

## The impact of acute caloric restriction on the metabolic phenotype in male C57BL/6 and DBA/2 mice

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### ARTICLE INFO

#### Article history:

Received 8 August 2009  
Received in revised form 18 December 2009  
Accepted 24 December 2009  
Available online 12 January 2010

#### Keywords:

Caloric restriction  
Metabolic rate  
Glucose homeostasis  
Insulin  
IGF-1

### ABSTRACT

Caloric restriction (CR) extends healthy lifespan in many organisms. DBA/2 mice, unlike C57BL/6 mice, are reported to be unresponsive to CR. To investigate potential differences underlying the CR response in male DBA/2 and C57BL/6 mice, we examined several metabolic parameters following acute (1–5 weeks) 30% CR. Acute CR decreased body mass (BM) in both strains, with lean and fat mass decreasing in proportion to BM. Resting metabolic rate (RMR) was unaltered by CR, following appropriate corrections for BM differences, although RMR was higher in DBA/2 compared to C57BL/6 mice. Acute CR decreased fed blood glucose levels in both strains, decreased fasting blood glucose in C57BL/6 mice but increased fasting levels in DBA/2 mice. Glucose tolerance improved after 1 week of CR in C57BL/6 mice but improved only after 4 weeks in DBA/2 mice. Acute CR had no effect on insulin levels, but lowered insulin sensitivity and decreased insulin-like growth factor-1 (IGF-1) levels in both strains. DBA/2 mice were hyperinsulinaemic and insulin resistant compared to C57BL/6 mice. These strain-specific differences in glucose homeostatic parameters may underlie the reported unresponsiveness of DBA/2 mice to CR. We also demonstrate delineation in the response of insulin and IGF-1 to acute CR in mice.

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### 1. Introduction

It is well established that caloric restriction (CR) delays ageing and extends both median and maximum lifespan in a range of model organisms (Masoro, 2005; Weindruch and Walford, 1988). Whilst CR slows disease onset and mortality in non-human primates (Colman et al., 2009) and induces several health benefits in humans (e.g. Fontana et al., 2004), the exact mechanism through which CR acts is currently not known. Moreover, CR does not appear to extend lifespan in all species or strains studied (Carey et al., 2002; Cooper et al., 2004; Harper et al., 2006; Harrison and Archer, 1987; Mockett et al., 2006; Sohal et al., 2009).

The impact of CR on mouse longevity has been studied in detail (Forster et al., 2003; Rikke et al., 2003, 2006; Rikke and Johnson, 2007; Turturro et al., 1999; Weindruch and Walford, 1988). Whilst CR in C57BL/6 mice, the most widely used mouse strain in ageing research, produces a significant and highly repeatable lifespan extension in both genders (Turturro et al., 1999; Weindruch and Walford, 1988), the effect of CR on lifespan in DBA/2 mice appears much more ambiguous. Turturro et al. (1999) in a study of over

1100 male and 500 female DBA/2 mice, reported that CR significantly increased lifespan, albeit more moderately than in C57BL/6 mice, with separation of the mortality curves between male *ad libitum* (AL) and CR DBA/2 mice only apparent after the mice reached ~600 days of age. In agreement, CR increased average lifespan in both male and female DBA/2 mice (Bronson and Lipman, 1991; Lipman, 2002) and in male and female F1 crosses (B6D2F1) derived from C57BL/6 and DBA/2 mice (Turturro et al., 1999). However, in contrast two additional studies have reported a lack (Fernandes et al., 1976) or indeed a negative effect (Forster et al., 2003) of CR on lifespan in DBA/2 mice, with significantly increased mortality in male DBA/2 mice under CR observed between 10 and 20 months of age (Forster et al., 2003). Whilst many factors may contribute to differences in mortality between studies (Selman et al., 2008b), the experimental protocols undertaken by Forster et al. (2003) and Turturro et al. (1999), for example, appear similar. However, despite the clear ambiguity in the lifespan effects in DBA/2 mice following CR, it was recently suggested that DBA/2 mice can be used as 'negative' controls (Ferguson et al., 2007, 2008; Sohal et al., 2009) in order to help identify the mechanisms underlying lifespan extension following CR.

Several strain-dependent differences in metabolism do exist between C57BL/6 and DBA/2 mice (Sohal et al., 2009). Alterations in adiposity following CR, specifically in visceral fat depots, may be important to life extension (Muzumdar et al., 2008), and DBA/2 mice with AL access to normal chow have higher weight gain and larger fat depots compared to age-matched C57BL/6 and 129T2

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mice (Funkat et al., 2004). Enhanced glucose tolerance, reduced insulin-like growth factor-1 (IGF-1) levels and enhanced insulin sensitivity all appear important to lifespan extension in mammals (see Masoro, 2005), and are observed following long-term CR (Anson et al., 2003; Argentino et al., 2005b) and in some (Bartke et al., 2007; Blüher et al., 2003) but not all (Selman et al., 2008a) long-lived genetically modified mice. In addition, DBA/2 mice are genetically predisposed to pancreatic islet failure, having enhanced glucose-mediated insulin secretion (Kooptiwut et al., 2002; Andrikopoulos et al., 2005), elevated fasting insulin levels, relative insulin resistance (Berghlund et al., 2008) but enhanced glucose tolerance at 2 months of age compared to C57BL/6 mice (Goren et al., 2004).

It has also been suggested that alterations in metabolic rate may underlie lifespan extension following CR (for review see Selman et al., 2005). In 5–6-month-old AL DBA/2 mice, mass-specific resting metabolic rate (RMR; expressed per gram body mass (BM)), skeletal muscle state 3 oxygen consumption and body temperature were all higher relative to C57BL/6 mice, despite similar food intake rates (Ferguson et al., 2008). Increased RMR was also reported in AL DBA/2 mice compared to AL C57BL/6 mice at 23 months of age (Sohal et al., 2009). However, in 3–4-month-old mice, mass-specific RMR and spontaneous activity levels were lower in DBA/2 mice than in C57BL/6 mice (Funkat et al., 2004). Following 40% CR for approximately 10–12 months, RMR (normalised per mouse, per gram BM, per gram lean mass, per organ weight (predicted mass of liver, kidney, heart, brain)), was reduced in DBA/2 mice relative to AL controls (Ferguson et al., 2007). In C57BL/6 mice, a similar reduction in RMR was observed, except when RMR was expressed per gram BM (Ferguson et al., 2007). After 19 months of 40% CR, RMR was again reduced in both strains when normalised as above, except that mass-specific RMR was again unaltered in C57BL/6 mice (Sohal et al., 2009). These authors suggest that because RMR was reduced in both strains and because DBA/2 mice are unresponsive to CR, then hypo-metabolism is unlikely to underlie CR-induced lifespan extension (Ferguson et al., 2007).

Acute CR, over days or weeks, in C57BL/6 mice is known to rapidly mirror many of the transcriptional and metabolic changes of longer-term CR (Dhahbi et al., 2004; Mulligan et al., 2008; Selman et al., 2006). However, it is currently not known how DBA/2 mice respond metabolically to acute CR. Therefore, we examined the response of age-matched male DBA/2 and C57BL/6 mice to acute (1–5 weeks) of 30% CR. RMR was determined at 22 °C (housing temperature) and 30 °C (thermoneutrality). Glucose homeostasis (glucose tolerance, fed and fasting blood glucose, fasting plasma insulin levels, insulin sensitivity and fasting plasma IGF-1 levels) and body composition (fat mass, lean mass, bone mineral content and bone mineral density, using dual-energy X-ray absorptiometry (DXA)) were determined as alterations in these metabolic parameters may underlie CR-induced lifespan extension (Anson et al., 2003; Argentino et al., 2005a; Muzumdar et al., 2008).

## 2. Materials and methods

### 2.1. Animals

16 Male DBA/2 and 16 male C57BL/6 mice were purchased (Charles River Laboratories, UK) at 4 weeks of age. Mice were maintained individually from 8 weeks of age onwards in shoebox cages (48 cm × 15 cm × 13 cm), with sawdust for bedding and enrichment provided (plastic houses). All animals were given *ad libitum* (AL) access to water and standard chow (D12450B, Research Diets Inc., USA; protein 20 kcal%, carbohydrate 70 kcal%, fat 10 kcal%), and maintained on a 12L/12D-light/dark cycle (lights on 07:00 h) at 22 ± 2 °C. At 10 weeks of age, mice were randomly assigned to an AL or caloric restricted (CR) group. No difference between groups was observed in body mass (BM) ( $p > 0.05$ ) at the start of the experiment. CR mice underwent a step-down protocol (Selman et al., 2006), with daily food intake reduced to 90% of AL levels at 10 weeks of age, 80% of AL levels at 11 weeks of age and maintained at 70% of AL levels from 12 weeks of age onwards. Food intake of AL mice

was determined daily (±0.01 g) between 16:30 and 17:00 h, with intake of CR mice adjusted weekly according to the intake of AL mice over the preceding week. CR mice were fed daily between 16:30 and 17:00 h. All experiments were carried out under local ethical review (University of Aberdeen), under licence from the UK Home Office and followed the "principles of laboratory animal care" (NIH Publication No. 86-23, revised 1985).

### 2.2. Resting metabolic rate

Resting metabolic rate (RMR) was determined following 1 week (13 weeks of age) and 4 weeks (16 weeks of age) of 30% CR using open-flow respirometry as previously described (Selman et al., 2001a). In summary, mice were weighed (±0.01 g) and then placed in an airtight Plexiglas chamber within a temperature-controlled incubator (INL-401N-010, Weiss-Gallenkamp, UK). Silica gel dried air was pumped (Charles Austin Ltd., Weybridge, UK) through the chamber at 600–800 ml min<sup>-1</sup> (DM3A, Alexander Wright Flow Meter, UK). A sub-sample (approximately 150 ml min<sup>-1</sup>) of dried air was then passed through a paramagnetic oxygen analyser (Series 1100, Servomex Group Ltd., UK) and an infrared carbon dioxide analyser (Series 1400, Servomex Group Ltd., UK). RMR (ml O<sub>2</sub> min<sup>-1</sup>), corrected for standard temperature and pressure, was calculated using the lowest 10 consecutive readings in oxygen concentration (equivalent to 5 min<sup>-1</sup> within the chamber (Hayes et al., 1992)). Animals did not have access to food and water during measurements. RMR was determined at 30 ± 0.5 and 22 ± 0.5 °C, with 30 °C being within the thermoneutral zone for adult mice (Selman et al., 2001a,b) and 22 °C the housing temperature. Each animal spent a minimum of 90 min<sup>-1</sup> at each temperature, with a 30 min<sup>-1</sup> period between each temperature change to allow the incubator temperature to stabilise (Selman et al., 2001a). Data collected during the periods of stabilisation were excluded from the subsequent analysis. The temperature at which measurements started was alternated daily between 22 and 30 °C. Respiratory quotient was calculated as the ratio between carbon dioxide production and oxygen consumption.

### 2.3. Dual-energy X-ray absorptiometry

Following 3 weeks of 30% CR (15 weeks of age), body composition of AL and CR mice was determined by dual-energy X-ray absorptiometry (DXA; Lunar PIXImus mouse densitometer, GE Medical Systems, UK). Mice were weighed and then anaesthetized by 3.5% isoflurane (NetTech, UK) inhalation for the duration of the X-ray scanning (~3.5 min<sup>-1</sup>). Lunar PIXIMUS 2.10 software was used to calculate total lean mass, total fat mass, total bone mineral density (BMD; g cm<sup>-2</sup>) and total bone mineral content (BMC; g) in the region of interest (defined as the sub-cranial body, as recommended by the manufacturer) using a previously described protocol (Johnston et al., 2005).

### 2.4. Glucose homeostasis

Glucose tolerance was determined after an overnight fast (19:00–08:00 h) after 1 week and 4 weeks of 30% CR. Mice were weighed, injected intraperitoneally (IP) with 20% D-glucose (2 g kg<sup>-1</sup>) and blood glucose levels, collected from tail vein samples, were determined using a glucometer (OneTouch Ultra, Lifescan, UK) as previously described (Cantley et al., 2009). CR mice were fed at ~15:00 h on the evenings immediately prior to a glucose tolerance test (GTT). Glucose tolerance is expressed as the area under the curve over a 120 min<sup>-1</sup> period following the IP injection of D-glucose. Food hoppers of CR mice were always empty by 19:00 h (Picchio and Selman, personal observation). Fed blood glucose was determined after 4 weeks of 30% CR. Blood from tail vein samples was collected from AL mice at 11:00 h and from CR mice at 18:30 h to ensure mice were post-prandial.

### 2.5. Fasting plasma insulin levels, IGF-1 levels and insulin sensitivity

Immediately at the start of 5 weeks of 30% CR (17 weeks of age) mice were fasted overnight, weighed and culled. Blood was collected and the resultant plasma stored at -80 °C. Fasting plasma insulin levels were determined using a mouse insulin ELISA kit (CrystalChem Inc., USA). Insulin sensitivity was estimated using the updated homeostatic model assessment (HOMA2) model (Wallace et al., 2004). Whilst HOMA2 is not formally validated for rodents, comparison between experimental groups allows a measure of insulin sensitivity (Taguchi et al., 2007). Fasting plasma IGF-1 levels were determined using a mouse IGF-1 ELISA kit (Quantikine, R and D Systems Inc., USA).

### 2.6. Statistical analysis

All statistical analyses were performed using SPSS (SPSS Inc., USA, version 16) and GraphPad Prism (GraphPad Inc., USA, version 5) software. One-way analysis of variance and general linear modelling (GLM) were used as appropriate. GLM was employed with strain (DBA/2 or C57BL/6) and treatment (AL or CR) introduced as fixed factors. RMR and body composition (fat and lean mass) co-vary with BM, and so BM was introduced into the GLM analysis as a covariate in order to control for BM effects. All non-significant interaction effects ( $p > 0.05$ ) in the GLM analyses were removed to obtain the best-fitted model in each case, with only significant interactions reported. Results are reported as mean ± standard error of the mean

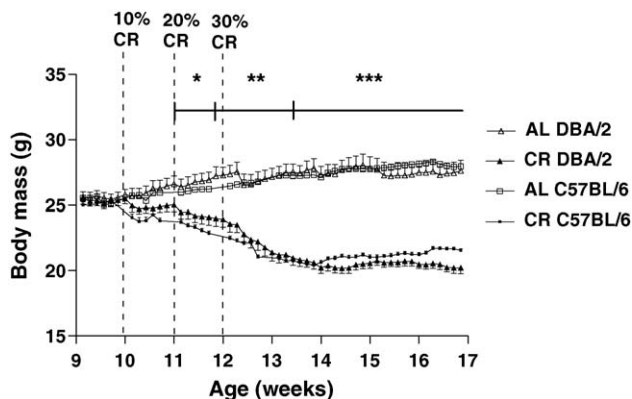
(SEM), with  $p < 0.05$  regarded as statistically significant. Significant treatment (AL or CR) effects are denoted by \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  and significant strain (C57BL/6 or DBA/2) effects are denoted by  $^s p < 0.05$ ;  $^{ss} p < 0.01$ ;  $^{sss} p < 0.001$ .

### 3. Results

Body mass (BM) in both DBA/2 and C57BL/6 mice decreased significantly from the introduction of 20% CR onwards (Fig. 1). By the completion of the study (Fig. 1), DBA/2 mice under CR were significantly lighter ( $20.2 \pm 0.4$  g) than AL controls ( $27.6 \pm 0.8$  g), as were C57BL/6 mice under CR ( $21.6 \pm 0.1$  and  $26.7 \pm 0.7$  g for CR and AL C57BL/6 mice, respectively). Whilst non-significant ( $p > 0.05$ ), the loss in BM following CR over the course of the experiment was greater in DBA/2 mice (~20%) than in C57BL/6 mice (~14%).

Following 1 week of 30% CR, no significant ( $p > 0.05$ ) treatment or BM effect on RMR was observed when RMR was measured at 22 or 30 °C (Table 1). However, a significant strain effect was observed with DBA/2 mice having significantly elevated RMR compared to C57BL/6 mice at both 22 and 30 °C (Table 1). Following 4 weeks of CR, no significant treatment or BM effect on RMR was observed at either 22 or 30 °C, but RMR of DBA/2 mice was again significantly higher than C57BL/6 mice at both temperatures (Table 1). Respiratory quotient (RQ) did not differ between AL and CR mice following 1 week ( $p = 0.074$ ; DBA/2, AL:  $0.731 \pm 0.025$ , CR:  $0.719 \pm 0.019$ ; C57BL/6, AL:  $0.844 \pm 0.007$ , CR:  $0.784 \pm 0.022$ ) or 4 weeks of CR ( $p = 0.057$ ; DBA/2, AL:  $0.753 \pm 0.020$ , CR:  $0.701 \pm 0.028$ ; C57BL/6, AL:  $0.763 \pm 0.026$ , CR:  $0.803 \pm 0.013$ ). However, RQ was significantly higher in C57BL/6 mice compared to DBA/2 mice (1 week ( $p < 0.001$ ) and 4 weeks ( $p = 0.026$ )).

Total fat mass (Fig. 2A) and total lean mass (Fig. 2B), as determined by DXA, were significantly reduced following 3 weeks of CR ( $p < 0.001$ , one-way ANOVA) in both strains. However, because BM differed significantly between AL and CR mice at this time (Fig. 1), the body composition data was reanalysed using GLM to account for these BM effects. GLM analysis indicated no significant strain or treatment effect on either fat or lean mass, although BM had a significant effect on both body composition parameters. This approach indicated that differences in body composition between AL and CR mice during acute CR were attributable to the CR mice being smaller, i.e. body composition was not altered in relative terms. Skeletal bone mineral density, as determined by DXA, was significantly higher in C57BL/6 mice relative to DBA/2 mice (Fig. 3A), although no



**Fig. 1.** Body mass (BM) of DBA/2 and C57BL/6 male mice fed an *ad libitum* (AL) or 30% caloric restricted (CR) diet. BM was not significantly different in mice assigned to the AL or CR groups immediately prior to the initiation of CR ( $25.7 \pm 0.6$  and  $25.4 \pm 0.6$  g,  $p = 0.711$  for AL and CR DBA/2 mice;  $25.4 \pm 0.3$  and  $25.1 \pm 0.2$  g,  $p = 0.137$  for AL and CR C57BL/6 mice, respectively). 10% CR was initiated at 10 weeks of age and increased to 30% by 12 weeks of age in a step-wise manner. Data are represented as mean  $\pm$  SEM (g). BM differed significantly between AL and CR groups from 20% onwards. One-way ANOVA, where \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .  $N = 8$  per group.

**Table 1**

Mean ( $\pm$ SEM) resting metabolic rate (RMR; ml O<sub>2</sub> min<sup>-1</sup>) measured at 22 and 30 °C in male DBA/2 and C57BL/6 mice following 1 week or 4 weeks of 30% caloric restriction (CR), compared to *ad libitum* (AL) controls. No significant differences ( $p > 0.05$ ) in RMR were observed between AL and CR mice of either strain following corrections for body mass (BM). At each temperature (22 or 30 °C) and each time-point (1 week or 4 weeks) DBA/2 mice had significantly higher resting metabolic rates than C57BL/6 mice. General linear model, with treatment (AL or CR) and strain (DBA/2 or C57BL/6) introduced as fixed factors and BM introduced as a covariate.  $N = 8$  per group.

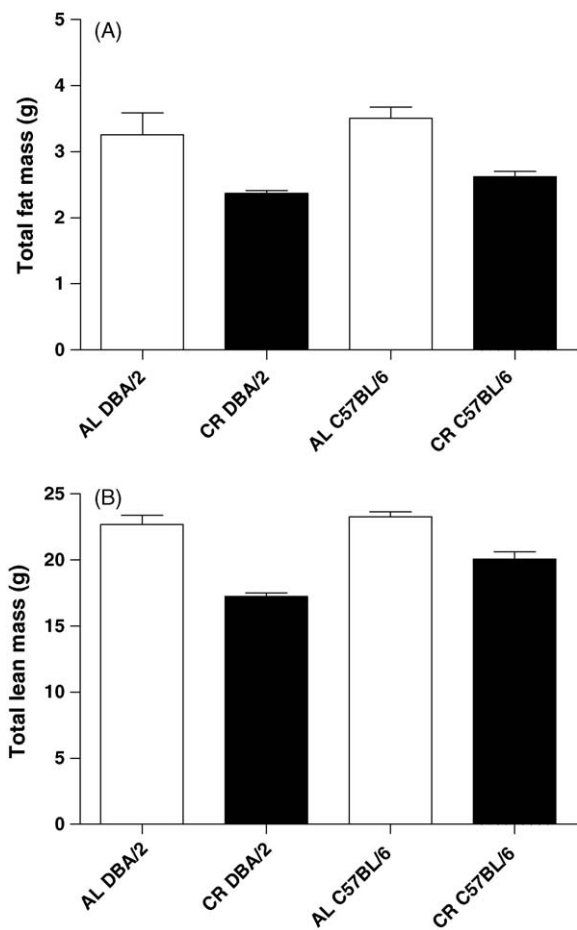
Strain	Time	Temperature	AL	CR
DBA/2	1 Week	22 °C <sup>sss</sup>	$1.538 \pm 0.199$	$1.331 \pm 0.084$
		30 °C <sup>ss</sup>	$0.884 \pm 0.154$	$0.688 \pm 0.099$
DBA/2	4 Weeks	22 °C <sup>sss</sup>	$1.570 \pm 0.144$	$1.311 \pm 0.069$
		30 °C <sup>sss</sup>	$1.029 \pm 0.141$	$0.759 \pm 0.070$
C57BL/6	1 Week	22 °C	$0.802 \pm 0.047$	$0.595 \pm 0.021$
		30 °C	$0.583 \pm 0.022$	$0.456 \pm 0.023$
C57BL/6	4 Weeks	22 °C	$0.711 \pm 0.044$	$0.656 \pm 0.033$
		30 °C	$0.547 \pm 0.065$	$0.382 \pm 0.019$

Significant strain effects denoted by:

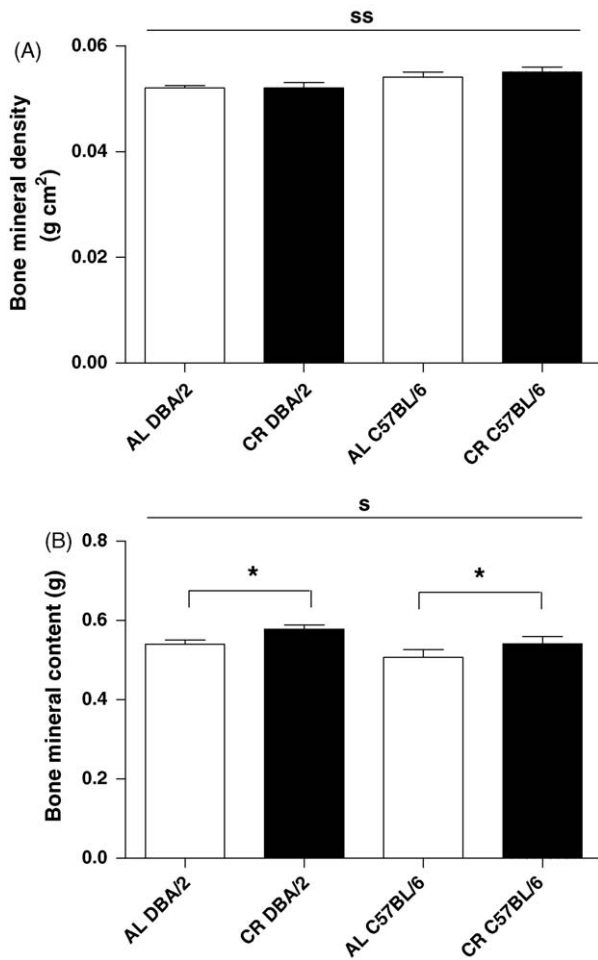
<sup>ss</sup>  $p < 0.01$ .

<sup>sss</sup>  $p < 0.001$ .

treatment effect was detected. Skeletal bone mineral content was increased in CR mice relative to AL mice across both strains (Fig. 3B), and was significantly higher in DBA/2 compared to C57BL/6 mice.

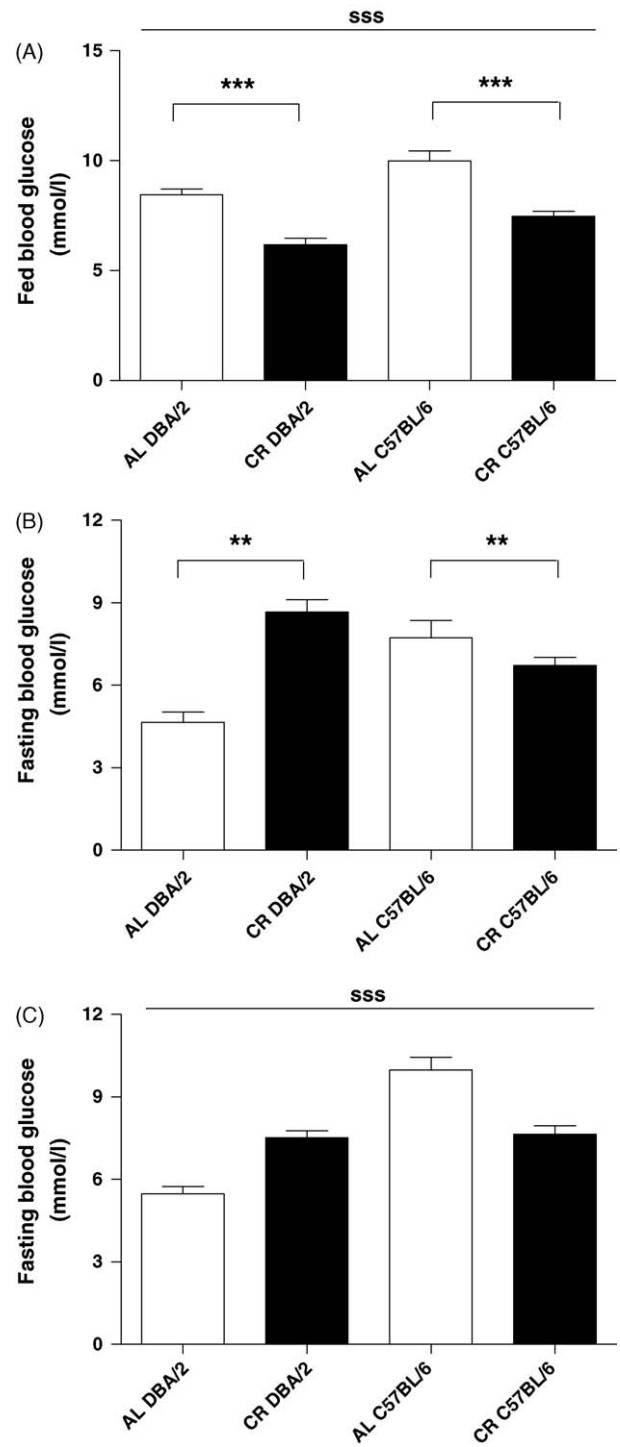


**Fig. 2.** (A and B) Total fat mass (A) and total lean mass (B), as determined by DXA, in DBA/2 and C57BL/6 mice following 3 weeks of 30% CR. After controlling for significant differences in body mass (BM) between AL and CR mice, neither treatment ( $p = 0.814$ ; AL or CR) nor strain ( $p = 0.562$ ; DBA/2 or C57BL/6) had any effect on total fat mass, although BM was significant ( $p = 0.01$ ). No strain ( $p = 0.467$ ) or treatment ( $p = 0.804$ ) effect on total lean mass was observed, but again BM was significant ( $p < 0.001$ ). General linear model with treatment (AL or CR) and strain (DBA/2 or C57BL/6) as fixed factors, and BM introduced as a covariate.  $N = 8$  per group.



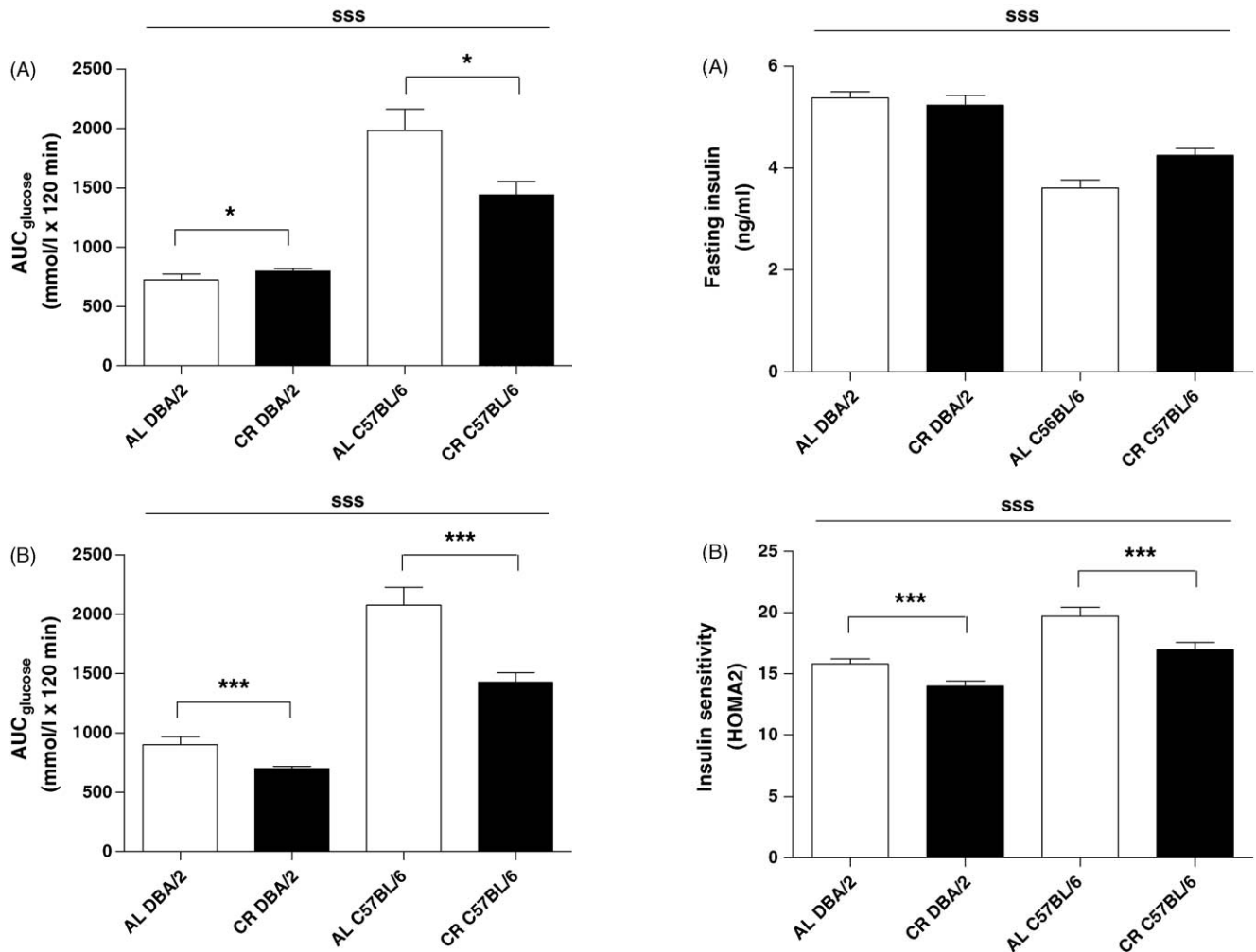
**Fig. 3.** (A and B) Bone mineral density (A; BMD) and bone mineral content (B; BMC), as determined by DXA, in DBA/2 and C57BL/6 mice following 3 weeks of 30% CR. BMD was significantly greater in C57BL/6 mice relative to DBA/2 mice ( $p = 0.002$ ), but no treatment effect was detected ( $p = 0.669$ ). BMC was significantly greater ( $p = 0.027$ ) in CR mice compared to AL mice ( $p = 0.027$ ), and greater ( $p = 0.032$ ) in DBA/2 compared to C57BL/6 mice. General linear model with treatment (AL or CR) and strain (DBA/2 or C57BL/6) as fixed factors.  $N = 8$  per group. Treatment effects: \* $p < 0.05$ . Strain effects: <sup>s</sup> $p < 0.05$ , <sup>ss</sup> $p < 0.01$ .

Fed blood glucose was significantly reduced after 4 weeks of CR in both strains (Fig. 4A). A significant strain difference was also observed with C57BL/6 mice having higher fed blood glucose levels compared to DBA/2 mice. Strain had no effect on fasting blood glucose levels after 1 week of CR (Fig. 4B). However, a significant treatment effect and strain  $\times$  treatment interaction were seen, with 1 week of CR increasing fasting blood glucose levels in DBA/2 mice but decreasing levels in C57BL/6 mice relative to AL controls. Following 4 weeks of CR, no significant effect of treatment on fasting blood glucose levels (Fig. 4C) were seen, although levels were higher in C57BL/6 than in DBA/2 mice. Glucose tolerance was determined by calculating the average area under the glucose clearance curve (AUC) following a glucose tolerance test. Following 1 week of CR (Fig. 5A), glucose tolerance showed a significant treatment effect. However, DBA/2 mice under CR had impaired glucose tolerance relative to AL mice, whilst C57BL/6 mice under CR had significantly improved glucose tolerance (Fig. 5A). Despite this, DBA/2 mice had enhanced glucose tolerance relative to C57BL/6 mice. Following 4 weeks of CR (Fig. 5B), CR mice of both strains had significantly enhanced glucose tolerance relative to AL mice, with DBA/2 mice having significantly improved glucose tolerance relative to C57BL/6 mice.



**Fig. 4.** (A–C) Fed and fasting blood glucose levels in DBA/2 and C57BL/6 mice following acute CR. Fed blood glucose levels (A) were reduced ( $p < 0.001$ ) following 4 weeks of 30% CR in both strains, with C57BL/6 mice having elevated ( $p < 0.001$ ) fed blood glucose levels relative to DBA/2 mice. Strain had no effect on fasting blood glucose levels after 1 week of 30% CR (B;  $p = 0.204$ ), but treatment ( $p = 0.002$ ) and strain  $\times$  treatment interaction ( $p < 0.001$ ) were significant. After 4 weeks of 30% CR (C), fasting blood glucose levels were higher in C57BL/6 mice compared to DBA/2 mice ( $p < 0.001$ ). No treatment effect ( $p = 0.656$ ), but a significant strain  $\times$  treatment interaction ( $p = 0.001$ ) was observed at this time. General linear model with treatment (AL or CR) and strain (DBA/2 or C57BL/6) fixed factors.  $N = 8$  per group. Treatment effects: \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Strain effects: <sup>sss</sup> $p < 0.001$ .

Fasting plasma insulin levels (Fig. 6A) were not significantly different between AL and CR mice following 5 weeks of CR, although DBA/2 mice were hyperinsulinaemic relative to C57BL/6 mice. Insulin sensitivity, as assessed using the homeostasis model

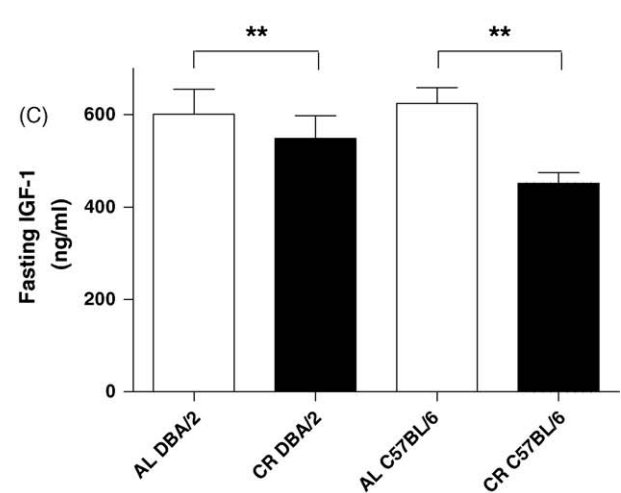


**Fig. 5.** (A and B) Glucose tolerance (denoted by area under the curve (AUC) following a glucose injection) in DBA/2 and C57BL/6 mice following acute CR. Glucose tolerance was significantly altered by treatment ( $p = 0.043$ ) after 1 week of 30% CR (A), being improved in C57BL/6 mice but reduced in DBA/2 mice, relative to AL controls. DBA/2 mice had significantly enhanced glucose tolerance compared to C57BL/6 mice ( $p < 0.001$ ), with a significant strain  $\times$  treatment interaction observed ( $p = 0.009$ ). Following 4 weeks of 30% CR (B), glucose tolerance was improved relative to AL controls in both strains ( $p < 0.001$ ). Glucose tolerance was enhanced in DBA/2 mice ( $p < 0.001$ ) compared to C57BL/6 mice at this time, with a significant strain  $\times$  treatment interaction also observed ( $p = 0.030$ ). General linear model with treatment (AL or CR) and strain (DBA/2 or C57BL/6) fixed factors.  $N = 8$  per group. Treatment effects: \* $p < 0.05$ ; \*\*\* $p < 0.001$ . Strain effects: sss $p < 0.001$ .

assessment of insulin (HOMA2), was significantly lower in CR mice compared to AL mice. DBA/2 mice also had lower insulin sensitivity compared to C57BL/6 mice (Fig. 6B) at this time. However, in contrast to the insulin results, plasma IGF-1 levels (Fig. 6C) were significantly reduced in both strains of mice following acute CR, although no strain differences were detected.

#### 4. Discussion

Whilst the positive effects of CR on lifespan and health have been described in a range of organisms ranging from yeast to mammals (Masoro, 2005; Weindruch and Walford, 1988), several studies suggest that these effects may not be universal across all organisms (Carey et al., 2002; Cooper et al., 2004; Harrison and Archer, 1987; Mockett et al., 2006; Sohal et al., 2009). The longevity response of the DBA/2 mouse strain following CR appears ambiguous, showing a lack (Fernandes et al., 1976) or a negative effect (Forster et al., 2003). Conversely, other studies have reported



**Fig. 6.** (A–C) Fasting plasma insulin levels, insulin sensitivity, and fasting plasma IGF-1 levels in DBA/2 and C57BL/6 mice following acute CR. Fasting plasma insulin levels (A) were unaffected by 5 weeks of 30% CR ( $p = 0.142$ ), although DBA/2 mice were hyperinsulinaemic ( $p < 0.001$ ) compared to C57BL/6 mice. Insulin sensitivity (B), estimated using the updated homeostatic assessment model (HOMA2), was lower in CR mice compared to AL mice ( $p < 0.001$ ), with DBA/2 mice having significantly ( $p < 0.001$ ) reduced insulin sensitivity compared to C57BL/6 mice ( $p < 0.001$ ). Fasting plasma IGF-1 levels (C) were significantly lower in CR mice of both strains ( $p = 0.009$ ) relative to AL controls, although no strain effect was detected ( $p = 0.320$ ). General linear model with treatment (AL or CR) and strain (DBA/2 or C57BL/6) fixed factors.  $N = 8$  per group. Treatment effects: \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Strain effects: sss $p < 0.001$ .

that DBA/2 mice display significant lifespan extension following CR (Bronson and Lipman, 1991; Turturro et al., 1999; Lipman, 2002). Those studies reporting a negative effect have led researchers to suggest that DBA/2 mice can be used as 'negative' controls in order to unravel the mechanisms related to lifespan extension following CR from those related to CR itself (Ferguson et al., 2007; Sohal et al., 2009).

In this study we compared the metabolic response of DBA/2 and C57BL/6 mice to acute CR. In agreement with previously published data using acute CR protocols (e.g. Mulligan et al., 2008; Selman et al., 2006), male mice of both strains lost body mass (BM) rapidly following CR. Acute CR induced a significant reduction in both lean and fat mass in both strains, although these reductions were simply due to reduced BM following CR, i.e. body composition was proportionally similar in CR and AL mice in agreement with our previous findings in Fischer 344 rats (Selman et al., 2005). No strain effect on body composition was observed, in contrast to previously published data in AL fed mice where DBA/2 mice possessed larger fat depots (epididymal, subcutaneous and retroperitoneal) compared to C57BL/6 mice (Funkat et al., 2004). The use of DXA in our study to estimate total fat mass may have missed changes in specific fat depots, e.g. visceral fat stores, suggested as being critical during CR to lifespan extension following CR (Muzumdar et al., 2008). Strain-specific differences in bone mineral density (BMD) and bone mineral content (BMC) existed. Whilst BMD was significantly greater in C57BL/6 mice, BMC was higher in DBA/2 mice and in mice under CR. The effects of CR on bone composition appear dependent on the CR regime employed and on the region of interest studied (Hamrick et al., 2008). For example, no difference in BMD or BMC was seen between C57BL/6 and DBA/2 mice following 6 months of 30% CR, although both increased in age-matched DBA/2 mice compared to C57BL/6 mice under AL feeding (Brochmann et al., 2003). In contrast, 8 weeks of 40% CR in mice (unknown strain) had no effect on whole-body BMC and BMD despite alterations in BMC and BMD within specific bone regions (Hamrick et al., 2008). The relevance of these strain differences in bone morphology on health are unknown but C57BL/6 mice, for example, are known to have more rapid fracture healing compared to DBA/2 mice (Manigrasso and O'Connor, 2008).

A lack of consensus exists on how exactly CR affects metabolic rate, with considerable debate on how best to correct for the confounding effects of altered BM and body composition following CR (Even et al., 2001; Greenberg and Boozer, 2000; Masoro, 2005; Selman et al., 2005; Speakman et al., 2002). CR lowers whole-animal metabolic rate, but different normalisation methods, different experimental protocols and different procedures employed to determine metabolic rate have led to confusion in the literature (for review see Selman et al., 2005). Here we show that acute CR (1 or 4 weeks) had no effect on resting metabolic rate (RMR) compared to AL mice, when measured either at the housing temperature (22 °C) or at thermoneutrality (30 °C). These data contrast with previously published studies reporting a reduction in RMR in these strains following CR (Ferguson et al., 2007; Sohal et al., 2009), although our finding of significantly higher RMR in DBA/2 mice compared to C57BL/6 mice is in agreement with previous data (Ferguson et al., 2008). The reasons for the differences between ours and previous data (Ferguson et al., 2007; Sohal et al., 2009) are unclear, but may be due in part, to differences in restriction levels (30% in this study vs. 40% CR) and/or duration of restriction (1–4 weeks in our study vs. 3 months, 10–12 months and 19 months). We suggest that different normalisation methods and the implicit assumptions underlying several of these methods (Selman et al., 2005) may partly explain the lack of agreement between studies. We recommend that researchers should describe in their methods the ambient temperature at which their study animals are maintained and the temperature at

which RMR is measured, as mice under CR are thermally stressed under normal housing temperatures (~20–24 °C; Overton and Williams, 2004). Availability of this information should help standardise experimental conditions for future comparisons.

The main metabolic differences we identified between DBA/2 and C57BL/6 mice following acute CR relate to aspects of glucose homeostasis. Improvements in glucose homeostasis, particularly insulin sensitivity, are suggested to be essential to longevity assurance (Bartke, 2008; Bartke and Brown-Borg, 2004, but see also Selman et al., 2008a), with reductions in plasma insulin and IGF-1 levels suggested as being critical to the lifespan extension observed (Masoro, 2005). We have shown previously in C57BL/6 mice that fasting blood glucose levels are reduced after 48 h of 30% CR (Selman et al., 2006). In agreement, acute CR lowered fasting blood glucose levels in C57BL/6 mice, but increased fasting levels in DBA/2 mice relative to AL controls. The reasons for these strain-specific differences are unclear. Stress, for example, can increase blood glucose levels in mice (Tabata et al., 1998). We saw no evidence to suggest that our DBA/2 cohort were significantly more stressed compared to our C57BL/6 mice (Selman, personal observation), and both strains underwent exactly the same experimental protocol. Differences in response to various stressors have been reported to exist between DBA/2 and C57BL/6 mice (Thoeringer et al., 2007), with DBA/2 mice found to have elevated corticosterone levels following restraint compared to C57BL/6 mice (Harizi et al., 2007). CR has been shown to increase peak plasma levels of free corticosterone in rodents (Sabatino et al., 1991), and interestingly hypercorticism is associated with insulin resistance in humans (Anagnostis et al., 2009). Therefore, strain-specific responses to stress may be an important factor in the magnitude of lifespan extension observed following CR.

We showed that glucose tolerance was enhanced following 1 and 4 weeks of CR in C57BL/6 mice. However, improved glucose tolerance in DBA/2 mice occurred only after 4 weeks of CR, being significantly poorer following 1 week of CR. These findings suggest that C57BL/6 mice respond more rapidly, in terms of improved glucose tolerance, following initiation of CR than DBA/2 mice. However, despite these temporal effects, overall C57BL/6 mice were significantly more glucose intolerant than DBA/2 mice. Fasting plasma insulin levels were also significantly elevated in DBA/2 compared to C57BL/6 mice, with isolated islets from DBA/2 mice known to hyper-secrete insulin in response to glucose (Berglund et al., 2008; Goren et al., 2004). However, surprisingly we observed no difference in fasting insulin levels between AL and CR mice, despite plasma insulin levels decreasing by ~50% after 1 week of 40% CR in C57BL/6 mice (Mulligan et al., 2008). The reasons for these differences are unclear but may be due to the severity of the restriction regime imposed and/or the absence of a step-wise reduction in food intake in the study of Mulligan et al. (2008). In addition to the fasting hyperinsulinaemia, DBA/2 mice were more insulin resistant than C57BL/6 mice. Long-term CR is known to enhance insulin sensitivity (Anson et al., 2003; Argentino et al., 2005a), but acute CR impaired insulin sensitivity in both strains, relative to AL controls. Again the reason for this is currently unclear and requires further investigation. Visceral adiposity appears to play a primary role in hepatic insulin resistance (Barzilai et al., 1998). Whilst we saw no change in total fat mass following CR, it is feasible that changes in specific fat depots, e.g. visceral depots, might only occur after longer-term and/or more restrictive CR regimes. Interestingly, elevated cortisol levels are associated with increased visceral fat accumulation in humans (Anagnostis et al., 2009). We suggest that tracking specific fat depots using *in vivo* imaging techniques (e.g. Calderan et al., 2006) following initiation of CR, alongside simultaneous measurements of insulin sensitivity and perhaps also corticosterone, may give further insights into this potential linkage.

In contrast to the effects of acute CR on insulin levels, plasma IGF-1 levels were significantly reduced in both strains following acute CR although no difference between strains was observed. IGF-1 secretion is principally regulated by nutrient intake (Clemmons, 2004) and IGF-1 levels are reduced following long-term CR and reduced in long-lived growth hormone (GH) deficient dwarf mice (for review see Masoro, 2005). In contrast to our findings, plasma IGF-1 levels were unaltered by 1, 4 or 16 weeks of ~40% in C57BL/6 mice (Mulligan et al., 2008). IGF-1 can enhance insulin sensitivity, primarily through metabolic effects mediated by skeletal muscle, and alterations in both IGF-1 and GH can influence insulin's ability to maintain carbohydrate homeostasis (Clemmons, 2004). Therefore, the observed decrease in insulin sensitivity in our study following acute CR may be partly explained by the lower IGF-1 levels at this time. However, this relationship is highly complex given that IGF-1 can suppress GH secretion (Clemmons, 2004) and because insulin regulates hepatic IGF-1 production independently from, and additively with, GH (Holt et al., 2003).

In summary, it has been suggested that DBA/2 mice are unresponsive to CR, although actually more studies report a positive effect of CR on lifespan in this strain (Bronson and Lipman, 1991; Turturro et al., 1999; Lipman, 2002) than do not (Fernandes et al., 1976; Forster et al., 2003). We report no effects of acute CR on RMR or body composition in either strain, after controlling for BM differences and suggest this disparity with previous results (Ferguson et al., 2007, 2008; Sohal et al., 2009) may be attributed, in part, to methodological and statistical differences between studies. However, strain-specific differences in several parameters relating to glucose homeostasis were observed. DBA/2 mice were hyperinsulinaemic, insulin resistant and demonstrated an attenuated CR-induced improvement in glucose tolerance, relative to C57BL/6 mice. Insulin signalling is an important candidate longevity assurance mechanism (Bartke, 2008; Bartke et al., 2007; Piper et al., 2008). We suggest that examination of the specific signalling components downstream of the insulin, IGF-1 and GH receptor pathways may provide insights in to how exactly acute and long-term CR act. In addition, this approach may help provide a mechanistic explanation for the apparent unresponsiveness of DBA/2 mice to CR (Fernandes et al., 1976; Forster et al., 2003). However, a final caveat to this research is that there is a requirement to re-examine longevity in DBA/2 mice following CR before this strain can irrefutably be called a 'negative' CR control.

## Acknowledgments

We are grateful to Ela Król for helpful comments on a previous draft of this manuscript. We thank Duncan Wood, Shona Fleming, Jerico Guzman Mejia, Luiza Duarte and Yuko Gamoto (all University of Aberdeen) for help and advice. This research was funded through University of Aberdeen start-up funds to CS, with LP supported, in part, by Université Blaise Pascal, France. The authors declare no competing financial interests.

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