amino acid profiling method reference: by performic acid oxidation followed by HPLC according to AOAC 994.12 mod and AOAC; Eurofins Nutrition Analysis Center, Des Moines, IA, USA), confirming target free GLY supplementation levels had been achieved and that the diets met the AAFCO nutrient profile for adult cats. At the start of the study, all cats began on the control diet and were fed this diet for two weeks as an acclimation phase. Following this, cats were subsequently fed each test diet in a randomised order for two weeks, discriminated by room location, with a two-week washout with the control diet in between. The animals were within 10% of ideal BCS and the diets provided were at levels to maintain ideal BCS throughout the study. Each two-week test block concluded with a 4mL fasted (overnight ³18 h) blood sample collected from the medial saphenous vein from each cat into a sodium heparin vacutainer (BD Vacutainer^{Ò}). Heparin anticoagulated WB was processed to determine plasma and RBC free amino acid (AA) profiles, of which GLY in both components formed the primary measure. Blood samples collected at the end of a washout block represented the baseline sample for the subsequent two-week test feeding block. Blood samples collected at the end of a test block represented the final, end point sample for the preceding two-week block of feeding test diet.

Impact of glycine supplementation on GSH and markers of oxidative stress (GLY feeding study)

Forty four senior cats (28 female, 16 male), of which 39 participated in the GLY inclusion level study, with a median age of 11.7 years (range 7.7 to 13.8 years), took part in the 16 week study, comprising a 4-week acclimation phase and a 12-week test phase (Figure 1b). For the four-week duration of the acclimation phase, cats were offered a control dry diet (IAMS Adult Cat Original Chicken, Mars Petcare, USA). Subsequently, for the test phase, the senior cohort was randomly divided into two groups of 22 cats, one group remained on the control dry diet and the other was immediately transferred to a test dry diet supplemented with 1.5% as-fed free GLY. The control group (14 female, 8 male) had a mean age of 11.4 (standard deviation = 1.9) years whilst the test group (14 female, 8 male) had a mean age of 11.5 (standard deviation = 2) years. Diets fed were analysed for nutrient composition and met the AAFCO nutrient profile for adult cats (Supplementary Table 1). Total GLY and CYS levels were 2.42% and 0.41% in the control diet, and 3.94% and 0.42% in the test diet, respectively (Supplementary Table 1). Blood and urine were collected at the end of the pre-test phase (from cats fasted overnight ³18 h) to act as a baseline for all measures. Blood was collected from the medial saphenous vein for the measurement of RBC glutathione profiles, WBC GSH, plasma and RBC AA profiles, mitogen-induced lymphoproliferative response, serum amyloid A (SAA), 8-hydroxy-2' -deoxyguanosine (8-OHdG), one of the major products of DNA oxidation widely used as a biomarker of oxidative stress[31], and biochemistry and haematology parameters (Figure 1b). Urine was collected in lodges, to which the cats had been habituated, for the measurement of F_2-IsoPs for additional quantification of oxidative damage. Subsequently blood was collected every 4 weeks to mirror the measurement of parameters conducted at the end of the acclimation phase, with the exception of WBC glutathione being omitted at week 4. Subsequent urine collection was conducted at the end of the test phase and study (week 12). Aliquots for the majority of the blood-based measures were obtained from 8mL heparin anticoagulated WB (BD Vacutainer^{Ò} 4 mL Heparin Tubes) with the exception of samples for biochemistry (1.2mL; serum separator tube, SST, SARSTEDT, Inc.), haematology (1mL; EDTA, BD Vacutainer^{Ò}), SAA (1.2mL; SST, SARSTEDT, Inc.) and WBC glutathione (1mL; EDTA, BD Vacutainer^{Ò}), which were collected into individual blood tubes.

Sample Analysis

Erythrocyte glutathione measurement: For the study evaluating the impact of ageing on glutathione, heparin anticoagulated WB was placed on a rocker for at least 1 min before a 2 mL aliquot was removed for amino acid analysis. The remaining 1 mL sample was centrifuged at 1,000 x g for 30 min at 4°C and the buffy coat was then removed from the RBC pellet. Determination of GSH and GSSG were carried out by enzymatic recycling of GSSG to GSH using a glutathione detection ELISA according to manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY, USA, Cat No. ADI-900-160) including pre-treatment of samples with metaphosphoric acid to remove interfering proteins. Absorbance measurements were made at 405 nm for 12 min at 1 min intervals with a microplate reader using kinetic mode (Cytation 3 multi-mode, BioTek, Winooski, VT, USA). WB and RBC total glutathione, GSH, GSSG and their ratio were determined; GSH was calculated as the difference between total glutathione and GSSH. Data were not normalised to haemoglobin based on the assumption that variation in RBC haemoglobin levels in healthy cats is minimal.

In the GLY supplementation study, 1 mL heparin anticoagulated WB was aliquoted and centrifuged at 2,000 x g for 10 min. The buffy coat was then removed from the RBC pellet and the pellet washed with PBS three times. The pellet was then flushed with nitrogen gas, snap frozen in liquid nitrogen and stored at -80°C. Samples were shipped on dry ice to Creative Proteomics (New York, USA) for the quantification of RBC glutathione. An internal standard (IS) solution containing 13C isotope-labelled GSH and 13C isotope-labelled GSSG, were prepared in an anti-oxidation buffer. Serially diluted IS solutions were also prepared in the anti-oxidation buffer. RBC pellets were thawed on ice and diluted 50x with the anti-oxidation buffer. 50µL of each RBC solution or IS calibration solution were mixed with 50 µL IS solution and 300 µL 40-mM N-ethylmaleimide-acetonitrile. After vortex-mixing for 10 mins at room temperature (RT), the resulting solutions were centrifuged, and the supernatant diluted 5x with water. 10µL aliquots of the diluted supernatant were analysed by UPLC-MRM MS on an Agilent 1290 UHPLC system coupled to an Agilent 6495B QQQ mass spectrometer with positive-ion detection. Separation was carried out using a 10cm long C18 UPLC column with an ammonium acetate buffer (A) and methanol (B) as the mobile phase for gradient elution (5% to 75% B in 10 min), at 40 °C and 0.20 mL/min. Concentrations of GSH and GSSG were calculated from the IS calibration by interpolating the constructed linear-regression curves, with the analyte to IS peak ratios measured from sample solutions.

***Plasma and RBC free amino acid quantification*:** Heparin anticoagulated WB was centrifuged at 4±2°C for 30 mins at 3000 g (cross-sectional study) and 10 mins at 2000 g (GLY inclusion level and supplementation studies) to separate the plasma, buffy coat, and RBCs. For the cross-sectional study, plasma was removed and deproteinised with 6% sulfosalicylic acid (1:1) to remove plasma proteins and enable reliable determination of free plasma CYS[32]. Samples were centrifuged at 4000 g for 25 mins. The supernatant was filtered through a 0.45 mm PTFE filter and pH adjusted to 2.2. frozen immediately at -80°C. For the GLY inclusion level and supplementation studies, plasma was removed and frozen immediately at -80°C. The RBC pellet was washed three times in cold phosphate buffered saline (PBS) before also being stored at -80°C. Complete free amino acid determination of plasma and RBCs was performed at the Amino Acid Laboratory, University of California (Davis), USA[33]. Amino acid quantification was conducted using a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK).

White blood cell glutathione measurement: Measurement of GSH concentration in WBC subsets was performed by The Ohio State University Veterinary Clinical Flow Cytometry Service (Columbus, OH, USA). Methods performed were adapted from Webb *et al.*[34] utilizing a Cytek^{Ò} Northern Lights (Cytek Biosciences Inc.) spectral flow cytometer. Markers of WBC subsets CD21 (B-cells; clone CA2.1D6, Bio-Rad Laboratories Inc., Hercules, CA, Cat No. MCA1781R), CD4 (helper T-cells; clone vpg34, Bio-Rad Laboratories Inc., Cat No. MCA1346F), CD8 (cytotoxic T-cells; clone vpg9, Bio-Rad Laboratories Inc., Cat No. MCA1347GA), CD14 (monocytes, clone TÜK4, Bio-Rad Laboratories Inc., Cat No. MCA1568GA), and a viability marker (propidium iodide, Sigma-Aldrich, Merck, Burlington, MA, Cat No. P4170) were used to segregate WBC subsets as part of a single multiplexed panel. In addition to the above subsets, granulocytes (neutrophils, eosinophils, and basophils together) were quantified and segregated according to characteristic light scatter properties and the absences of expression of CD21, CD4, CD8, or CD14. Briefly: to assess GSH in the cells, a non-fluorescent substrate, monochlorobimane (mBCl, Thermo Fisher Scientific Inc., Waltham, MA, Cat No. M1381MP) was included in the panel, which forms fluorescent adducts with glutathione when catalyzed by the enzyme glutathione-S-transferase. The median florescence intensity (MFI) of the mBCl-glutathione complex was recorded for each WBC population subset (B-cell, CD4+ T-cells, CD8+ T-cells, monocytes, and granulocytes). MFI were then correlated to the amount of GSH per cell using a standard curve generated by serial dilutions of known quantities of feline peripheral blood WBCs assessed by both flow

cytometry and a commercial glutathione assay kit (Cayman Chemical, Ann Arbor, MI). Pairwise contrasts were performed for each of the WBC subsets considered both as the number of each subset per µL of blood (absolute cell counts) and as the amount of GSH per cell within each subset.

***Mitogen-induced lymphoproliferative response*:** Heparin anticoagulated WB was centrifuged at RT for 30 mins at 390 g. The buffy coat was removed and WBCs isolated by Histopaque gradient density separation (Histopaque®-1077, Merck, Cat No. 10771 and Histopaque®-1119, Merck Cat No. 11191). Isolated WBCs were counted using a Beckman Z2 particle cell counter, plated at $1x10^6$ cells/well in triplicate for each stimulation condition. Concanavalin A (Con A; from Jack Bean, Merck, Cat No. C5275) was added to the wells at either 0, 1 or 10 mg/mL. Cells were incubated at 37°C in a 5% CO_2 enriched environment for 96 hrs. After incubation, plates were centrifuged at RT for 10 mins at 350 g and a tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Merck, Cat No. M5655) performed as per manufacturer's instructions to quantify viable cells as a proxy of proliferation relative to the control.

***Serum Amyloid A*:** Measured using the Multispecies "PHASE"™ Serum Amyloid A ELISA Assay Kit (TriDelta Development Ltd. Boonton, NJ, USA, Cat No. TP-802) according to the manufacturer's instructions.

Oxidative DNA damage: 8-hydroxy-2' -deoxyguanosine (8-OHdG) was measured using the OxiSelect™ Oxidative DNA Damage ELISA Kit (Cell Biolabs, Inc., USA, Cat No. STA-320) according to the manufacturer's instructions. Urine samples collected for the measurement of F_2-IsoPs were stored at −80 °C prior to shipment to Vanderbilt Eicosanoid Core Laboratory (Nashville, Tennessee, USA) for quantification of F_2-IsoPs $F_{2\alpha}$ ($PGF_{2\alpha}$), free 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP; and 5-series F_2-IsoP by gas chromatography-negative ion chemical ionization-mass spectrometry employing stable isotope dilution as described in Milne *et al.*[35] and Milne *et al.*[36].

Biochemistry and haematology: Performed at IDEXX laboratories, USA. See Supplementary Table 4 and Supplementary Table 5 for biochemistry and haematology parameters, respectively.

Statistical Methods

The sample size for the cross-sectional study was determined using variance estimates for RBC total glutathione from a previous unpublished study in dogs at the Waltham Petcare Science Institute. In order to use these data to power for this cross-sectional study, variability in cats was assumed to be comparable to dogs. The variance components were used to simulate 1000 data sets, with the 25% effect size in the senior group. A linear mixed effects model was fit to each data set, with age as the fixed effect and animal as the random effect. Planned comparisons for this study were between the young adult and senior groups.

Data on GLY from a previous, unpublished study conducted in cats were used to estimate the variance components for calculating the sample size for the GLY inclusion level study. The variance components for plasma GLY and RBC GLY were estimated and used to simulate 1000 data sets, with fold changes of 23% and 30% induced on the final sampling occasion in one diet group for plasma GLY and RBC GLY, respectively. A linear mixed effects model was fit to each data set for each measure with diet, sampling occasion and their interaction as the fixed effects and individual cat as the random variable. Planned comparisons for the GLY inclusion level study were the change from baseline at each time point compared between groups.

Data collated from the cross-sectional study were used to power for the GLY feeding study by estimating the variance components of RBC total glutathione in pmoles and simulating 1000 data sets, with a 40%-fold change induced on final sampling occasion. A linear mixed effects model was fit to each data set, for each measure, with diet group, sample occasion and their interaction as the fixed effects and individual cat as the random effect. Planned comparisons for the GLY feeding study were between the diet groups at each sampling occasion.

For all analyses, power was calculated as the percentage of simulated data sets where all planned contrasts involving the fixed effect level where the effect size was induced were statistically significant. The sample size was determined as the minimum number of animals required to detect the desired effect size with a minimum of 80% power.

For the cross-sectional study all sampling occasions were combined prior to being fit to a linear mixed effects model, with age group as the fixed effects and individual cat as the random effects. The model residuals were examined visually to assess the assumptions of the

model (linearity of predictors, normality of residuals, independence of variables and homoscedasticity). To improve agreement with model assumptions, a log10 transformation was applied to some parameters, prior to model-fitting. Plasma GSH to GSSG ratio data was log10 transformed after the addition of 1 (the minimum value to remove negative values). The mean of each measure for each age group and the difference between age groups (or fold change where data were log10 transformed) were estimated with 95% confidence intervals (CI).

For all measures in the GLY inclusion level and feeding studies, the data were fit to a linear mixed effects model (to account for repeated measures), with diet, sampling occasion and their interaction as the fixed effects, and individual cat as the random effect. The model residuals were examined visually to assess the assumptions of the model. To improve agreement with model assumptions, a log10 transformation was applied to some parameters, prior to model-fitting. The estimated means and 95% family-wise CI were extracted from the model for each diet at each sampling occasion.

In the GLY inclusion level study, the change from baseline to each subsequent time point was compared between the diets.

With the data from the GLY feeding study, the baseline value for each animal was included in the model as a covariate to account for any differences between the groups that may exist. Contrasts were made between diets at each of the sampling occasions.

For both the cross-sectional and GLY feeding studies the primary measure was RBC total glutathione, whilst the primary variables for the GLY inclusion level study were plasma and RBC GLY. For all comparisons, the estimated differences or fold changes were reported alongside the 95% family-wise CI and single-step adjusted *P*-values. Statistically significant differences were determined when the *P*-value ≤ 0.05 for all measures. Statistical analyses were performed in R v4.1.2[37] using libraries *nlme*[38], *multcomp*[39], *lme4* and *ggplot2*[40].

Results

Impact of ageing on glutathione (cross-sectional study)

Of the 32 cats enrolled for the cross-sectional study, one of the young adult cats, aged 2.8 years, was removed for health reasons unrelated to the study prior to any data being recorded. In addition, three cats (2 young and 1 senior) had one measure missing for WB glutathione due to not obtaining sufficient blood during sampling.

Red blood cell and whole blood glutathione: Significantly lower RBC total glutathione was observed in the senior cats (> 9 years) compared to the young adult (< 3 years) age group (fold change 0.7, 95% CI (0.6, 0.9); P = 0.008) (Figure 2a, Table 1). Mean RBC total glutathione was 672.3 (549.8, 821.9) mM in the senior cats and 946.6 (769.1, 1165) mM in the younger adult group. Additionally, significantly lower WB total glutathione was observed in the senior compared to the young adult age group (difference -4.1, 95% CI (-7.3, -0.8) mM; P < 0.02) (Figure 2b, Table 1). Furthermore, lower concentrations of GSH (RBC: fold change 0.4, 95% CI (0.2, 0.6); WB: difference -6.9, 95% CI (-10.6, -3.2) mM) and higher concentrations of GSSG (RBC: fold change 3.8, 95% CI (2.5, 5.7); WB: fold change 2.8, 95% CI (1.9, 4.2)) were observed in the senior animals, leading to further significant differences compared to the younger adult cohort in RBC and WB (all P < 0.001) (Table 1).

Plasma glycine, cysteine and methionine profiles: No significant differences between age groups were observed in the plasma concentrations of free GLY (P = 0.81), CYS (P = 0.88) and methionine (P = 0.58) (Table 1). Free concentrations of all three AA were within reference ranges for plasma AA in healthy adult cats[33].

Comparing different levels of glycine supplementation (GLY inclusion level study)

Of the 52 senior cats enrolled for the dosing study, one cat was removed due to healthcare concerns unrelated to the study.

Plasma and red blood cell glycine: For plasma, free GLY levels were significantly different between the final and baseline timepoints at levels of 1.5% (difference 47.1, 95% CI (24.4, 69.8) mM) and 6.0% (difference 179, 95% CI (156, 201) mM) free GLY dietary supplementation (all P < 0.001) (Table 2). A difference was not observed in free plasma GLY for the 0.5% free GLY inclusion level (P = 0.9) (Table 2). Dietary free GLY supplementation

at 0.5% (difference 94.2, 95% CI (55.2, 133.0) mM), 1.5% (difference 86, 95% CI (46.9, 125.0) mM) and 6.0% (difference 174, 95% CI (136, 213.0) mM) led to significant differences in free RBC GLY levels between final and baseline timepoints (all $P < 0.001$) (Table 2).

These data informed the selection of the 1.5% GLY as-fed dietary supplementation level for the subsequent feeding study.

Impact of glycine supplementation on GSH and markers of oxidative stress (GLY feeding study)

Of the 44 senior cats recruited to the main feeding study ten (7 female, 3 male) were removed during the 16-week trial, all due to poor consumption of the study diets, leading to weight loss that exceeded the threshold limits defined for the study (-10% ideal BCS). This comprised the removal of two control cats (both female) during the acclimation phase, and three control cats (2 female, 1 male) and five test cats (3 female, 2 male) during the test phase, totalling five control and five test cats. This meant the feeding study completed with >75% power against the primary measure, RBC total glutathione, to detect a 40% difference between the groups. Data from cats removed during the feeding study were excluded from the statistical analysis of the study measures. Bodyweights of the 34 senior cats were not significantly different between the diet groups throughout the supplementation study ($P = 0.62$) (Supplementary Figure 1).

Red blood cell glutathione: The dietary intervention influenced RBC glutathione; total glutathione concentrations were found to be significantly higher in the GLY supplemented group than the control group at week 4 (difference 0.7, 95% CI (0.1, 1.3) nM; $P = 0.02$) (Figure 3a, Table 3). Additionally, at week 4, significantly higher RBC GSH levels were observed with 1.5% free GLY supplementation (difference 1.2, 95% CI (1, 1.5) ng; $P = 0.03$) (Figure 3b, Table 3). No further statistically significant differences were identified at the remaining test phase sampling points for RBC total glutathione or GSH; although a trend not meeting the statistical level of significance, was observed for total glutathione, with higher concentrations in the test diet group compared to the control diet group at week 12 ($P = 0.06$). Statistically significant differences were also not found for RBC GSSH or GSH:GSSG between the control and free GLY supplemented groups (P ³ 0.12) (Figure 3c, 3d, Table 3).

White blood cell glutathione: The impact of 1.5% free GLY supplementation on glutathione concentration within WBCs was assessed in specific cell populations: CD4+ T-cells, CD8+ T-cells, CD14+ monocytes, CD21+ B-cells, and granulocytes, via flow cytometry (Supplementary Table 2). One statistically significant difference was identified; the absolute number of CD21+ cells (a marker for B-cells) was higher in the test group compared to the control group at week 8 of the test phase (difference 463, 95% CI (188, 738.0) cells/mL; $P <$ 0.001). No further statistically significant differences in glutathione concentration were observed, measured either via the number of cells of each subset per µL or the quantity of GSH per cell.

Red blood cell and plasma amino acids: Levels of free GLY were found to differ between the test and control diet groups in the GLY supplementation study (Figure 4) (Table 3). Free plasma GLY levels were significantly higher in the test group at all sampling timepoints in the test phase compared to the control group (*P* £ 0.009, Table 3) (Figure 4a, Table 3). Free RBC GLY was found to be significantly higher for the test group at the first and last test phase timepoints compared to the control group (*P* £ 0.004, Table 3 (Figure 4b) (Table 3). Aside from GLY, several AAs profiled from plasma and RBCs indicated a statistically significant difference between diet groups only at one or two of the study's sampling timepoints (Supplementary Table 3). For plasma, these instances were observed at week 8 for free L–isoleucine, L–leucine, L–lysine, L–ornithine and L–valine, with levels of these amino acids being significantly higher in the test group compared to the control group (all *P-values* £ 0.024 and maximum difference of 19.2 mM) (Supplementary Table 3a). Additionally, concentrations of free L–serine at week 4 (difference 1.1, 95% CI (1, 1.3) mM; $P = 0.04$) and L–ornithine at week 12 (difference 1.2, 95% CI (1, 1.4) mM; $P = 0.02$) were significantly higher in the test group compared to the control group within plasma (Supplementary Table 3a). In contrast, levels of free L–asparagine, L–histidine, methionine and L–proline in RBCs were found to be lower at week 8 in the test compared to the control study group (all *P-values* £ 0.044 and maximum difference of -5.3 mM) (Supplementary Table 3b).

Lymphoproliferative response: Cats offered the diet supplemented with 1.5% free GLY had ConA-induced lymphoproliferative activities not significantly different to control group cats throughout the test phase (all *P* ³ 0.35) (Table 4).

Serum Amyloid A: Concentrations of SAA were not significantly different between the diet groups throughout the supplementation study (all *P* ³ 0.13) (Table 4).

***Oxidative damage markers**:* Dietary supplementation of GLY was found to influence 8-OHdG and some of the urine F_2-IsoPs measured as markers of oxidative damage (Table 4). The concentration of 8-OHdG was observed to be significantly lower for the test cats compared to the control group at week 8 (difference 0.7, 95% CI (0.6, 0.9) nG/mL; P = 0.004), but was not found to be significantly different between the groups at the beginning and end of the dietary supplementation phase (P ³ 0.13) (Table 4). Of the four F_2-IsoPs quantified, comparisons between the study groups identified statistically significant differences for both $PGF_{2\alpha}$ (difference 0.6, 95% CI (0.4, 1.0) ng/mg Creatinine (Cr); P < 0.05) and 2,3-dinor-5,6-dihydro-8isoPGF$_{2\alpha}$ (difference 0.7, 95% CI (0.5, 1.0) ng/mg Cr; P = 0.04), with levels lower for the GLY supplemented group compared to the control group (Table 4).

***Biochemistry and haematology**:* A small number of parameters within the biochemistry panel were statistically significant between diet groups (Supplementary Table 4). Cholesterol levels were significantly lower for the test group compared to the control group at weeks 4 and 8 (P £ 0.01). At week 8, symmetric dimethylarginine (SDMA), Na:K ratio and creatine kinase were significantly higher (P £ 0.03), while phosphorous and cholesterol levels were significantly lower for the test group compared to the control group. At week 12, levels of SDMA and blood urea nitrogen (BUN) were found to be significant higher in the test group compared to the control group (P £ 0.03).

Amongst the spectrum of parameters measured in the haematology analysis, only four significant differences were identified (Supplementary Table 5). These were two basophil parameters at week 4, where both Basophils and % Basophils were significantly higher in the test group compared to the control group (P £ 0.004), and at week 12 where MCV was significantly higher and MCHC was significantly lower in the test group compared to the control group (P £ 0.04).

Discussion

Glutathione is an antioxidant, important to the maintenance of oxidative defence, which, in turn, governs cellular survival. This series of investigations set out to establish whether glutathione is impacted by ageing in cats, and if supplementation with glutathione precursor GLY could alter circulating GLY and glutathione levels, and markers of oxidative stress over a 12-week dietary supplementation period in healthy, senior cats.

The preliminary, cross-sectional study indicated that senior cats have reduced WB and RBC levels of glutathione compared with younger adult cats. Both forms of the antioxidant, GSH and GSSG, were determined. In the reduced state, GSH can react with CYS within proteins to maintain their reduced forms or with ROS to neutralise them. The latter causes GSH to become reactive and form a disulphide bridge with another reactive GSH molecule to form a GSSH disulphide[41]. GSH can be regenerated from GSSG by glutathione reductase (GSR) in the presence of NADPH. In healthy cells, approximately 90% of the total glutathione pool is GSH, roughly 10% GSSG and decreased GSH: GSSG is considered indicative of oxidative stress[42]. Significantly lower RBC GSH:GSSG was observed for the senior cats compared to their younger counterparts, which may be indicative of greater oxidative stress. An imbalance in glutathione metabolism, defined by lower GSH:GSSG, has been linked to a number of chronic diseases including feline chronic renal failure[43] and in humans, cancer, neurodegenerative disorders, cystic fibrosis, viral infection, diabetes mellitus, renal failure and liver disease[44]. Cats are thought to be particularly susceptible to oxidative injury because feline haemoglobin contains 8-10 reactive sulfhydryl groups rather than 4 as in the dog and other mammalian species[16,41,45,46]. Therefore, cats may have a higher requirement for GSH to maintain sulfhydryl groups in the GSH form for the maintenance of normal function.

No significant age-related differences were observed in plasma levels of GLY, CYS or methionine. This may reflect the fact that all of the animals in the cross-sectional study were healthy and offered appropriate amounts of complete and balanced diets. As the plasma levels of GLY and CYS were within normal range for both age groups, the lower levels of glutathione in the senior cats may be due to age related changes in the activity of enzymes involved in glutathione synthesis[17]. Reports in humans suggest that older individuals have lower intracellular levels of GLY and CYS due to slower body protein turnover or decreased *de novo* synthesis[21,47]. However, protein turnover in cats is suggested to be higher than in

humans due to the reliance on protein metabolism for energy[48]. There is evidence for a deceleration of overall protein turnover with ageing in humans[49], which suggests that the supply of GLY and CYS could be decreased. Impaired protein digestibility has been reported with age in cats[50,51] which could affect intracellular amino acid levels and hence glutathione metabolism. In future studies, measuring RBC levels of these amino acids could be more informative than plasma levels, particularly if investigating changes due to supplementation.

For the GLY feeding study, 1.5% free GLY was selected for supplementation from the inclusion level study. Using the average body weight of a cat and average consumption, 1.75 mM/kg/day total GLY was fed in the GLY feeding study. This GLY level is roughly equivalent to the 1.33 mM/kg/day dose used in human dietary intervention studies[21,23]. In the GLY feeding study, levels of RBC total glutathione and GSH were significantly elevated in the test group of senior cats receiving 1.5% free GLY supplementation compared to the control group at week 4. No further statistically significant observations were identified in the feeding study. However, RBC total glutathione indicated a trend towards higher concentration in the test group compared to the control group at week 12. Collectively, these findings suggest that supplementation with free GLY, has some capacity to increase intracellular glutathione concentrations in healthy senior cats. Early in the supplementation phase, an excess of GLY may drive the reaction with c-glutamylcysteine (c–GC), increasing *de novo* generation of glutathione in the second part of a two-step reaction catalysed by GSH synthase[52]. The drive towards glutathione biosynthesis may continue until either one of two circumstances arise. Substrate availability of c–GC declines, impacting the rate of the secondary enzymatic reaction via GSH synthase, further reducing glutathione synthesis. Alternatively, the glutathione pool could increase until a threshold has been reached which elicits a competitive feedback inhibition, in which glutathione competes with glutamate for glutamate cysteine ligase (GCL), limiting the rate of the primary synthesis reaction[53]. Either consequence restricts further glutathione synthesis and could explain the subsequent decline in RBC intracellular levels at week 8, where the study groups were found to have comparable glutathione levels. The trend observed at week 12 where higher RBC total glutathione was observed in the test group might suggest the rate limiting condition being overcome, enabling the loop to cycle back to re-establish glutathione synthesis and increase intracellular concentrations once again.

Levels of glutathione observed in the GLY feeding study were not found to be comparable to the cross-sectional study. None of the individuals within the study cohorts were the same

between the studies. Glutathione concentrations in the cross-sectional study were not normalised to haemoglobin based on the assumption that variation in RBC haemoglobin levels in healthy cats, including seniors, is small[54,55]. This could provide an explanation for the sizeable difference observed. Another important consideration is the difference in methods for the measurement of glutathione. The mass spectrometry-based approach (UPLC-MRM MS) used in the feeding study and the ELISA method utilised in the cross-sectional study are not comparable. Furthermore, it is acknowledged that the measurement of glutathione may be subject to inaccuracies due to the development of post-sampling artefacts[56]. Oxidation biases the pool of GSSG, leading to its overestimation[56]. Concentrations reported elsewhere for clinically normal client-owned cats with mean age 10.62 ± 0.76 years are 4.24 ± 0.67 mM for WB GSH and 19.66 ± 2.75 mM for WB GSSH[26].

The feeding study included the measurement of glutathione in WBCs and markers of immune function, such as lymphocyte proliferation. There is evidence in humans that dietary supplementation with pressurized whey protein isolate can alter lymphocyte glutathione[57]. Glutathione has also been shown to play an important role in the activation of T-lymphocytes[58]. In addition, depletion of intracellular glutathione reduced lymphocyte differentiation and proliferation in humans and rodents[59,60]. Of particular relevance is the age-associated reduction in lymphoproliferative response to the T-lymphocyte specific polyclonal lymphocyte activator, ConA which has been shown to be partially restored by dietary glutathione supplementation in mice[61]. However, here attempts to increase WBC glutathione (GSH) via dietary GLY supplementation were largely unsuccessful, although one statistically significant observation was noted for CD21+ cells at week 8. This suggests elevations in glutathione brought about by free GLY supplementation may not be limited to RBCs.

Along with other AAs, levels of GLY were measured in both plasma and RBCs. This confirmed that the supplementation level selected was sufficient to significantly elevate plasma and RBC GLY concentrations in the test group of senior cats throughout the 12-week test phase. RBC GLY levels were found to significantly differ between the study groups post-supplementation at weeks 4 and 12. However, statistical significance was not achieved at the intermediate sampling timepoint. These findings align with the observations for RBC total glutathione and the associated hypothesis proposed. Higher RBC GLY levels were observed at the same time as the elevations in intracellular glutathione concentrations at week 4 (P =0.02) and week 12 (P =0.063) of the supplementation phase. In between (week 8),

concentrations of RBC GLY were also higher, but not significantly different from the control group of senior cats and RBC total glutathione was not statistically significant. Taken together, the rate of synthesis of the antioxidant may be declining. A mechanism to moderate AA uptake into RBCs could also be at play. Adaptive regulation for transport of neutral AAs, including GLY, across membranes, has been reported for mammalian cells[62]. Another plausible explanation for the difference in the observations in RBC GLY between the test phase timepoints is fluctuation in the intake of the test diet, with a possible decline after week 4, which is subsequently restored prior to week 12. However, diet consumption data collected for the feeding study confirmed the relative consistency in the intake of both diets (Supplementary Figure 2). Dietary supplementation of GLY significantly impacted levels of other AA in plasma and RBCs, predominantly at the intermediate timepoint measure. Similarly, enrichment of numerous AAs including leucine, isoleucine, valine, ornithine and proline were determined in plasma in a study of GLY metabolism conducted via oral administration of ^{15}N-GLY in young adult men[63]. Given the hypothesis proposed regarding reduced glutathione biosynthesis around this timeframe within our study, conversion of excess GLY to other amino acids offers a plausible explanation.

To explore the impact of dietary GLY supplementation beyond glutathione, several markers of oxidative stress, shown to be clinically relevant in ageing[64] were explored. Acute phase proteins (APPs) have been shown to positively correlate with RBC glutathione concentration in humans[65]. One such APP, SAA, has been reported to be a more responsive marker of inflammation than C-reactive protein in cats[66]. Levels of SAA could not be differentiated between the feeding study groups. Other markers, namely F_2-IsoPs, have been publicised as the best available indicators of oxidative stress for humans. Furthermore, F_2-IsoPs have been shown to increase in different biofluids over the human lifespan[67] and to be higher in elderly verses younger subjects[21]. Measurement of F_2-IsoPs is commonly performed in urine due to their chemical stability brought about by a lack of artificial auto-oxidation[68]. Urine $PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8iso $PGF_{2\alpha}$, were significantly lower in the test group compared to the control group, following 12 weeks of 1.5% free GLY supplementation. Produced during periods of inflammation and oxidative stress, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is an eicosanoid, which promotes the formation of 8-iso-$PGF_{2\alpha}$ and 2,3-dinor-5,6-dihydro-8iso $PGF_{2\alpha}$[69,70]. In addition, although not meeting the level of statistical significance (P = 0.06), there was a trend towards lower urinary 8-iso-$PGF_{2\alpha}$, in the test group. Our findings indicate a reduction in these markers of oxidative stress in senior cats eating a diet supplemented with free GLY. This is consistent with the human intervention study by Sekhar *et al.* in which dietary GLY and CYS supplementation, targeting age-associated glutathione decline, significantly lowered F_2-IsoPs[21]. Further oxidative damage, to DNA specifically, was investigated via plasma 8-OHdG, one of the best measures of

the mutagenic consequences of oxidative stress[31]. Human studies provide evidence that 8-OHdG levels increase with age[28,71]. A significant decrease in 8-OHdG was identified at week 8 in the test group compared to the control group, which was not apparent at week 4 or 12. As with lymphocyte glutathione, these findings align with intervention studies conducted in other species; dietary supplementation with glutathione[72] and vitamins E and C[73] have been shown to reduce 8-OHdG in diabetic patients[72] and aged rats[73], respectively. Taken together, these results suggest dietary supplementation of GLY alters markers of oxidative stress in senior cats, which appears to be dependent on an increase in RBC glutathione. These findings align with a review by Zhong *et al.*[74], who discuss the protective effects of GLY, suggesting it as a novel anti-inflammatory, immunomodulatory, and cytoprotective agent. For oxidative stress, they propose various mechanisms by which GLY prevents ROS formation[74].

The results of the feeding study suggest dietary supplementation with 1.5% free GLY, a precursor of glutathione, may offer a viable route to alleviating the age associated reduction in glutathione observed in senior cats. Further work is needed elucidate the mechanism(s) by which this occurs. As discussed earlier, *de novo* glutathione synthesis is dependent on sufficient availability of GLY and c–GC. In turn, the pool of c–GC is dependent on a prior reaction with GCL, requiring glutamate and CYS[51]. Supplementation with c–GC in human and rodent studies results in increased glutathione, confirming biosynthesis of glutathione can occur without the first enzyme reaction[75-77]. Therefore, CYS could be another limiting factor in the synthesis of glutathione, although supplementation with CYS must be carefully considered due to its possible toxicity[78]. Unfortunately, this could not be assessed as for technical reasons, levels of CYS were not determined in the feeding study. Exploring mechanisms by which GLY may reduce oxidative stress in senior cats in conjunction with other glutathione precursors is therefore warranted.

Other than the limitations discussed thus far, increased numbers of animals completing the study would have improved the ability to detect a difference in the supplementation study. Notably, two trends were observed within the study, one for RBC total glutathione ($P = 0.06$) and another for one of the F_2-IsoPs ($P = 0.06$), both week 12 (end) measures. Such trends might have led to statistically significant differences if a larger senior feline cohort had been used.

In summary, we have been able to confirm that levels of glutathione, the most abundant antioxidant, are significantly lower in WB and RBCs in older, senior cats compared to younger adult cats. Offering older cats a dry diet supplemented with 1.5% as-fed free GLY for 12 weeks induced significant elevations in RBC total glutathione and GSH, although this was limited to early in the supplementation phase. Supplementation of GLY was also found to influence WBC GSH levels and markers of oxidative stress, although only at certain timepoints within the study.

Overall, the results suggest age-associated reduction in RBC glutathione in cats may be partially resolved by dietary free GLY supplementation at the concentration tested. Further studies investigating the mechanism(s) of action of GLY and the impact of supplementation with other glutathione precursors are needed.

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Conflict of Interest

This work was funded by Mars Petcare; all authors except for SJME were employees of Mars Petcare at the time of the study. Data in this manuscript have been used to support a patent application.

Authorship

AR conducted the analysis and interpretation of data for the GLY feeding study, and led drafting of the manuscript. JEA designed, organised and conducted the cross-sectional study, including analysis and interpretation of data. RE designed, organised and conducted the GLY inclusion level and feeding studies, including analysis and interpretation of data for the GLY inclusion level study. LCM conducted statistical analysis of data including powering analyses. YBL supported with study design, analysis and interpretation of data for the GLY inclusion level and feeding studies. SJME conducted method development and analysis of leukocyte glutathione. LH supported the analysis and interpretation of data for the GLY feeding study. MH led the conception of the work. PW supported the conception, design, and interpretation of data for all studies. All authors critiqued the manuscript, and read and approved the final version.

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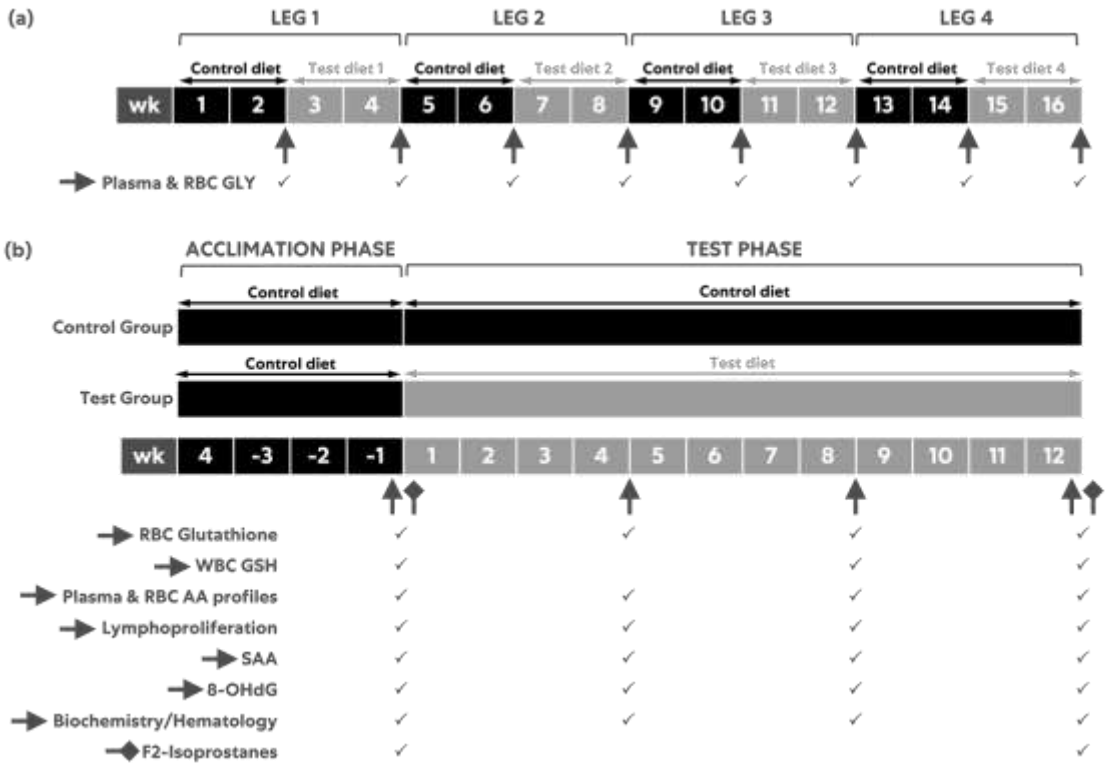

Fig 1: Glycine (a) inclusion level and (b) feeding study design schematics. Blood (triangular arrows) and urine (diamond arrows) sample collection time points and associated sample measures (ticked where measured).

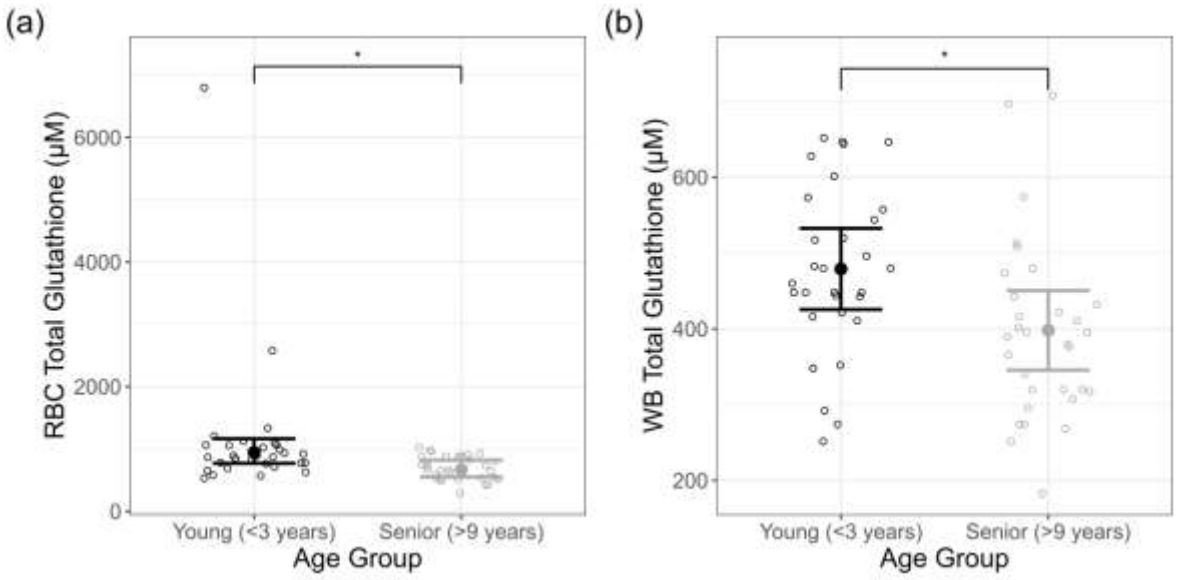

Fig 2: Mean (a) red blood cell (RBC) and (b) whole blood (WB) total glutathione (mM) by age group. Individual data are shown as open circles and means as solid circles with 95% confidence intervals. * indicates significance between groups (*P* £ 0.05).

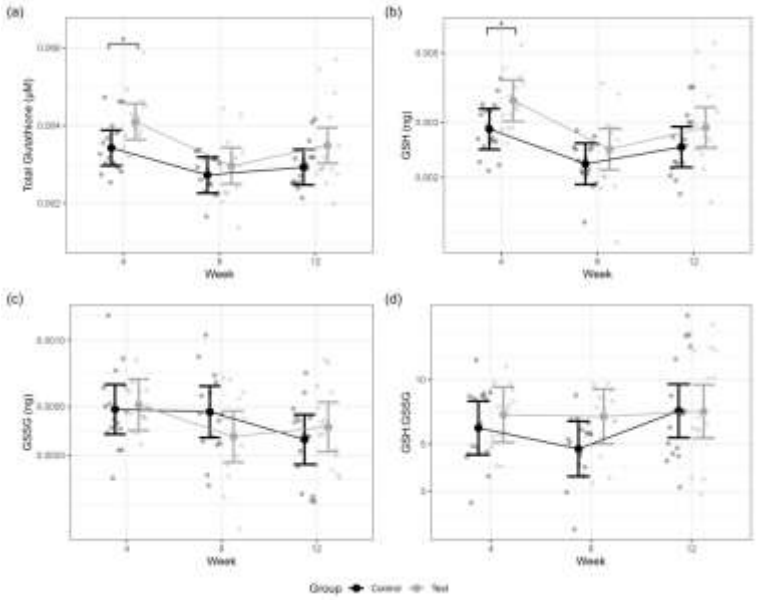

Fig 3: Mean red blood cell (RBC) glutathione concentrations for the GLY feeding study test (supplemented) and control (unsupplemented) senior cats. RBC glutathione represented as (*a.*) total glutathione (*b.*) reduced glutathione (GSH), (*c.*) oxidised glutathione (GSSG), and (d.) GST:GSSG. Individual data are shown as open circles and means as solid circles with 95% confidence intervals. * indicates significance between groups (P £ 0.05).

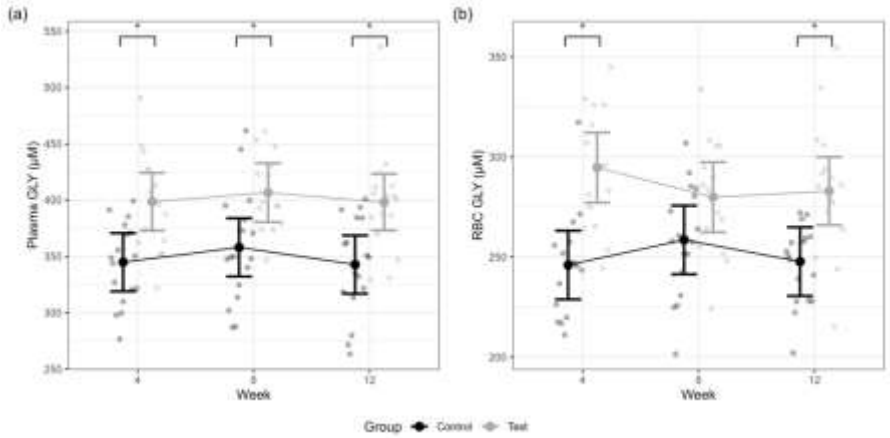

Fig 4: Mean (a) plasma and (b) red blood cell (RBC) glycine (GLY) levels from the effect of the GLY feeding study diet groups. Individual data are shown as open circles and means as solid circles with 95% confidence intervals. * indicates significance between groups (*P* £ 0.05).

Table 1. Red blood cell (RBC) and whole blood (WB) glutathione concentrations, and free plasma glycine (GLY), cysteine (CYS) and methionine levels in the cross-sectional study young adult and senior cats.

Measure	Young Adult (<3 y)	Senior (>9 y)	Difference (Senior – Young Adult) in Means	Fold Change (Senior / Young Adult) in Means	*P* value
RBC total glutathione (mM)	946.6 (769.1, 1165)	672.3 (549.8, 821.9)		0.7 (0.6, 0.9)	0.008
RBC GSH (mM)	858.3 (586.5, 1256.1)	302.7 (209.4, 437.8)		0.4 (0.2, 0.6)	<0.001
RBC GSSG (mM)	71.4 (51.1, 99.7)	268 (193.9, 370.3)		3.8 (2.5, 5.7)	<0.001
RBC GSH:GSSG	12 (6.9, 21.1)	1.12 (0.65, 1.93)		0.1 (0.1, 0.2)	<0.001
WB total glutathione (mM)	479.1 (425.6, 532.7)	398.1 (345.6, 450.5)	-81.1 (-146.7, -15.4)		0.016
WB GSH (mM)	446.1 (386.3, 505.8)	308.7 (249.7, 367.6)	-6.9 (-10.6, -3.2)		<0.001
WB GSSG (mM)	26.9 (19.5, 37.3)	75.2 (54.5, 103.5)		2.8 (1.9, 4.2)	<0.001
WB GSH:GSSG	16 (10.5, 24.2)	3.7 (2.4, 5.6)		0.2 (0.1, 0.4)	<0.001
Plasma GLY (mM)	373.8 (352.4, 395.1)	377 (356.3, 397.7)	3.2 (-22.9, 29.3)		0.810
Plasma CYS (mM)	21 (19.2, 23.1)	20.8 (19.1, 22.8)		1 (0.9, 1.1)	0.876
Plasma methionine (mM)	52.4 (46.6, 58.2)	50.4 (44.7, 56)	-2.03 (-9.13, 5.08)		0.577

All values are means and brackets indicate 95% confidence intervals of the mean ($P \leq 0.05$). GSH, reduced glutathione; GSSG, oxidised glutathione; GSH:GSSG, reduced to oxidised glutathione ratio.

Table 2. Free plasma and red blood cell (RBC) glycine (GLY) levels from the effect of the GLY inclusion level study diet groups.

% Dosing Free GLY	GLY (mM)	Baseline	Final	Difference (Final – Baseline) in Means	*P* value
0%	Plasma GLY	355 (334, 376)	355 (334, 376)	0.1 (-22.4, 22.6)	1
0%	RBC GLY	282 (251, 312)	280 (250, 311)	-1.1 (-39.8, 37.6)	1
0.5%	Plasma GLY	359 (337, 380)	380 (358, 401)	20.8 (-1.8, 43.4)	0.086
0.5%	RBC GLY	253 (222, 283)	347 (316, 378)	94.2 (55.2, 133)	<0.001
1.5%	Plasma GLY	354 (333, 376)	401 (380, 423)	47.1 (24.4, 69.8)	<0.001
1.5%	RBC GLY	246 (215, 277)	332 (301, 363)	86 (46.9, 125)	<0.001
6.0%	Plasma GLY	365 (343, 386)	544 (522, 565)	179 (156, 201)	<0.001
6.0%	RBC GLY	268 (237, 298)	442 (411, 473)	174 (136, 213)	<0.001

All values are means and brackets indicate 95% confidence intervals of the mean ($P \leq 0.05$).

Table 3. Red blood cell (RBC) glutathione and glycine (GLY) concentrations, and free plasma GLY and cysteine (CYS) levels in the GLY feeding study test (supplemented) and control (unsupplemented) senior cats.

Measure	Test Phase Week	Test	Control	Difference (Test – Control) in Means	Fold Change (Test / Control) in Means	*P* value
RBC total glutathione (mM)	4	0.0041 (0.0037, 0.0046)	0.0034 (0.0030, 0.0039)	0.0007 (0.0001, 0.0013)		0.020
RBC total glutathione (mM)	8	0.0030 (0.0025, 0.0034)	0.0027 (0.0023, 0.0032)	0.0002 (-0.0004, 0.0008)		0.659
RBC total glutathione (mM)	12	0.0035 (0.0030, 0.0039)	0.0029 (0.0025, 0.0034)	0.0006 (0.0000, 0.0011)		0.063
RBC GSH (ng)	4	0.0035 (0.0030, 0.0041)	0.0029 (0.0025, 0.0033)	1.23 (1.0200, 1.5000)		0.029
RBC GSH (ng)	8	0.0025 (0.0021, 0.0029)	0.0022 (0.0019, 0.0026)	1.11 (0.9170, 1.3500)		0.410
RBC GSH (ng)	12	0.0029 (0.0025, 0.0034)	0.0025 (0.0025, 0.0029)	1.15 (0.954, 1.4000)		0.182
RBC GSSG (ng)	4	0.0005 (0.0004, 0.0007)	0.0005 (0.0004, 0.0006)		1.0500 (0.7500, 1.4700)	0.982
RBC GSSG (ng)	8	0.0004 (0.0003, 0.0005)	0.0005 (0.0004, 0.0006)		0.7700 (0.5500, 1.0900)	0.201
RBC GSSG (ng)	12	0.0004 (0.0003, 0.0005)	0.0004 (0.0003, 0.0005)		1.1400 (0.8160, 1.5900)	0.720
GSH:GSSG	4	6.86 (5.10, 9.24)	5.95 (4.45, 7.94)		0.965 (0.701, 1.33)	0.987
GSH:GSSG	8	6.73 (5.00, 9.06)	4.75 (3.52, 6.39)		1.15 (0.796, 1.67)	0.689
GSH:GSSG	12	7.11 (5.32, 9.49)	7.15 (5.35, 9.54)		1.13 (0.781, 1.64)	0.770
RBC GLY (mM)	4	295 (277, 312)	246 (229, 263)	48.7 (26.5, 70.8)		<0.001
RBC GLY (mM)	8	280 (262, 297)	259 (241, 276)	21.3 (-0.9, 43.5)		0.063
RBC GLY (mM)	12	283 (266, 300)	248 (231, 265)	35.2 (13.3, 57.1)		<0.001
Plasma GLY (mM)	4	399 (373, 424)	345 (319, 371)	53.7 (20.9, 86.6)		<0.001
Plasma GLY (mM)	8	407 (380, 433)	358 (332, 384)	48.6 (15.4, 81.8)		0.002
Plasma GLY (mM)	12	398 (373, 423)	343 (317, 369)	55.3 (22.8, 87.8)		<0.001

All values are means and brackets indicate 95% confidence intervals of the mean ($P \leq 0.05$). GSH, reduced glutathione; GSSG, oxidised glutathione; GSH:GSSG, reduced to oxidised glutathione ratio.

Table 4. Oxidative stress measure concentrations in the GLY feeding study test (supplemented) and control (unsupplemented) senior cats.

Measure	Test Phase Week	Test	Control	Difference (Test – Control) in Means	*P* value
ConA (10 mg/mL)	4	0.95 (0.80, 1.13)	1.08 (0.91, 1.28)	0.88 (0.70, 1.10)	0.385
ConA (10 mg/mL)	8	0.87 (0.73, 1)	0.88 (0.74, 1.05)	0.99 (0.79, 1.25)	1.000
ConA (10 mg/mL)	12	0.97 (0.80, 1.17)	0.99 (0.82, 1.18)	0.99 (0.78, 1.25)	0.988
ConA (1 mg/mL)	4	1.12 (0.97, 1.28)	1.24 (1.08, 1.42)	0.908 (0.75, 1.07)	0.352
ConA (1 mg/mL)	8	1.08 (0.94, 1.25)	1.07 (0.93, 1.23)	1.01 (0.84, 1.21)	0.998
ConA (1 mg/mL)	12	1.24 (1.07, 1.45)	1.17 (1.01, 1.36)	1.06 (0.88, 1.29)	0.804
SAA	4	1.92 (1.23, 3.02)	3.1 (1.98, 4.87)	0.621 (0.35, 1.11)	0.128
SAA	8	1.65 (1.04, 2.64)	2.45 (1.51, 3.97)	0.68 (0.37, 1.24)	0.286
SAA	12	1.49 (0.93, 2.37)	2.3 (1.45, 3.67)	0.65 (0.36, 1.17)	0.198
8-OHdG (nG/mL)	4	30.3 (24.8, 37)	37.4 (30.7, 45.6)	0.81 (0.63, 1.05)	0.130
8-OHdG (nG/mL)	8	26.6 (21.8, 32.5)	37.6 (30.8, 45.8)	0.71 (0.56, 0.91)	0.004
8-OHdG (nG/mL)	12	31.4 (25.8, 38.2)	33.9 (27.8, 41.3)	0.93 (0.72, 1.19)	0.821
8-iso-$PGF_{2\alpha}$ (nG/mG Cr)	12	0.84 (0.57, 1.10)	1.16 (0.88, 1.43)	-0.32 (-0.65, 0.01)	0.058
$PGF_{2\alpha}$ (nG/mG Cr)	12	4.71 (3.20, 6.94)	7.66 (5.14, 11.40)	0.62 (0.38, 1.00)	0.049
5-series F2-IsoP (nG/mG Cr)	12	1.68 (1.27, 2.21)	2.09 (1.58, 2.77)	0.80 (0.57, 1.13)	0.197
2,3-dinor-5,6-dihydro-8iso$PGF_{2\alpha}$ (nG/mg Cr)	12	6.65 (5.02, 8.80)	9.63 (7.21, 12.9)	0.69 (0.49, 0.98)	0.040

All values are means and brackets indicate 95% confidence intervals of the mean. ConA, concanavalin A; Cr, creatinine; SAA, serum amyloid A. 8-OHdG, 8-hydroxy-2'deoxyguanosine.