



# Life-long vitamin C supplementation in combination with cold exposure does not affect oxidative damage or lifespan in mice, but decreases expression of antioxidant protection genes

Colin Selman<sup>a,1,\*</sup>, Jane S. McLaren<sup>a,1</sup>, Claus Meyer<sup>b</sup>, Jackie S. Duncan<sup>c</sup>, Paula Redman<sup>a</sup>, Andrew R. Collins<sup>d,2</sup>, Garry G. Duthie<sup>d</sup>, John R. Speakman<sup>a</sup>

<sup>a</sup>Aberdeen Centre for Energy Regulation and Obesity (ACERO), School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK

<sup>b</sup>BioSS, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK

<sup>c</sup>ACERO, Division of Energy Balance and Obesity, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK

<sup>d</sup>Division of Cellular Integrity, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK

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## Abstract

Oxidative stress is suggested to be central to the ageing process, with endogenous antioxidant defence and repair mechanisms in place to minimize damage. Theoretically, supplementation with exogenous antioxidants might support the endogenous antioxidant system, thereby reducing oxidative damage, ageing-related functional decline and prolonging life- and health-span. Yet supplementation trials with antioxidants in animal models have had minimal success. Human epidemiological data are similarly unimpressive, leading some to question whether vitamin C, for example, might have pro-oxidant properties *in vivo*. We supplemented cold exposed ( $7 \pm 2$  °C) female C57BL/6 mice over their lifespan with vitamin C (ascorbyl-2-polyphosphate), widely advocated and self administered to reduce oxidative stress, retard ageing and increase healthy lifespan. No effect on mean or maximum lifespan following vitamin C treatment or any significant impact on body mass, or on parameters of energy metabolism was observed. Moreover, no differences in hepatocyte and lymphocyte DNA oxidative damage or hepatic lipid peroxidation was seen between supplemented and control mice. Using a DNA macroarray specific for oxidative stress-related genes, we found that after 18 months of supplementation, mice exhibited a significantly reduced expression of several genes in the liver linked to free-radical scavenging, including Mn-superoxide dismutase. We confirmed these effects by Northern blotting and found additional down-regulation of glutathione peroxidase (not present on macroarray) in the vitamin C treated group. We suggest that high dietary doses of vitamin C are ineffective at prolonging lifespan in mice because any positive benefits derived as an antioxidant are offset by compensatory reductions in endogenous protection mechanisms, leading to no net reduction in accumulated oxidative damage.

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## 1. Introduction

The biochemical, physiological and functional changes characteristic of advancing age, are believed to be caused by, in

part, reactive oxygen species (ROS) damage (Beckman and Ames, 1998; Golden et al., 2002). To stem this potential damage, a cascade of endogenous antioxidants, including superoxide dismutase, catalase, glutathione peroxidase (Gpx) and glutathione have evolved (Beckman and Ames, 1998). Theoretically, one might anticipate that dietary supplementation with exogenous antioxidants may support the endogenous defence system by scavenging additional ROS, thus reducing oxidative damage. Vitamin C is a powerful water soluble antioxidant (Carr and Frei, 1999) which may protect against lipid damage (Amer, 2002; Huang et al., 2002), protein oxidation (Carty et al., 2000), DNA oxidation (Lenton et al.,

\* Corresponding author. Present address: Centre for Diabetes and Endocrinology, Department of Medicine, University College London, Rayne Institute, 5 University Street, London WC1E 6JJ, UK. Tel.: +44 2076796588; fax: +44 2076796583.

E-mail address: [c.selman@ucl.ac.uk](mailto:c.selman@ucl.ac.uk) (C. Selman).

<sup>1</sup> Both authors contributed equally to this paper.

<sup>2</sup> Present address: Institute for Nutrition Research, School of Medicine, University of Oslo, PO Box 1046 Blindern, N-0316 Oslo, Norway.

1999 but see Cadenas et al., 1997), protect against cognitive impairment (Paleologos et al., 1998) and may reduce incidence of certain cancers (Carr and Frei, 1999; Chen et al., 2000). However, there are very variable effects of life-long vitamin C supplementation in animal studies. A life-long supplementation of 1% ascorbic acid in the drinking water of C57BL/6 male mice was reported to increase mean lifespan significantly (Massie et al., 1984), but a mixed antioxidant diet, which included vitamin C, did not have an effect on lifespan of voluntary wheel running rats (Holloszy, 1998). However, a mixed antioxidant supplemented diet, which included vitamin C, did significantly increase mean lifespan and reduce hippocampal inclusion bodies in ApoE-deficient mice (Veurink et al., 2003). Epidemiological data in humans is similarly confused although some minor benefits to overall mortality risk, coronary heart disease and subsequent risk of stroke, in particular, appear associated with vitamin C supplementation (Enstrom et al., 1992; Gale et al., 1995; Osganian et al., 2003), despite markers of oxidative damage generally being unaffected (Prieme et al., 1997).

The general inability of antioxidants, including vitamin C, in supplementation studies to consistently deliver the promise of increased lifespan in animal models, or reduced disease risk in humans, could have a varied causality (see McCall and Frei, 1999). Perhaps, *in vivo* vitamin C acts more as a pro-oxidant than an antioxidant (Carr and Frei, 1999; Childs et al., 2001; Rehman et al., 1998), possibly necessitating increased activation of the defence system to maintain the status quo. Alternatively, vitamin C may successfully scavenge ROS (Carr and Frei, 1999), but this may not be translated into reduction in damage and lifespan enhancement because of compensatory effects, which may take two forms. Firstly, we know that body size and energy metabolism may have effects on free-radical production (Speakman, 2005; Speakman et al., 2004). If vitamin C supplementation modulates either, or both of these factors, then the effects could offset any direct impact on free-radical scavenging resulting in no net benefit in reduced oxidative damage. Alternatively, vitamin C may negatively affect the endogenous scavenging and repair systems, either directly (Nemoto et al., 1997; Podmore et al., 1998), or indirectly via systems that sense reduced radical production and retarded damage accumulation. These reductions may also offset any benefits leading to no net change in oxidative damage. Finally, oxidative damage might be significantly reduced, but this might have no impact on ageing and lifespan because we have overestimated the importance of these processes, or because the benefits are offset by other effects—for example the generation of genotoxic chemicals which, for example, promote cancer (Sowell et al., 2004).

Cold exposure elevates metabolic rate in mammals (Selman et al., 2001a, 2002a), enhances levels of various antioxidant enzymes and endogenous antioxidants (Barja de Quiroga et al., 1991; Selman et al., 2000; Siems et al., 1999; Spasic et al., 1993), and increases both ROS production (Barja de Quiroga et al., 1991; Venditti et al., 2004) and oxidative stress (Selman et al., 2002a; Topp et al., 2000; Venditti et al., 2004). Therefore, we employed a life-long cold exposure ( $7 \pm 2^\circ\text{C}$ ) paradigm in the

following experiment in order to increase metabolic rate, ROS production and oxidative stress, consequently allowing us to be more likely to detect a beneficial impact of antioxidant treatment if one existed. We measured lifespan in both control and vitamin C (ascorbyl-2-polyphosphate) supplemented mice maintained in the cold ( $7 \pm 2^\circ\text{C}$ ), and examined the impact of supplementation on body mass, parameters of energy balance (resting metabolic rate, daily energy expenditure, food intake), lymphocyte and hepatocyte DNA oxidative damage. In addition, we used an HPLC method to evaluate hepatic lipid peroxidation, as assessed by thiobarbituric acid-reactive substances (TBARS) (Magwere et al., 2006; Navarro et al., 2002). A cDNA-based microarray specific for oxidative stress genes, was used to identify hepatic gene expression changes with both age and vitamin C supplementation, and these findings were verified using standard Northern blotting techniques.

## 2. Materials and methods

### 2.1. Study design

C57BL/6 mice were purchased at 8 weeks of age from a commercial breeder (Harlan, UK Limited, UK) and were individually housed in cages (48 cm  $\times$  15 cm  $\times$  13 cm) at an ambient temperature of  $22 \pm 2^\circ\text{C}$ . From 12 weeks of age onwards, mice were maintained on a 16L/8D light/dark cycle (lights on 06:00), at  $7 \pm 2^\circ\text{C}$  within a controlled temperature room (Atlantic Cooling Services, UK), with access to bedding material and *ad libitum* access to standard mouse diet (Rat and Mouse Maintenance (RM1), Special Diets Services, BP Nutrition, UK) and water. Over a period of 4 weeks of acclimation to  $7 \pm 2^\circ\text{C}$ , bedding material was gradually removed and individuals randomly assigned a 'control' RM1 diet (10 mg kg<sup>-1</sup> ascorbyl-2-polyphosphate) group or on a vitamin C supplemented diet group (RM1 + 180 mg kg<sup>-1</sup> of ascorbyl-2-polyphosphate, equivalent to 18 $\times$  the unsupplemented dietary level). Thus, vitamin C supplementation commenced at 16 weeks of age. All experiments followed institutional guidelines for laboratory animal care and the UK Home Office.

### 2.2. Lifespan analysis

All mice were housed at  $7 \pm 2^\circ\text{C}$ , with a total of 21 female mice maintained on the control diet and 20 females maintained in the vitamin C supplemented diet. All mice were monitored daily but otherwise left undisturbed until they died naturally. If death appeared imminent, individuals were sacrificed following Home Office guidelines to minimize suffering. Kaplan–Meier survival curves were constructed using the known of birth and death dates of each individual. Additional groups of female mice, following the identical protocol described above, were used to examine the effects of vitamin C supplementation on oxidative stress (Comet assay and TBARS) and stress-related gene expression in the liver.

### 2.3. Energy balance

Resting metabolic rate (RMR) was determined at 6 and 22 months of age, i.e. 2 and 18 months following initiation of vitamin C supplementation, using open-flow respirometry (Selman et al., 2001b). All individuals had *ad libitum* access to food and water prior to, but not during measurements. Mice were weighed (0.01 g) immediately before being placed in an airtight Plexiglas chamber within a temperature-controlled incubator (INL-401N-010, Gallenkamp, UK) at  $7 \pm 0.1^\circ\text{C}$ . Air, dried by passing through silica gel, was pumped (Charles Austin Pump Ltd., UK) at 600–800 ml min<sup>-1</sup> (DM3A, Alexander Wright Flow Meter, UK), re-dried and a sub-sample ( $\sim 150$  ml min<sup>-1</sup>) passed through a paramagnetic oxygen and an infrared carbon dioxide analyser (Series 1400, Servomex Group Ltd., UK). Energy expenditure was calculated, corrected for standard temperature and pressure, and the lowest 10 consecutive

readings (equivalent to 5 min within the chamber) in oxygen concentration used to calculate resting metabolic rate ( $\text{ml O}_2 \text{ min}^{-1}$ ).

Daily energy expenditure (DEE) was estimated using the doubly-labelled water technique (Lifson et al., 1955; Speakman, 1997, 1998). In brief, a sub-sample of mice, at 6 and 22 months of age, were weighed (0.01 g) and deuterium (enrichment = 4.63 atom%) and  $^{18}\text{O}$  (enrichment = 9.44 atom%) was administered IP (weighed to 0.0001 g). The isotopes equilibrated for 60 min and then an initial blood sample was taken (50  $\mu\text{l}$  Vitrex pre-calibrated capillaries). All blood samples were immediately flame-sealed and stored until analysis. Final blood samples were collected exactly 24 h after the initial sample and background samples were also collected. Blood samples were distilled into glass Pasteur pipettes (Volac, John Poulten Ltd., UK) and the distillates used for mass spectrometric analysis (Micromass Ltd., UK) of deuterium and  $^{18}\text{O}$ . Data analysis was carried out following the two-sample method, using the plateau technique (Speakman, 1997).

#### 2.4. Comet assay—single cell gel electrophoresis

We employed the modified Comet assay containing an extra post-lysis DNA digestion step using lesion-specific enzymes, to determine DNA oxidative damage in lymphocytes and hepatocytes (Collins, 2004; Collins and Dusinska, 2002). In summary, isolated lymphocytes and hepatocytes were suspended in 85  $\mu\text{l}$  of 1% low melting point agarose and pipetted on to pre-coated standard agarose slides. Cells were lysed for 1 h, via immersion in 2.5 M NaCl, 0.1 M  $\text{Na}_2\text{EDTA}$ , 10 mM Tris-HCl (pH 10) and 1% Triton X-100, leaving only residual nucleoids embedded on the slides. The slides were then washed three times post-lysis with enzyme buffer [0.1 M KCl, 0.5 mM  $\text{Na}_2\text{EDTA}$ , 40 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES) KOH, 0.2 mg  $\text{ml}^{-1}$  bovine serum albumin pH 8.0] and incubated with endonuclease III (ENDO III converts oxidised pyrimidines to strand breaks) or formamido-pyrimidine-DNA glycosylase (FPG recognizes and breaks altered purines). Endo III and FPG are site-specific bacterial repair endonuclease enzymes that enhance the sensitivity and specificity of the Comet assay through their ability to recognize specific types of DNA damage and convert lesions to DNA breaks, thus increasing Comet tail intensity. Slides were electrophorised (30 min, 25 V, 300 mA, 4 °C), in 0.3 M NaOH, 1 mM  $\text{Na}_2\text{EDTA}$ , neutralised with Trizma base (pH 7.5), stained with 20  $\mu\text{l}$  4,6-diamidino-2-phenylindole (DAPI) and viewed by fluorescence microscopy. Damage was scored visually, with 100 Comets on each slide assigned a score from 0 to 4, depending on the fraction of DNA pulled out into the tail. The overall score for each slide was therefore between 0 (undamaged) and 400 (completely damaged).

#### 2.5. Thiobarbituric acid-reactive substances

Liver tissue homogenates (0.1 ml) in 0.05 M potassium phosphate (pH 7.4) were incubated (37 °C) in 2.5 ml buffer (final volume) as previously described (Selman et al., 2002b). Incubation samples were mixed with 0.5 ml 15% TCA and peroxidation products then determined after reaction with 0.67% thiobarbituric acid. Samples were measured on a HPLC (Sphersorb 5 ODS2 (C18)), with fluorimeter excitation set at 532 nm and emission at 553 nm. The results are expressed as  $\mu\text{mol}$  malonaldehyde mg protein $^{-1}$ .

#### 2.6. Hepatic ascorbic acid determination

Ascorbic acid in liver homogenates was measured by isocratic reverse phase HPLC, with an ion-pairing reagent (Ross, 1994). In brief, standards of ascorbic acid and the MPA/acetic acid solution were prepared and run at concentrations of 10  $\mu\text{g ml}^{-1}$ , 25  $\mu\text{g ml}^{-1}$ , 50  $\mu\text{g ml}^{-1}$ , 75  $\mu\text{g ml}^{-1}$  and 100  $\mu\text{g ml}^{-1}$ . Samples were defrosted on ice and transferred to gold chromacol vials before application to the HPLC column (Waters 486UV detector), with UV detection set at 263 nm. The results are expressed as  $\mu\text{mol}$  ascorbic acid mg protein $^{-1}$ .

#### 2.7. Macroarray analysis

Female mice ( $n = 6$  in each case) were sacrificed from the vitamin C supplemented group and control group at 6 and 22 months of age, i.e. 2 and 18 months after initiation of vitamin C supplementation, and at the same time

each day to avoid any circadian changes in liver metabolism that may confound any effects observed due to vitamin C supplementation. Liver samples were collected, immediately frozen in liquid  $\text{N}_2$  and stored at  $-80$  °C. No pathological conditions were evident in any experimental animals. Total RNA was extracted using a guanidium isothiocyanate/phenol method (Chomczynski and Sacchi, 1987), suspended in 300  $\mu\text{l}$  of ultra-pure water and frozen at  $-80$  °C. Each sample (250  $\mu\text{l}$  of total RNA) was treated following the Clontech Atlas Pure System<sup>TM</sup> (Clontech Laboratories Inc., USA <http://www.clontech.com/clontech>) protocol, including digestion using 25  $\mu\text{l}$  of RNase-free DNase 1. Isolated RNA was re-suspended in 30  $\mu\text{l}$  ultra-pure water, yield and purity determined spectrophotometrically by capillary electrophoresis (Agilent Technologies, UK), and stored at  $-80$  °C until use. Clontech Atlas<sup>TM</sup> mouse stress cDNA expression arrays (Cat #7749-1) contained a grid of 140 cDNAs spotted in duplicate on a nylon membrane. cDNA probe preparation, using [ $\alpha$ - $^{32}\text{P}$ ] dATP (3000 Ci  $\text{mmol}^{-1}$ , Amersham Pharmacia Biotech, UK), and hybridisation to the Atlas<sup>TM</sup> array followed the manufacturer's protocol. However, during incubation of the total RNA sample in the course of probe synthesis, a modification was introduced whereby all samples were incubated at 48 °C for 4 min and the master-mix then incubated at 48 °C for 2 min. Hybridisation of the labelled arrays occurred overnight at 68 °C. Membranes were then washed three times in wash 1 ( $2\times$  SSC, 1% SDS) at 68 °C, and once in wash 2 ( $0.1\times$  SSC, 0.5% SDS) at 68 °C and again at RT. Membranes were subsequently exposed to Kodak BIOMAX MS film (Kodak Ltd., UK) at  $-80$  °C, using a Kodak BIOMAX intensifying screen for 2–5 days and then scanned and analysed using Clontech Atlas Image 2.0 software.

#### 2.8. Northern blotting

Total RNA was extracted from the same liver samples used for the macroarrays using a guanidium isothiocyanate/phenol method, fractionated on a 1.4% denaturing agarose gel before being transferred overnight to a positively charged nylon membrane (Amersham Biosciences, UK). Following cross-linking, membranes were hybridised O/N at 42 °C and probed sequentially for Mn-superoxide dismutase (5' CATTGCCAGGTCTCCAACATGCCTCTCTT), Cu-Zn-superoxide dismutase (5' CTGCACGCCGCCGACACAACATTATTGAGGT), catalase (5' GAGAATCCATCCAGCGTTGATTACAGGTGA) and glutathione peroxidase (5' AGACCAAATGATGTACTTGGGGTCGGTCAT) mRNA using 5' Digoxigenin end-labelled oligonucleotides (Eurogentec Ltd., UK). Membranes were subsequently stripped and hybridised for 18S rRNA (5' CGCCTGCTG-CCTTCCTTGATGTGGTAGCCG). Signals were detected by chemiluminescence using CDP-Star as the substrate (Applied Biosystems, UK). The signals were scanned and quantified by densitometry using ImageJ (Microsoft Java Image, USA).

#### 2.9. Statistical analysis

All values reported are mean  $\pm$  standard error (S.E.M.), except where indicated and data were analysed using Minitab (Minitab Inc., PA, USA, version 13) statistical software. Significance is indicated by *p*-values  $<0.05$ . For macroarray analysis, all spot intensities were log-transformed with respect to the base 2, resulting in differences of 1, 2 and 3 on the log-scale corresponding to fold-changes of 2, 4 and 8 on the original scale.

Normalisation methods for gene expression arrays usually assume that the majority of spotted genes will show no differential expression between arrays. This assumption is still reasonable in our experiment for the six slides within one group, but not for between-group comparisons. For this reason we employed a two-stage strategy. First normalisation stage: For the six slides within the same group we calculated a *baseline array* by averaging the log-intensities across the six hybridisations. The Clontech macroarrays include 12 scale and housekeeper genes. We selected those which were unaffected by saturation effects and standardized the four baseline arrays to have the same mean and standard deviation among these unsaturated controls. Second normalisation stage: Within an experimental group, there was no reason to assume that genes were systematically differentially expressed between individual animals. For this reason, we used the standardized group average (see first stage) and examined the six scatter plots of this average against each of the individual arrays and assumed the data to scatter around the 45° line of this plot. A

non-linear robust regression (loess regression/normalisation) was employed to remove any systematic departure from this expected behaviour. The data generated from the 6-month- and 22-month-old mice were analysed separately, where the effect of vitamin C supplementation on gene expression was tested by a two-sample *t*-test for each gene. The Bonferroni method was applied to correct for multiple testing.

### 3. Experimental results

There was no extension in the lifespan of cold exposed mice ( $7 \pm 2^\circ\text{C}$ ) following life-long vitamin C supplementation compared to those individuals maintained on the control diet (Fig. 1, mean  $\pm$  S.D. lifespan:  $747 \pm 139$  days compared to  $710 \pm 131$  days; range 287–887 days and 403–891 days for vitamin C supplemented and control mice, respectively;  $P > 0.05$ ). Kaplan–Meier analysis of survival revealed no difference in mortality rate between the two groups (Log Rank  $X^2$ ,  $P > 0.05$ ).

Both body size and metabolic rate have been implicated as important factors in the ageing process. There was no treatment effect on body mass (Fig. 2A,  $P > 0.05$ ), although body mass did significantly increase in both control mice and vitamin C mice with age ( $P < 0.001$ ). Daily food intake between the control and the vitamin C supplemented mice did not differ between the two experimental groups at either 13 months (control  $7.8 \pm 0.9 \text{ g day}^{-1}$  compared with vitamin C  $8.0 \pm 0.7 \text{ g day}^{-1}$ ,  $P > 0.05$ ) or 22 months of age (control  $8.4 \pm 1.2 \text{ g day}^{-1}$  compared with vitamin C  $9.1 \pm 1.5 \text{ g day}^{-1}$ ,  $P > 0.05$ ). Body mass had a significant effect on resting metabolic rate ( $\text{RMR} = 0.964 + 0.0431 \times \text{body mass}$ ,  $P < 0.001$ ). Using a general linear model with body mass as a covariate, neither age nor treatment had significant effects on RMR (Fig. 2B;  $P > 0.05$ ). In addition, we measured daily energy expenditure at 6 and 22 months of age using the doubly-labelled water method and similarly both age and treatment did not have any significant effect on DEE when body mass was included as a covariate in the analysis (Fig. 2C,  $P > 0.05$ ).

We investigated the levels of lipid peroxidation in the liver by measuring thiobarbituric acid-reactive substances by HPLC at 22 months of age, and showed that there was no treatment effect on hepatic lipid peroxidation (Fig. 3A). Employing the

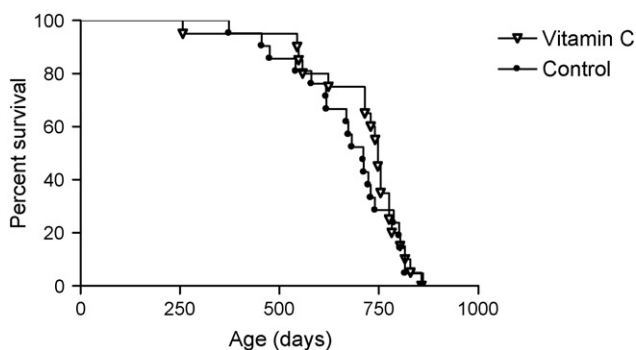


Fig. 1. Lifespan of life-long vitamin C supplemented ( $N = 20$ ) C57BL/6 female mice compared to non-supplemented controls ( $N = 21$ ) maintained at  $7 \pm 2^\circ\text{C}$ . No significance in median lifespan ( $747 \pm 139$  days compared with  $710 \pm 131$  days) between the vitamin C supplemented group and the control group, respectively, was observed (Log Rank  $X^2$ ,  $P = 0.575$ ).

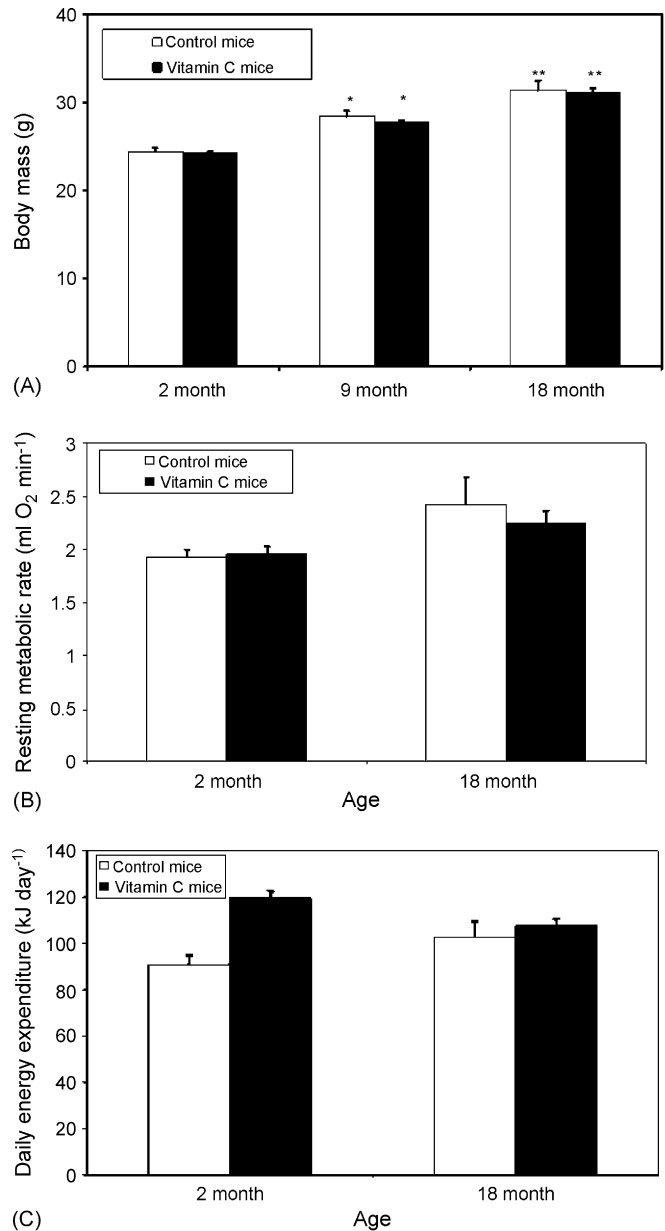


Fig. 2. All mice are denoted by time on vitamin C supplemented diet, i.e. 2, 9 or 18 months following initiation of supplementation, with treatment starting at 4 months of age, thus corresponding to 6, 13 and 22 months of age, respectively. (A) A significant increase ( $P < 0.001$ ) was observed in body mass (g) with age following vitamin C supplementation in both control and treatment groups, although no difference in body mass was observed between groups at any supplementation initiation age ( $P > 0.05$ ). Asterisk denotes significant difference to 2 months of supplementation ( $P < 0.001$ ). Double asterisk denotes significant difference to 2 and 9 months of supplementation,  $P < 0.001$ . Mean  $\pm$  S.E.M. (B) No significant age (2 and 18 months of supplementation) or treatment effect was observed in resting metabolic rate at  $7 \pm 0.1^\circ\text{C}$  (RMR,  $\text{ml O}_2 \text{ min}^{-1}$ ), when body mass was introduced as a covariate employing a general linear model ( $P > 0.05$ ). Mean  $\pm$  S.E.M. (C) Daily energy expenditure ( $\text{kJ day}^{-1}$ ) was determined using the doubly-labelled water technique. No significant age (2 and 18 months of vitamin C supplementation) or treatment effect was observed in daily energy expenditure, when body mass was introduced as a covariate when employing a general linear model ( $P > 0.05$ ). Mean  $\pm$  S.E.M.  $N = 6$  in all cases.



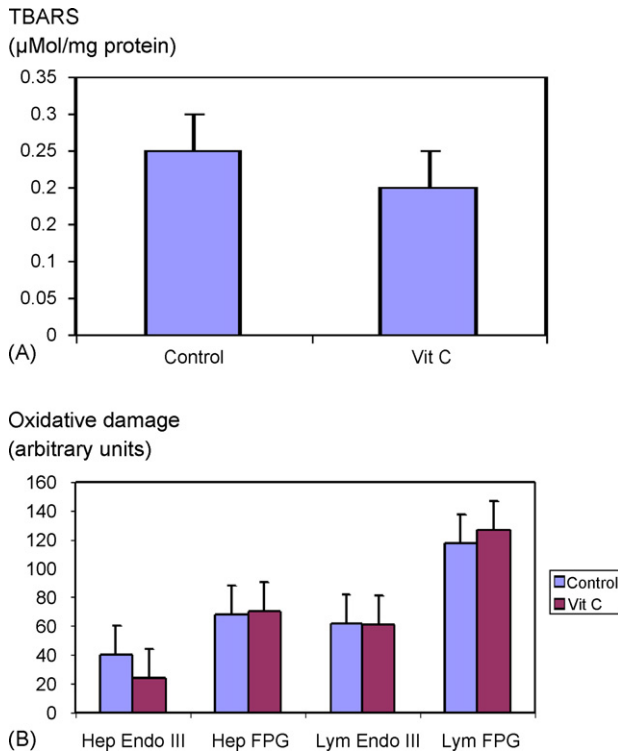


Fig. 3. Oxidative stress markers following 18 months of vitamin C supplementation (22 months of age). (A) No treatment effect was observed on the levels of hepatic lipid peroxidation (thiobarbituric-acid-reactive substances) by HPLC ( $\mu\text{mol mg}^{-1}$  protein). Mean  $\pm$  S.E.M. (B) No difference in the amount of hepatocyte (Hep) or lymphocyte (Lym) DNA oxidative DNA damage using either Endo III (Endonuclease III-Pyrimidine damage) or FPG (Formamidopyrimidine-DNA glycosylase-Purine damage) observed using the modified Comet assay. Mean  $\pm$  S.E.M.  $N = 5$  in all cases.

modified Comet assay, no difference in oxidative DNA damage was seen between treatment groups at 22 months of age in either hepatocytes or lymphocytes when using the lesion-specific enzymes endonuclease III and formamidopyrimidine-DNA glycosylase (Fig. 3B).

Hepatic ascorbic acid levels of vitamin C supplemented mice were higher in the treatment group at both 13 months ( $5.4 \pm 0.7 \mu\text{mol mg protein}^{-1}$  versus  $3.9 \pm 0.5 \mu\text{mol mg protein}^{-1}$ ) and 22 months of age ( $5.3 \pm 1.0 \mu\text{mol mg protein}^{-1}$  versus  $4.9 \pm 0.8 \mu\text{mol mg protein}^{-1}$ ) compared to control mice ( $P < 0.05$ , one tailed test).

Applying the Bonferoni correction to the significance levels that individual comparisons needed to attain to be considered significant at the 0.05 level ( $P < 0.00033$ ), none of the differences in hepatic gene expression at 6 months of age (2 months of vitamin C supplementation), for all 153 genes on the array, reached statistical significance. Using the same criteria at 22 months of age (18 months of vitamin C supplementation) three genes were significantly up-regulated in the vitamin C treated group (Table 1); cyclooxygenase2 which was up-regulated 2.8-fold, the tumour suppressor p21 which was up-regulated 3.3-fold and microsomal UDP-glucuronosyltransferase which was up-regulated 3.1-fold. Two other genes, FK506-binding protein and calnexin precursor, reached a less stringent criterion of  $P < 0.001$ . In addition, eight genes were significantly down-regulated in the vitamin C treatment group ( $P < 0.05$  using the Bonferoni corrected limit of  $P = 0.00033$ ). These eight genes included manganese-superoxide dismutase (Mn-SOD) down-regulated (1.9-fold), and quinone NAD(P)H dehydrogenase1 which was down-regulated 1.3-fold. Two other genes reached the less stringent criterion of  $P < 0.001$  (Table 1).

Table 1

Oxidative stress-related genes that were (A) up-regulated and (B) down-regulated in the livers of mice following 18 months of vitamin C supplementation (started at 4 months of age), compared to age matched unsupplemented control mice ( $n = 6$  in both groups)

<i>t</i>	<i>P</i>	Control intensity	Treatment intensity	Fold-change	Gene
<b>(A) Up-regulated genes</b>					
-24.24	0.000000002	9.43	10.93	2.8	Cyclooxygenase2 (Cox2)
-10.93	0.00000170	9.34	11.07	3.3	Cyclin-dependent kinase inhibitor 1 (p21)
-9.1	0.00000987	15.41	16.81	2.6	Microsomal (UDPGT)
-5.18	<i>0.00057817</i>	<i>14.84</i>	<i>15.78</i>	<i>1.9</i>	<i>FK506-binding protein (Fkbp1a)</i>
-4.75	<i>0.00077594</i>	<i>15.01</i>	<i>15.94</i>	<i>1.9</i>	<i>Calnexin precursor (Canx)</i>
<b>(B) Down-regulated genes</b>					
15.45	0.000000026	13.32	11.93	2.6	Soluble epoxide hydrolase (Ephx2)
9.23	0.000003306	12.80	11.94	1.8	Manganese-superoxide dismutase 2 (Sod2)
6.86	0.000044099	13.72	12.27	2.7	Cytochrome P450 IVA12 (Cyp4a12)
6.74	0.000051334	12.92	11.78	2.2	T-complex protein 1 zeta subunit (Tcp1-zeta)
6.15	0.000108881	12.19	11.77	1.3	Quinone NAD(P)H dehydrogenase1 (Ndh1)
5.98	0.000135165	13.49	12.67	1.8	NADPH-cytochrome P450 reductase (CPR)
5.93	0.000145857	12.29	11.39	1.9	Multidrug resistance protein 1 (Mdr1)
5.44	0.000286152	14.04	13.17	1.8	Liver carboxylesterase 22 precursor (Es22)
4.86	<i>0.000658848</i>	<i>13.20</i>	<i>12.17</i>	<i>2.0</i>	<i>T-complex protein 1 delta subunit (Tcp1-delta)</i>
4.74	<i>0.000791409</i>	<i>13.17</i>	<i>11.95</i>	<i>2.3</i>	<i>Nucleophosmin (Npm)</i>

Gene expression was measured using the Clontech Atlas stress gene array. The tables list, in order of significance, differences in expressions of genes that met the Bonferoni adjusted significance criterion for  $P < 0.05$  ( $P = 0.00033$ ) and, in italics, additional genes that reached a less stringent criterion of  $P < 0.001$ . Column values list the *t* value for the mean expression difference, the significance of the difference (*P*), the mean intensity of the expression for control and vitamin C treatment groups, respectively, and the fold-difference in expression.

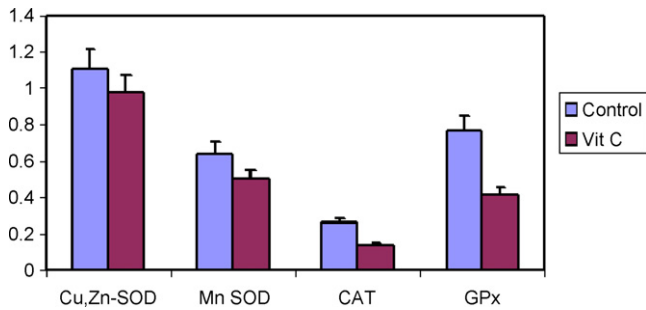


Fig. 4. Hepatic gene expression levels by Northern blotting for four antioxidant genes, relative to 18S ribosomal RNA levels. In all cases, analyses were made for vitamin C supplemented and control animals at 18 months following initiation of treatment (22 months of age). No significant changes were observed for Cu–Zn-SOD. Mean  $\pm$  S.D. Treatment effects were significant for the other three enzymes.  $N = 6$  in all cases.

Hepatic gene expression changes observed on the macroarrays were verified by Northern blotting using three genes (Mn-SOD, Cu–Zn-SOD, catalase) at both 6 and 22 months of age, i.e. 2 and 18 months of vitamin C supplementation. In addition, we measured gene expression of glutathione peroxidase, an antioxidant enzyme not present on our macroarray. At 6 months of age, the directions and significance of the expression differences on the arrays for the three genes were 0.233 and 0.318, respectively (i.e. all clearly not significantly altered) and the lack of difference was also observed by Northern analysis. At 22 months of age both Mn-SOD and Cu–Zn-SOD genes were significantly down-regulated at  $P = 0.0000331$  and  $P = 0.0066$ , respectively. Only this first difference in gene expression was statistically significant at  $P = 0.05$  when our Bonferoni correction was applied to the array data. The differences in expression by Northern blotting are illustrated in Fig. 4 for all four genes at 22 months of age. For Cu–Zn-SOD we found no significant effect of treatment on the gene expression relative to 18S (Fig. 4;  $P = 0.823$ ). However, Mn-SOD, catalase and Gpx showed significant decreases in gene expression following 18 months of vitamin C supplementation (Fig. 4) relative to 18S (Mn-SOD  $P = 0.034$ , catalase  $P = 0.016$ , Gpx  $P = 0.005$ ). For these same four genes, we observed no difference in gene expression between control and vitamin C supplemented animals after 2 months of vitamin C supplementation (6 months of age). In addition, we observed a significant effect when using Northern blotting that was not significant on the arrays, for catalase. This may suggest that using the Bonferoni procedure on the arrays may be too stringent a criterion—leading to false negative results. The largest effect among the antioxidant enzymes was for Gpx—a gene not represented on the array. In the light of these confirmatory and extended analyses we are confident that the gene expression changes reported as significant using the conservative Bonferoni criterion are real.

#### 4. Discussion

Although the primary purpose of our experiments was not to determine the impact of cold exposure (hence the absence of a

warm exposed group), the mean lifespan of 710–747 days in our C57BL/6 female mice at  $7 \pm 2^\circ\text{C}$  was similar to, or only slightly less than, that reported for conventionally housed C57BL/6 female mice on a pure genetic background (mean lifespan 692–810 days (Blackwell et al., 1995; Kunstyr and Leuenberger, 1975; Storer, 1966)). These data suggest that life-long cold exposure ( $7 \pm 2^\circ\text{C}$ ) only marginally impacted on the mean lifespan of these animals. *A priori* we had expected that life-long cold exposure might significantly shorten lifespan due to elevated oxidative metabolism in the cold, thereby generating more reactive oxygen species and enhancing our ability to detect an effect of vitamin C. However, as the understanding of links between ROS, metabolism and ageing have evolved in the past few years (Brand, 2000; Hulbert et al., 2004; Speakman, 2005; Speakman et al., 2004), the absence of a negative effect of cold exposure is less surprising.

The key aspect from the perspective of the present study was the complete absence of any impact of life-long dosing with vitamin C on lifespan or oxidative stress. The absence of an effect of vitamin C on lifespan, despite its supposed antioxidant properties, might occur for several reasons. Our data provide some evidence which may distinguish between these alternatives. First, we detected no effect of the vitamin C treatment on body mass, food intake, resting metabolism or daily energy expenditure. This strongly suggests that antioxidant effects of the vitamin were not being compensated for by modulations in the rate of oxidative metabolism, which might impact on total rates of reactive oxygen species production. Moreover, the vitamin C supplemented food was not more palatable than the control diet, thus we did not have a covert ‘caloric restriction’ effect in the controls offsetting any benefits in the treatment group. The supplementation significantly increased ascorbic acid levels in the livers of mice following 9 and 18 months of supplementation, despite mouse (but not human) hepatocytes being able to synthesize ascorbate *de novo* from glucose. Because we observed an increase in the tissue levels (liver) of ascorbate even though mice are able to synthesise their own, it seems possible that the other observations we have made would also translate to other species that are incapable of synthesising ascorbate endogenously (such as humans). However, this hypothesis would need to be tested.

Our measures of oxidative damage to both lipids and DNA, which have previously been indicated as primary macromolecular targets protected by vitamin C (Amer, 2002; Block et al., 2002; Huang et al., 2002; Lenton et al., 1999) indicated no treatment effect on the levels of oxidative damage, supporting previous studies (e.g. Prieme et al., 1997). We used TBARS as a marker of lipid peroxidation in the liver. Although TBARS as a measure of oxidative stress has been criticised and more specific assays are now available (compared to when this work was performed), the assay based on HPLC is still widely used as a measure of lipid oxidative damage (Magwere et al., 2006; Navarro et al., 2002). The absence of an impact of treatment on our measured parameters of oxidative stress allowed us to eliminate two possibilities. We can exclude the possibility that vitamin C had a significant positive benefit by reducing oxidative damage that is offset by negative effects

on other processes unrelated to oxidative stress (for example the generation of genotoxins (Sowell et al., 2004)). This does not mean vitamin C in our experiments did not generate genotoxins, just that if it did, then the impact was insufficient to negatively affect lifespan. The observed absence of an effect on oxidative damage also allows us to exclude the possibility that an effect on oxidative damage did occur, but rather that oxidative damage may not be a significant contributor to the ageing process. We are therefore left with two alternatives. One possibility is that *in vivo* vitamin C exerts pro-oxidant effects thereby increasing damage. However, the animals can enhance their endogenous protection and repair system resulting in no negative impact on measured oxidative stress and lifespan. Alternatively, vitamin C may act as an antioxidant but it may simultaneously negatively impact on the endogenous protection and repair system. Our gene expression data suggested that there was a significant down-regulation of key genes connected with free-radical scavenging and repair in the vitamin C supplemented group. We did not observe a down-regulation of catalase expression, which was unchanged, but this is perhaps because a too stringent criterion was utilised in the array analysis, as we detected a significant reduction by Northern blotting. We did not observe any significant up-regulation in expression of various components of the endogenous defence and repair mechanisms. This directional bias indicates that vitamin C was not acting as a pro-oxidant to which the animals had to respond by up-regulating their endogenous defence systems – supporting other studies that indicate vitamin C, at levels present in our manipulation, does not exert pro-oxidant effects in mice.

## 5. Summary

Although we did not confirm any of these hepatic gene expression changes at the protein or enzyme activity level, these data do provide a preliminary working model for understanding why treatment with a powerful antioxidant like vitamin C does not bring the anticipated benefits in reduced oxidative stress or increases in either mean or maximum lifespan (in this and in common with other supplementation studies) in cold exposed mice. We suggest that the absence of such effects may come about primarily because, despite its proven antioxidant capabilities, there are compensatory changes in the endogenous protection system. The mechanism by which the endogenous antioxidant system is down-regulated will be of key significance. If the protective genes are down-regulated because the cells sense a reduction in ROS being generated, and hence a lower requirement for protection, the prognosis for any antioxidant based treatment that aims to extend lifespan via a reduction in oxidative damage will be poor. In this context it is perhaps significant that a recent meta-analysis of supplementation treatments with vitamin E in humans concluded there was no evidence of significant benefits (Pham and Plakogiannis, 2005). However, if the mechanism involves a more direct effect of vitamin C on gene expression then the hope remains that alternative antioxidant therapies might be found that reduce free-radical production, but do not trigger the same responses.

We suggest that our novel findings in cold exposed mice following vitamin C supplementation should now be extended to mice under more ‘conventional’ housing temperatures, or indeed to mice maintained within their thermoneutral zone as this is likely to be the most similar to those experienced by the majority of humans during their lifespan.

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## References

- Amer, M.A., 2002. Modulation of age-related biochemical changes and oxidative stress by vitamin C and glutathione supplementation in old rats. *Ann. Nutr. Metab.* 46, 165–168.
- Barja de Quiroga, G., Lopez-Torres, M., Perez-Campo, R., Abelenda, M., Paz Nava, M., Puerta, M.L., 1991. Effect of cold acclimation on GSH, anti-oxidant enzymes and lipid peroxidation in brown adipose tissue. *Biochem. J.* 277 (Part 1), 289–292.
- Beckman, K.B., Ames, B.N., 1998. The free radical theory of aging matures. *Physiol. Rev.* 78, 547–581.
- Blackwell, B.N., Bucci, T.J., Hart, R.W., Turturro, A., 1995. Longevity, body weight, and neoplasia in ad libitum-fed and diet-restricted C57BL6 mice fed NIH-31 open formula diet. *Toxicol. Pathol.* 23, 570–582.
- Block, G., Dietrich, M., Norkus, E.P., Morrow, J.D., Hudes, M., Caan, B., Packer, L., 2002. Factors associated with oxidative stress in human populations. *Am. J. Epidemiol.* 156, 274–285.
- Brand, M.D., 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp. Gerontol.* 35, 811–820.
- Cadenas, S., Barja, G., Poulsen, H.E., Loft, S., 1997. Oxidative DNA damage estimated by oxo8dG in the liver of guinea-pigs supplemented with graded dietary doses of ascorbic acid and alpha-tocopherol. *Carcinogenesis* 18, 2373–2377.
- Carr, A., Frei, B., 1999. Does vitamin C act as a pro-oxidant under physiological conditions? *FASEB J.* 13, 1007–1024.
- Carty, J.L., Bevan, R., Waller, H., Mistry, N., Cooke, M., Lunec, J., Griffiths, H.R., 2000. The effects of vitamin C supplementation on protein oxidation in healthy volunteers. *Biochem. Biophys. Res. Commun.* 273, 729–735.
- Chen, K., Suh, J., Carr, A.C., Morrow, J.D., Zeind, J., Frei, B., 2000. Vitamin C suppresses oxidative lipid damage *in vivo*, even in the presence of iron overload. *Am. J. Physiol. Endocrinol. Metab.* 279, E1406–E1412.
- Childs, A., Jacobs, C., Kaminski, T., Halliwell, B., Leeuwenburgh, C., 2001. Supplementation with vitamin C and *N*-acetyl-cysteine increases oxidative stress in humans after an acute muscle injury induced by eccentric exercise. *Free Radic. Biol. Med.* 31, 745–753.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Collins, A.R., 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotechnol.* 26, 249–261.
- Collins, A.R., Dusinska, M., 2002. Oxidation of cellular DNA measured with the comet assay. *Methods Mol. Biol.* 186, 147–159.
- Enstrom, J.E., Kanim, L.E., Klein, M.A., 1992. Vitamin C intake and mortality among a sample of the United States population. *Epidemiology* 3, 194–202.

- Gale, C.R., Martyn, C.N., Winter, P.D., Cooper, C., 1995. Vitamin C and risk of death from stroke and coronary heart disease in cohort of elderly people. *Br Med J* 310, 1563–1566.
- Golden, T.R., Hinerfeld, D.A., Melov, S., 2002. Oxidative stress and aging: beyond correlation. *Aging Cell* 1, 117–123.
- Holloszy, J.O., 1998. Longevity of exercising male rats: effect of an antioxidant supplemented diet. *Mech. Ageing Dev.* 100, 211–219.
- Huang, H.Y., Appel, L.J., Croft, K.D., Miller III, E.R., Mori, T.A., Puddey, I.B., 2002. Effects of vitamin C and vitamin E on in vivo lipid peroxidation: results of a randomized controlled trial. *Am. J. Clin. Nutr.* 76, 549–555.
- Hulbert, A.J., Clancy, D.J., Mair, W., Braeckman, B.P., Gems, D., Partridge, L., 2004. Metabolic rate is not reduced by dietary-restriction or by lowered insulin/IGF-1 signalling and is not correlated with individual lifespan in *Drosophila melanogaster*. *Exp. Gerontol.* 39, 1137–1143.
- Kunsty, I., Leuenberger, H.G., 1975. Gerontological data of C57BL/6J mice. I. Sex differences in survival curves. *J. Gerontol.* 30, 157–162.
- Lenton, K.J., Therriault, H., Fulop, T., Payette, H., Wagner, J.R., 1999. Glutathione and ascorbate are negatively correlated with oxidative DNA damage in human lymphocytes. *Carcinogenesis* 20, 607–613.
- Lifson, N., Gordon, G.B., Mc, C.R., 1955. Measurement of total carbon dioxide production by means of D<sub>2</sub>O<sup>18</sup>. *J. Appl. Physiol.* 7, 704–710.
- Magwere, T., Pamplona, R., Miwa, S., Martinez-Diaz, P., Portero-Otin, M., Brand, M.D., Partridge, L., 2006. Flight activity, mortality rates, and lipoxidative damage in *Drosophila*. *J. Gerontol. A Biol. Sci. Med. Sci.* 61, 136–145.
- Massie, H.R., Aiello, V.R., Doherty, T.J., 1984. Dietary vitamin C improves the survival of mice. *Gerontology* 30, 371–375.
- McCall, M.R., Frei, B., 1999. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic. Biol. Med.* 26, 1034–1053.
- Navarro, A., Sanchez Del Pino, M.J., Gomez, C., Peralta, J.L., Boveris, A., 2002. Behavioral dysfunction, brain oxidative stress, and impaired mitochondrial electron transfer in aging mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 282, R985–R992.
- Nemoto, S., Otsuka, M., Arakawa, N., 1997. Effect of high concentration of ascorbate on catalase activity in cultured cells and tissues of guinea pigs. *J. Nutr. Sci. Vitaminol. (Tokyo)* 43, 297–309.
- Osganian, S.K., Stampfer, M.J., Rimm, E., Spiegelman, D., Hu, F.B., Manson, J.E., Willett, W.C., 2003. Vitamin C and risk of coronary heart disease in women. *J. Am. Coll. Cardiol.* 42, 246–252.
- Paleologos, M., Cumming, R.G., Lazarus, R., 1998. Cohort study of vitamin C intake and cognitive impairment. *Am. J. Epidemiol.* 148, 45–50.
- Pham, D.Q., Plakogiannis, R., 2005. Vitamin E supplementation in cardiovascular disease and cancer prevention: Part 1. *Ann. Pharmacother.* 39, 1870–1878.
- Podmore, I.D., Griffiths, H.R., Herbert, K.E., Mistry, N., Mistry, P., Lunec, J., 1998. Vitamin C exhibits pro-oxidant properties. *Nature* 392, 559.
- Prieme, H., Loft, S., Nyssonen, K., Salonen, J.T., Poulsen, H.E., 1997. No effect of supplementation with vitamin E, ascorbic acid, or coenzyme Q10 on oxidative DNA damage estimated by 8-oxo-7,8-dihydro-2'-deoxyguanosine excretion in smokers. *Am. J. Clin. Nutr.* 65, 503–507.
- Rehman, A., Collis, C.S., Yang, M., Kelly, M., Diplock, A.T., Halliwell, B., Rice-Evans, C., 1998. The effects of iron and vitamin C co-supplementation on oxidative damage to DNA in healthy volunteers. *Biochem. Biophys. Res. Commun.* 246, 293–298.
- Ross, M.A., 1994. Determination of ascorbic acid and uric acid in plasma by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Appl.* 657, 197–200.
- Selman, C., Grune, T., Stolzing, A., Jakstadt, M., McLaren, J.S., Speakman, J.R., 2002a. The consequences of acute cold exposure on protein oxidation and proteasome activity in short-tailed field voles, *Microtus agrestis*. *Free Radic. Biol. Med.* 33, 259–265.
- Selman, C., Korhonen, T.K., Bunger, L., Hill, W.G., Speakman, J.R., 2001a. Thermoregulatory responses of two mouse *Mus musculus* strains selectively bred for high and low food intake. *J. Comp. Physiol. [B]* 171, 661–668.
- Selman, C., Lumsden, S., Bunger, L., Hill, W.G., Speakman, J.R., 2001b. Resting metabolic rate and morphology in mice (*Mus musculus*) selected for high and low food intake. *J. Exp. Biol.* 204, 777–784.
- Selman, C., McLaren, J.S., Collins, A.R., Duthie, G.G., Speakman, J.R., 2002b. Antioxidant enzyme activities, lipid peroxidation, and DNA oxidative damage: the effects of short-term voluntary wheel running. *Arch. Biochem. Biophys.* 401, 255–261.
- Selman, C., McLaren, J.S., Himanka, M.J., Speakman, J.R., 2000. Effect of long-term cold exposure on antioxidant enzyme activities in a small mammal. *Free Radic. Biol. Med.* 28, 1279–1285.
- Siems, W.G., Brenke, R., Sommerburg, O., Grune, T., 1999. Improved anti-oxidative protection in winter swimmers. *Q J M* 92, 193–198.
- Sowell, J., Frei, B., Stevens, J.F., 2004. Vitamin C conjugates of genotoxic lipid peroxidation products: structural characterization and detection in human plasma. *Proc. Natl. Acad. Sci. U.S.A.* 101, 17964–17969.
- Spasic, M.B., Saicic, Z.S., Buzadzic, B., Korac, B., Blagojevic, D., Petrovic, V.M., 1993. Effect of long-term exposure to cold on the antioxidant defense system in the rat. *Free Radic. Biol. Med.* 15, 291–299.
- Speakman, J.R., 1997. Doubly labelled water. Theory and practice. Chapman and Hall, London.
- Speakman, J.R., 1998. The history and theory of the doubly labeled water technique. *Am. J. Clin. Nutr.* 68, 932S–938S.
- Speakman, J.R., 2005. Body size, energy metabolism and lifespan. *J. Exp. Biol.* 208, 1717–1730.
- Speakman, J.R., Talbot, D.A., Selman, C., Snart, S., McLaren, J.S., Redman, P., Krol, E., Jackson, D.M., Johnson, M.S., Brand, M.D., 2004. Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* 3, 87–95.
- Storer, J.B., 1966. Longevity and gross pathology at death in 22 inbred mouse strains. *J. Gerontol.* 21, 404–409.
- Topp, H., Lengger, C., Schoch, G., Werner, J., Mietzsch, E., 2000. Renal excretion of 8-oxo-7,8-dihydro-2'-deoxyguanosine in Wistar rats with increased O<sub>2</sub> consumption due to cold stress. *Arch. Biochem. Biophys.* 376, 328–332.
- Venditti, P., De Rosa, R., Portero-Otin, M., Pamplona, R., Di Meo, S., 2004. Cold-induced hyperthyroidism produces oxidative damage in rat tissues and increases susceptibility to oxidants. *Int. J. Biochem. Cell Biol.* 36, 1319–1331.
- Veurink, G., Liu, D., Taddei, K., Perry, G., Smith, M.A., Robertson, T.A., Hone, E., Groth, D.M., Atwood, C.S., Martins, R.N., 2003. Reduction of inclusion body pathology in ApoE-deficient mice fed a combination of antioxidants. *Free Radic. Biol. Med.* 34, 1070–1077.