The effects of caloric restriction on adipose tissue and metabolic health are sex- and age-dependent

Karla J. Suchacki[1#] & Ben J. Thomas[1#], Claire Fyfe[3], Adriana A.S. Tavares[1,2], Richard J. Sulston[1], Andrea Lovdel[1], Holly J. Woodward[1], Xuan Han[1], Domenico Mattiucci[1], Eleanor J. Brain[1], Carlos J. Alcaide-Corral[1,2], Gillian A. Gray[1], Phillip D. Whitfield[4,5], Roland H. Stimson[1], Nicholas M. Morton[1], Alexandra M. Johnstone[3] and William P. Cawthorn[1*].

[1]University/BHF Centre for Cardiovascular Science and [2]Edinburgh Imaging, University of Edinburgh, The Queen's Medical Research Institute, Edinburgh BioQuarter, 47 Little France Crescent, Edinburgh, EH16 4TJ, UK. [3]Rowett Institute, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK. [4]Division of Biomedical Sciences, University of the Highlands and Islands, Centre for Health Sciences, Inverness, UK. [5]Institute of Infection, Immunity & Inflammation, University of Glasgow, Glasgow, G12 8TA, UK.

[#]These authors contributed equally to this manuscript.

*__*Correspondence to*__: William Cawthorn, University/BHF Centre for Cardiovascular Science, The Queen's Medical Research Institute, Edinburgh BioQuarter, 47 Little France Crescent, Edinburgh, EH16 4TJ. W.Cawthorn@ed.ac.uk

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HIGHLIGHTS

- Caloric restriction (CR) decreases fat mass and improves glucose homeostasis in young male mice, but young females resist these effects.
- These effects are associated with improved hepatic insulin sensitivity and suppressed gluconeogenesis, without marked differences in peripheral glucose uptake.
- CR's effects on fat loss and glucose homeostasis are comparable in aged male and female mice, implicating oestrogen as the driver of the sexually dimorphic effects in young mice.
- In humans, females resist CR-induced fat loss in an age-dependent manner, further supporting the role of oestrogen in the sexually dimorphic effects of CR.

SUMMARY

Caloric restriction (CR) is a nutritional intervention that reduces the risk of age-related diseases in numerous species, including humans. CR's metabolic effects, including decreased fat mass and improved insulin sensitivity, play an important role in its broader health benefits. However, the extent and basis of sex differences in CR's health benefits are unknown. We found that 30% CR in young (3-month-old) male mice decreased fat mass and improved glucose tolerance and insulin sensitivity, whereas these effects were blunted or absent in young female mice. Resistance to fat and weight loss in females was not explained by decreased energy expenditure compared to males. Positron emission tomography-computed tomography (PET/CT) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) showed that peripheral glucose uptake was comparable between sexes. Instead, CR caused a pronounced suppression of hepatic gluconeogenesis and altered liver ceramide content in males but not in females. In aged mice (18-months old), when females are anoestrus, CR decreased fat mass and improved glucose homeostasis to a similar extent in both sexes. CR-induced fat loss in humans was also sex- and age-dependent: younger females (<45 years) resisted fat loss compared to younger males while in older subjects (>45 years) this sex difference was absent. Collectively, these studies identify age-dependent sex differences in the metabolic effects of CR and highlight adipose tissue, the liver and oestrogen as key determinants of CR's metabolic benefits. This has important implications for understanding the interplay between diet and health and for maximising the benefits of CR in humans.

INTRODUCTION

Caloric restriction (CR) is a therapeutic nutritional intervention involving a sustained decrease in calorie intake whilst maintaining adequate nutrition. CR extends lifespan and reduces the risk of age-related diseases in numerous species, ranging from yeast to primates (1-3). Trials of CR in humans show numerous health benefits, including the prevention of cardiovascular disease, hypertension, obesity, type 2 diabetes, chronic inflammation, and risk of certain cancers (4). Thus, the ability of CR to promote healthy ageing is now also recognised in humans. In addition to these health benefits, many effects of CR reflect evolutionary adaptations that confer a survival advantage during periods of food scarcity (5). Establishing the extent and basis of CR's effects may thereby reveal new knowledge of healthy ageing and the interplay between food, nutrition, and health.

A key contributor to the health benefits of CR is its impact on metabolic function. Ageing is characterised by hepatic insulin resistance, hyperinsulinaemia and excessive accumulation of white adipose tissue (WAT), particularly visceral WAT (6-8). The latter is coupled with adipose dysfunction, whereby WAT becomes unable to meet the demands for safe lipid storage. This results in ectopic lipid accumulation in the liver and other tissues, contributing to insulin resistance and metabolic dysregulation (6, 8). CR counteracts these effects, decreasing WAT mass, increasing fatty acid oxidation, preventing ectopic lipid deposition and enhancing insulin sensitivity and glucose tolerance (5). Notably, studies in rodents show that removal of visceral WAT, independent of CR, is sufficient to prevent insulin resistance, improve glucose tolerance and increase lifespan (9-11). Visceral WAT loss in humans is also strongly associated with CR's metabolic benefits (12). Thus, the ability of CR to decrease visceral WAT, and the resultant improvements in hepatic insulin sensitivity, are likely central to CR's effects on metabolic function and healthy ageing.

This importance of adiposity and metabolic function raises the possibility of sex differences in the CR response. Indeed, it is now well established that metabolic homeostasis and adipose biology differ substantially between males and females (13-16); however, most preclinical rodent studies have used males only (17), suggesting that much of the CR literature may have overlooked sex as a potential determinant of CR's effects. Nevertheless, some clinical and preclinical CR studies from our lab and others have identified sexually dimorphic responses (18-21), whereby males lose fat mass to a greater extent than females. Oestrogens underlie many metabolic sex differences (13, 22), suggesting that oestrogen may contribute to females' resistance to fat loss during CR. However, the extent and basis of sexual dimorphism in CR's metabolic effects remains to be firmly established.

Herein, we first systematically review the CR literature to establish the degree to which sex has been overlooked a determinant of the CR response. To further elucidate the extent and basis of sex differences, we studied CR in male and female mice and humans at ages where oestrogen is physiologically active or absent. Together, our findings show that the CR field has largely overlooked sex differences and reveal that both mice and humans display age-dependent sexual dimorphism in the metabolic effects of CR.

RESULTS

Sex is routinely overlooked as a biological variable in the CR literature.

One review of the recent CR literature found that most rodent studies use males only, with females typically used only to address female-specific experimental questions (18); however, the extent of this bias in earlier CR studies, and whether it applies to human CR research, has not been assessed. To address these issues, we first systematically quantified the use of males and females, and the consideration of sex as a biological variable, in mouse and human CR studies (Fig. 1, S1). We focussed on research published since 2003, when the European Commission first highlighted the importance of addressing sex as a biological variable (23). We also excluded studies that necessarily focussed on a single sex, such as those addressing effects of CR on female reproductive function, leaving only studies in which there is no scientific rationale for ignoring potential sexual dimorphism. We found that male-only studies predominated for mice, accounting for ~64% of all mouse CR studies since 2003 (Fig. 1A, S1B). This is consistent with previous analyses of the more-recent CR literature (18). Fewer than 20% of studies used females only, while around 7.3% combined males and females, suggesting an assumption that sex would not influence experimental outcomes. In contrast, ~60% of human studies combined males and females, while ~23% used only females and ~12% only males (Fig. 1C, S1B). Strikingly, by the end of 2021, studies that included both sexes and analysed data with sex as a variable were in a minority for both mice (~3.4%) and humans (~4.5%) (Fig. 1A, 1C). Moreover, the proportion of studies using each sex or combination of sexes has remained relatively constant since 2003 (Fig. 1A, 1C). Thus, efforts to increase the consideration of sex in experimental design, as promoted by the European Commission (23), Canadian Institutes of Health Research (CIHR) and National Institutes of Health (NIH) (24), appear to have had little impact on the field of CR research.

We next focussed on the minority of studies that did consider sex in their experiments ("M&F separate") and that included analysis of metabolic parameters. We found that results in ~74% of mouse studies (Fig. 1B) and ~62% of human studies (Fig. 1D) indicated a sex difference in the CR response. Thus, sex differences have been described in the CR literature, but the continuing dearth of studies that include both sexes suggests that this issue continues to be overlooked in the CR field.

Females resist CR-induced weight loss, fat loss, and adipocyte hypertrophy

To explore sex differences in the CR response, we assessed the effects of 30% CR, implemented from 9-15 weeks of age, in C57BL/6J and C57BL/6N mice. As expected, CR decreased body mass in both males and females (Fig. 2A) but this effect was greater in males, with ANOVA confirming a significant sex-diet interaction. This sex difference in response to CR is particularly clear when body mass is presented relative to baseline, pre-CR levels (Fig. 2B).

To determine how fat and lean mass contribute to these diet and sex effects, body composition was assessed weekly using time-domain nuclear magnetic resonance (TD-NMR; Fig. 2C-E, S2A-B). CR decreased fat and lean mass in males, whereas females maintained fat mass and lost only lean mass (Fig. 2C-E). A significant sex-diet interaction was apparent for absolute fat mass (Fig. 2C) and for fat mass relative

158 to baseline (Fig. 2D); the latter showed that CR decreased fat mass in males, whereas
159 AL males and AL or CR females increased fat mass to a similar extent over the six-
160 week duration (Fig. 2D). For lean mass, a significant sex-diet interaction occurred for
161 absolute mass, with losses being greater in males than in females (Fig. 2E). However,
162 when compared to baseline lean mass, the CR vs AL effect was similar between the
163 sexes, in part because AL females continued to increase lean mass over time (Fig.
164 2F).
165
166 Given that these body composition changes coincide with changes in overall body
167 mass, we also assessed fat and lean mass as % body mass. Males preferentially lost
168 fat mass and preserved lean mass in response to CR, with % lean mass being greater
169 in CR vs AL-fed males (Fig. S2). In contrast, diet did not alter % fat or % lean mass in
170 females, indicating that changes in body composition were proportionate to the overall
171 changes in body mass (Fig. S2).
172
173 After 6 weeks of AL or CR diet, tissue-specific changes in response to CR were
174 assessed (Fig. 2G; Fig. S3). We found that in males but not females CR decreased
175 the absolute mass of gonadal (gWAT), inguinal (iWAT), mesenteric (mWAT) and
176 perirenal (pWAT) WAT depots, as well as brown adipose tissue (BAT; Fig. S3A). CR
177 also tended to decrease the absolute mass of pericardial WAT (pcWAT) in males only
178 (Fig. S3A). To determine if these changes were proportionate to changes in overall
179 body mass, we further analysed the mass of each adipose depot as % body mass
180 (Fig. 2G). This showed that the significant sex-diet interaction persisted for gWAT,
181 iWAT, mWAT and pWAT. CR did not affect % BAT mass in either sex, though % BAT
182 mass was significantly greater in males than in females (Fig. 2G). Thus, both in
183 absolute terms and as % body mass, CR decreases WAT mass in males but not in
184 females.
185
186 We next analysed the masses of other tissues to determine if this sex-dependent effect
187 of CR was unique to WAT. CR significantly decreased the absolute mass of the liver,
188 pancreas, kidneys, gastrocnemius muscle (gastroc), heart, spleen and thymus (Fig
189 S3B). Significant sex-diet effects were detected for the liver and kidney, with CR
190 causing greater decreases in males than in females; however, for each of the other
191 tissues the CR effect was similar between the sexes (Fig. S3B). In contrast to these
192 effects on absolute mass, CR did not significantly affect the relative masses of each
193 of these tissues, nor did sex influence the CR effect (Fig. S3C). This indicates that the
194 absolute masses of these tissues decreased in proportion to the changes in overall
195 body mass. One notable exception is the adrenal glands, the mass of which was
196 increased with CR to a greater extent in males than in females (Fig. S3B-C). Together,
197 these data show that the sex differences in CR-induced loss of body mass and fat
198 mass were driven primarily by decreased WAT mass in males, which females robustly
199 resist.
200
201 Differences in WAT mass can be driven by changes in adipocyte size and/or adipocyte
202 number. Thus, given the marked sex differences in the effect of CR on WAT mass, we
203 next investigated this effect at the level of adipocyte size (Fig. 2H-I). In gWAT, CR
204 significantly decreased average adipocyte area in males but not in females (Fig. 2H&I).
205 The same relationships were observed in mWAT, another visceral adipose depot (Fig.
206 S4). These observations suggest that differences in lipolysis and/or lipogenesis
207 underpin the sex differences in CR's effects on WAT mass.

Females resist CR-induced improvements in glucose homeostasis

We next investigated if other metabolic effects of CR also differ between the sexes. We found that CR decreased blood glucose to a greater extent in males than in females (Fig. 3A). Consistent with this, oral glucose tolerance tests (OGTT) revealed that CR improved glucose tolerance in both sexes but this effect was greater in males (Fig. 3B). These diet and sex effects were reflected by the OGTT total area under the curve (tAUC), calculated against 0 mM blood glucose; however, they were not apparent for the incremental AUC (iAUC), calculated against fasting blood glucose for each mouse. This suggests that differences in fasting glucose are the main driver of CR-induced improvements in glucose tolerance and the sex differences therein.

To further assess the basis for these effects on glucose tolerance, we analysed plasma insulin during the OGTT. CR decreased plasma insulin in males but not in females, largely because AL females were already relatively hypoinsulinaemic (Fig. 3D). Based on these blood glucose and insulin concentrations we calculated the homeostasis model assessment for insulin resistance (HOMA-IR) (25) and the Matsuda Index of insulin sensitivity (26). Across both sexes CR significantly decreased HOMA-IR and increased the Matsuda Index; however, within each sex the CR effect was significant for males only, indicating that females resist the CR-induced improvements in insulin sensitivity (Fig. 3E).

Time of CR feeding does not alter the sex differences in CR's metabolic effects

Time of feeding can influence the metabolic effects of CR (5, 27). Therefore, to test if our feeding regime influenced the observed sex differences we studied a cohort of mice that received their daily ration of CR diet in the evening instead of the morning (Fig. S5-6). Consistently, we found that the sex-dependent effect of CR on body mass, fat mass, lean mass and WAT mass was conserved (Fig. S5A-H). Similarly, an OGTT revealed that the diet and sex effects on glucose homeostasis persisted (Fig. S6A-B).

CR decreases overall energy expenditure to a similar extent in male and female mice

We next investigated whether altered energy expenditure explained the sex differences in the CR response. As expected, CR significantly decreased absolute energy expenditure, with a greater decrease at night than in the day (Fig. 4A-B). The daytime diet differences disappeared when normalised to lean body mass (Fig. 4C), suggesting that they are driven primarily by the loss of lean mass and a consequent reduction in basal metabolic rate. In contrast, CR-induced decreases in nighttime and total energy expenditure persisted even when normalised to lean body mass (Fig. 4C), indicating that factors beyond altered lean mass contribute to this CR effect. The relationship between lean mass and energy expenditure (*P,* Slope) did not differ among the groups; however, the intercepts of the best-fit lines (*P,* Intercept) did differ significantly (Fig. 4D). Thus, for a given lean body mass, CR-fed males and females had significantly lower energy expenditure than their AL-fed counterparts (Fig. 4D). Within the CR-fed groups, females also had significantly lower total energy expenditure than males for a given lean mass (Fig. 4D). CR females also tended to have lower nighttime energy expenditure than CR males, but this did not reach

statistical significance (Fig. 4B). Indeed, within each diet there were no sex differences in absolute energy expenditure and no significant sex-diet interactions. Importantly, total energy expenditure did not differ between CR-fed males and females (Fig. 4B), indicating that females' resistance to overall weight loss is not a result of decreased energy expenditure during CR.

Sex differences in glucose homeostasis are not explained by differential glucose uptake

The basis for the sex differences in CR-improved glucose homeostasis was next investigated. We first used positron emission tomography-computed tomography (PET/CT) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) to assess glucose uptake *in vivo* (Fig. 5, S7). We focussed on tissues with high basal and/or insulin-stimulated glucose uptake, including the heart, brain, skeletal muscle, BAT, WAT, bone, and the bone marrow (BM) (28); the latter was also included because CR increases bone marrow adipose tissue (BMAT), another site of high basal glucose uptake (29).

Consistent with previous observations (29), we found that glucose uptake was highest in BAT, followed by the brain, bones, BM, the heart and skeletal muscle (Fig. 5A-B, S7A). However, CR did not stimulate glucose uptake in any of these tissues and decreased tibial glucose uptake (Fig. 5A), including in the BMAT-enriched distal tibia (Fig. S7A). Gamma counting further revealed no significant effect of CR in the gastrocnemius muscle, arm bones, liver, pancreas, spleen or thymus (Fig. 5C, S7B); however, across both sexes, CR tended to increase glucose uptake in iWAT and gWAT and this effect was significant when assessed across all WAT depots (Fig. 5C). CR also significantly increased glucose uptake in the kidneys (Fig. 5C). Several sex differences were further detected, independent of diet. Thus, females had lower glucose uptake into gastrocnemius muscle and higher glucose uptake into BAT, the heart, gWAT, the humerus, the forearm and the kidneys (Fig. 5A-C). Notably, however, sex did not significantly alter the effect of CR in any of the tissues assessed. Thus, the sexually dimorphic effects of CR on glucose homeostasis are not a result of CR increasing glucose disposal to a greater extent in males than in females.

CR exerts sexually dimorphic effects on hepatic gene expression

The lack of CR-driven differences in peripheral uptake suggested that reduced hepatic glucose production may underpin the sex differences in glucose homeostasis. To test this, we analysed transcript expression in the livers of AL and CR mice, focussing on factors important for the regulation of gluconeogenesis, lipid metabolism and insulin sensitivity. We found that CR significantly increased mRNA levels of the rate-determining gluconeogenic enzyme phosphoenolpyruvate carboxykinase (*Pck1)* in females but not in males (Fig. 6A). A similar profile occurred for peroxisome proliferator-activated receptor-alpha (*Ppara*) (Fig. 6A), a key regulator of hepatic lipid metabolism that prevents hypoglycaemia during fasting (30). Compared to CR males, CR females also had higher mRNA levels for peroxisome proliferator-activated receptor-gamma (*Pparg*), carbohydrate response element-binding protein (*Chrebp*) and *Mt-nd1,* which encodes a subunit of mitochondrial complex I (Fig. 6A). In contrast,

mRNA for *Mt-co1*, encoding a subunit of mitochondrial complex IV, did not differ between males and females but was increased with CR in both sexes. Regardless of diet, mRNA levels for fatty acid-binding protein 5 (*Fabp5*) and glycerol-3-phosphate dehydrogenase 1 (*Gpd1*) were higher in females than males, with the latter being significantly decreased by CR in each sex (Fig. 6A). Finally, principal component analysis of these qPCR data identified CR females as the most transcriptionally distinct group, further highlighting the overall sex differences in the CR response (Fig. 6B).

CR exerts sexually dimorphic effects on hepatic sphingolipid content

We next tested whether altered lipid storage, implicated in sex-dependent differences in hepatic glucose production (22), might contribute to the sexually dimorphic metabolic effects of CR. Gross hepatic triglyceride content was not affected by diet or sex (Fig. 6C). Therefore, we undertook a more-detailed molecular investigation of lipid species that may underpin altered hepatic glucose regulation, focussing on sphingolipids (31, 32). Targeted lipidomics revealed that total ceramide content was significantly lower in females than in males but was not influenced by CR (Fig. 6D); however, individual ceramide species were modulated by CR in a sex-dependent manner. Thus, 22:0 and 22:1 ceramides were lower in females than in males and were decreased by CR, with this diet effect being stronger in males (Fig. 6D, S8A). Conversely, 23:0 ceramides were greater in AL females than in AL males and were increased by CR in males only (Fig. 6D); a similar pattern occurred for 14:0, 16:0, 18:0, 18:1, 20:1, 26:0 and 26:1 ceramides (Fig. S8A).

Ceramides are generated from dihydroceramides (DHCs) and an elevated hepatic ceramide:DHC ratio promotes metabolic dysfunction and insulin resistance (32, 33). Thus, we further analysed DHCs and the ceramide:DHC ratio as potential mediators of CR's sexually dimorphic metabolic effects. As for total ceramides, total DHCs were lower in females than in males; however, unlike for total ceramides, CR increased total DHCs in males only (Fig. 6E). Differential effects of sex and/or diet also occurred for the specific DHC species. A common pattern was for CR to increase DHC concentrations in males only, such that these were lowest in AL males but similar in CR males, CR females and AL females; this pattern occurred for 16:0, 18:0, 18:1, 20:0, 23:0, 24:0, and 24:1 DHC species (Fig. 6E, S8B). CR increased 14:0 DHCs in both sexes, albeit more strongly in males, and decreased 22:0 DHCs across both sexes (Fig. 6E, S8B). The overall ceramide:DHC ratio was decreased by CR in males but not in females (Fig. 6E) and this also occurred for many of the individual species, including 16:0, 18:0, 20:0, 22:0, 22:1, 23:0, 24:0 and 24:1 (Fig. S8B); however, unlike for the total ceramide:DHC ratio, the ratio for each of these species was highest in the AL males and significantly lower in females, such that CR males had a similar ratio to females on either diet (Fig. S8B). Consistent with this, principal component analysis of these ceramide data identified AL males as the most distinct group, with CR males being more similar to females on either diet (Fig. 6F).

Together, these data indicate that CR suppresses ceramide synthesis and gluconeogenesis to a greater extent in males than in females, highlighting these as potential mechanisms for CR's sexually dimorphic effects on glucose homeostasis.

355 ***Sex differences in CR's metabolic effects are absent when CR is initiated in aged***
356 ***mice***
357 To test if ageing influences sex differences in the CR response, we next investigated
358 the effects of CR initiated in 18-month-old mice, when females are anoestrous (34).
359 Unlike in young mice, CR decreased body mass and fat mass to a similar extent in
360 aged males and females, while the decreases in lean mass were greater in aged
361 females (Fig. 7A-C). Consistent with this, CR's effects on the masses of individual
362 WAT depots did not differ between the sexes (Fig. 7D, S9A). Among non-adipose
363 tissues CR significantly decreased pancreas mass only in aged females, but there
364 were no sex differences in the effects of CR on the masses of other tissues (Fig. S9B-
365 C). Thus, unlike in young mice, aged males and females show a similar effect of CR
366 on overall adiposity and this is driven predominantly by loss of WAT.
367
368 We then investigated CR's effects on glucose homeostasis and insulin sensitivity. As
369 shown in Figure 8A, in aged mice the ability of CR to decrease fasting blood glucose
370 was not significantly influenced by sex. Similarly, CR improved glucose tolerance to a
371 similar extent in males and females, with tAUC but not iAUC being significantly
372 decreased with CR (Fig. 8B-C). The latter indicates that, as for young mice, CR
373 improves glucose tolerance in aged mice primarily by decreasing fasting glucose.
374 Despite no sex differences in glucose tolerance, both sex and diet decreased glucose-
375 stimulated insulin secretion, with this being greatest in aged AL males and decreased
376 by CR more so in males than in females (Fig. 8D). Interestingly, a sex-diet effect was
377 observed for HOMA-IR but not for the Matsuda Index (Fig. 8E); this is similar to the
378 effects observed in young mice (Fig. 3E) and suggests that in aged mice there remain
379 some sex differences in the relationship between plasma insulin and gluconeogenesis
380 in the fasted state. However, overall, these studies demonstrate that sex differences
381 in the metabolic effects of CR are highly age dependent, with CR being equally
382 effective in aged males and females whereas young females resist these aspects of
383 the CR response.
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385
386 ***In humans CR-induced fat loss is sex- and age-dependent***
387 We next investigated if the metabolic effects of CR in humans are also sex- and age-
388 dependent. To do so we analysed data from forty-two men and women who
389 participated in a weight loss study involving a 4-week CR intervention. As in young
390 mice, body mass and fat-free (lean) mass decreased more so in males than in females,
391 with males also showing a trend for greater fold-decreases in total fat mass (Fig. 9A-
392 F). When adjusted for age (Fig. 9G), fold-change in body mass differed significantly
393 between males and females (*P,* Intercept = 0.025). This demonstrates that overall
394 weight loss was greater in males than females, independent of age. In contrast, the
395 decreases in fat mass and fat-free mass were sex- and age-dependent. Thus, fat loss
396 in younger individuals was greater in males than females but, with age, fat loss
397 increased in females but diminished in males (Fig. 9H) (*P,* Slope = 0.007). Conversely,
398 the loss of fat-free mass in younger individuals was greater in females than in males
399 and this relationship reversed in older individuals (Fig. 9I). As for fat mass, the
400 relationship between age and loss of fat-free mass differed significantly between the
401 sexes (*P,* Slope = 0.0004).
402
403 These age-sex interactions were particularly apparent when the cohort was divided
404 into younger and older groups (<45 vs >45 years of age). Total weight loss was greater

405 in males than females regardless of age (Fig. 9J), but only in the younger age group
406 did males show greater fat loss than females (Fig. 9K). Conversely, males' greater
407 loss of fat-free mass occurred only in the older age group (Fig. 9L). Notably, a similar
408 pattern of age-dependent fat loss was apparent for CR in young vs aged mice (Fig.
409 S10). Thus, our data demonstrate that the ability of CR to decrease body mass, fat
410 mass and lean mass is sex- and age-dependent in both mice and humans.

DISCUSSION

Herein we aimed to determine the extent and basis of sex differences in the metabolic effects of CR. We reveal that 96.6% (mouse) and 95.7% (human) of published CR research overlooks potential sex differences, with most mouse studies using males only whereas most human studies combine data from males and females. We show that, in young mice, CR decreases fat mass and improves glucose homeostasis to a greater extent in males than in females, whereas these sex differences are blunted or absent when CR is initiated in old mice. The effects of CR on hepatic gene expression and sphingolipid content also differ between young male and female mice, highlighting the liver as a key mediator of CR's sexually dimorphic metabolic effects. Finally, we reveal that humans also exhibit age-dependent sex differences in the CR response, with younger age groups showing greater fat loss in males than females whereas, with increasing age, CR-induced fat loss becomes similar between the sexes. Together, these data identify sex and age as factors that have a major influence on the metabolic health outcomes of CR.

Age-dependent sex differences in CR's metabolic benefits

Our observation that young females resist overall weight loss during CR is consistent with some previous findings in mice (20, 21, 35), monkeys (3), and humans (36-41). Young females' resistance to CR-induced fat loss has also been reported in mice (20, 21, 42-44) and humans (19, 38, 45-47); however, fewer studies have directly assessed sex differences in the effect of CR on glucose homeostasis and insulin sensitivity. Many studies have included males and females but did not sex-stratify their analyses, thereby overlooking the potential impact of sex on these important metabolic outcomes (12, 48). Nevertheless, CR in monkeys and humans has been found to improve glucose homeostasis and insulin sensitivity more so in males than in females (3, 39), consistent with our findings in young mice.

Our observations fundamentally extend this previous research by demonstrating, for the first time, that these sex differences are age dependent. The novelty of this finding highlights that relatively few studies have investigated CR when initiated in aged subjects. In 2004, Dhahbi *et al* showed that CR's health benefits persist even when initiated in aged mice (49), and subsequent research has compared the metabolic effects of CR when implemented in young vs aged animals (50, 51). Unfortunately, these studies did not include females, an oversight that has persisted in the CR field (Figs. 1,S1). Other notable research has comprehensively compared aged males and females, but only in the context of lifelong CR that was initiated in younger mice (52). Two recent studies have also highlighted age-dependent sex differences in the effects of methionine restriction or time-restricted feeding, but these dietary interventions were limited to young vs middle-aged mice (53, 54). The most relevant previous research is perhaps from Brandhorst *et al,* who observed significant visceral fat loss in female mice fed a 'fasting-mimicking diet' from 16-20.5 months of age (55). While this is consistent with our observations in aged females (Fig. 7,S9), it is limited by having not assessed glucose homeostasis nor including male counterparts for direct comparison. Thus, our study is the first to compare CR when initiated in young or old males and females and to thereby identify age-dependent sex differences in CR's metabolic effects.

Our findings have several implications for interpreting previous CR research. Although most studies show that young females resist overall weight loss and fat loss during CR, such sex differences have not been universally observed. For example, in young female rats some studies report that CR decreases body mass and/or fat mass to a similar extent in both sexes (56-58), while others find fat loss to be even greater in females than in males (59, 60). CR-induced fat loss has also been noted in young female mice (61-64), albeit in studies that lack males for direct comparison. Several human studies also show no differences in total fat loss with CR, even in younger women who are unlikely to be menopausal (39, 41, 65). What are the reasons for these inconsistent results, and can they further inform the basis for sex differences in the CR response?

Considering our present findings, one possibility is that, even in younger subjects, age differences might have influenced the outcomes of these CR studies. Indeed, both Janssen *et al* and Das *et al* studied women who, while pre-menopausal, were significantly younger than their male counterparts (41, 65). Given that males' fat loss decreases as they get older (Figs. 9H,K), this disparity could have undermined the ability of these studies to detect sex differences in fat loss. A related, broader issue is that much human CR research has studied females who are over 45 years old (36, 38, 39, 45, 47, 66-68). This is important because it is the age at which oestrogen levels typically begin to decline (69) and after which we demonstrate no sex differences in fat loss (Fig. 9K). Thus, our findings underscore the importance of properly age-matching males and females, and ideally confirming menopausal status, if the response to CR is to be reliably assessed.

While age is clearly an important variable, many animal studies still find no resistance to fat loss in young females (61-64), even when appropriately age-matched to male counterparts (56-60). Therefore, other factors must also be influencing CR's sexually dimorphic effects. One possibility is that males show a greater CR response because they are usually heavier and fatter than females at the onset of CR, and thus have more fat available to lose. Consistent with this 'baseline adiposity' hypothesis, during CR in rats Hill *et al* found total fat loss to be greater in young females than young males and, unlike in mice, pre-CR adiposity was greater in the females (59, 60). Moreover, we show that sex differences in baseline adiposity become less pronounced with ageing, such that % fat mass is similar in aged male and female mice (Fig. 7D). Therefore, this hypothesis could explain why CR decreases fat mass in the fatter, aged females, whereas young, leaner females resist this effect. However, two key points argue against this hypothesis. Firstly, we show that young females' resistance to fat loss still occurs in obese and overweight humans, among whom % fat mass is greater in females than in males, irrespective of age (Fig. S11). This is consistent with numerous previous studies of overweight or obese humans, in which CR causes a greater degree of weight loss and/or fat loss in males than females (19, 36-40, 45, 47, 66). Secondly, in young, non-obese humans, sex differences in fat loss persist even after controlling for variation in baseline fat and body mass (41). Thus, sex differences in the CR response are a not simply a consequence of baseline differences in these parameters.

A third possibility is that young females' resistance to weight and fat loss can be overcome if the extent and/or duration of CR is great enough. Our CR protocol involves a 30% decrease in daily caloric intake over six weeks. In contrast, those studies

511 reporting fat loss in young females typically used CR of a greater extent and/or
512 duration. For example, Hill *et al* studied 60% CR for 11 weeks (59, 60) while Valle *et*
513 *al* used 40% CR for 14 weeks (57, 58). Others have tested CR of a similar extent to
514 our studies (20-30%) but for longer durations, ranging from ten weeks to six months
515 (56, 61, 63). It is now well established that the CR response is dose and duration
516 dependent (5, 21, 43, 52, 70, 71), with the former having been systematically
517 investigated in male mice (72-75). Thus, a key goal for future studies will be to
518 determine how sex and age influence these dose- and duration-dependent effects.
519
520 A final important consideration is the distinction between total versus regional
521 adiposity. Indeed, the CALERIE trial finds no sex differences in the loss of total fat
522 mass but does show that, compared to men, women resist loss of trunk fat during CR
523 (41). This is consistent with our data showing that fat loss in young male mice is
524 greatest for the visceral depots (Figs. 2G, S3A, S5G), and with previous observations
525 for CR in obese humans (19, 45, 47, 66). Some CR studies may therefore have failed
526 to detect sex differences because they measured only total adiposity, missing out on
527 possible site-specific differences (39, 76). Thus, any studies seeking to fully
528 understand sex differences in CR's metabolic effects, whether in preclinical models or
529 in humans, should always aim to assess adiposity on both whole-body and regional
530 levels.
531
532
533 ***Basis for young females' resistance to weight loss and fat loss***
534 Our studies also shed light on the mechanistic basis for these sex differences. The
535 ability of young females to resist weight loss and fat loss suggests that, compared to
536 males, females are better able to mitigate negative energy balance during CR.
537 Consistent with this, previous murine CR studies show that females decrease total
538 energy expenditure more than males, possibly resulting from greater suppression of
539 BAT activity (20, 57, 58). In contrast, we find that CR suppresses energy expenditure
540 similarly in young males and females (Fig. 4) without suppressing BAT activity (Fig.
541 5). This suggests that our females resist weight loss through other means. However,
542 a key consideration is the timing of these analyses: our mice underwent indirect
543 calorimetry from weeks 4 to 6 of CR, when body masses and adiposity are no longer
544 declining; instead, the sexually dimorphic decreases in body mass and composition
545 occur during the first two weeks of CR (Fig. 2A,C). Hence, we propose that females
546 do suppress energy expenditure more than males during this initial period, thereby
547 helping them to resist weight loss. Future studies should therefore ensure that any
548 calorimetry analyses coincide with relevant metabolic responses of CR.
549
550 Despite this limitation, our calorimetry data, together with our histological analyses,
551 provide further insights about why young females resist fat loss specifically. We show
552 that, compared to CR males, CR females tend to have greater decreases in energy
553 expenditure during the night (Fig. 4B), a time when, with our CR regimen, energy is
554 derived predominantly from increased fat oxidation (77). Thus, one possibility is that
555 young CR females maintain fat mass because of decreased fat oxidation. Previous
556 studies have also shown that CR in young female mice enhances WAT lipogenesis
557 without increasing lipolysis (62), consistent with our finding that CR does not decrease
558 adipocyte size in young females (Figs. 2H-I, S4). The situation in humans differs
559 slightly: CR enhances WAT lipolysis in both sexes but females resist this effect (38).
560 Together, these differences in lipid metabolism likely explain why fat loss in young

561 females is absent or blunted during CR. Whether ageing alters these sex differences
562 in lipid metabolism remains to be determined.
563
564
565 ***Importance of visceral fat loss for CR's metabolic effects***
566 These effects on fat mass and lipid metabolism likely contribute to young females'
567 resistance to improved glucose homeostasis during CR. Human studies have shown
568 that inefficient WAT lipolysis is associated with impaired glucose metabolism in
569 women (78) and that, during CR, decreased fat mass is linked to the improvements
570 in insulin sensitivity (12). Similarly, studies of CR or lipectomy in animal models
571 suggest that visceral fat loss contributes to CR's broader metabolic benefits (6, 9-
572 12). However, no previous research has investigated if this relationship between fat
573 loss and insulin sensitisation differs between the sexes. Our work therefore supports
574 and extends these previous studies by demonstrating that CR-induced fat loss
575 coincides with improved glucose homeostasis and insulin sensitivity in males and
576 aged females, whereas young females' resist these metabolic effects.
577
578
579 ***The liver as a key driver of sex differences in glucose tolerance***
580 Several of our findings highlight the importance of the liver as a key mediator of CR's
581 sexually dimorphic metabolic effects. Firstly, our PET/CT data show that CR improves
582 glucose tolerance without any marked increases in peripheral glucose uptake (Fig. 5).
583 This includes the BMAT-rich distal tibia, suggesting that, although BMAT has high
584 basal glucose uptake (29), increased BMAT glucose uptake is not required for
585 improved glucose tolerance during CR. Moreover, although CR does increase glucose
586 uptake in WAT and the kidneys this effect does not differ between males and females
587 and therefore is unlikely to explain the sex differences in glucose tolerance (Fig. 5B).
588 Together our data are consistent with human studies showing that, in the fasted state,
589 CR does not affect overall glucose disposal (79, 80). Secondly, during an OGTT we
590 show that CR decreases the tAUC but not the iAUC, highlighting decreases in fasting
591 glucose as the main driver of the improvements in glucose tolerance. Thirdly, we show
592 that CR's effects on hepatic gene expression differ between young male and female
593 mice. One notable finding is that CR increases *Pck1* expression in females only (Fig.
594 6A), consistent with CR females having greater fasting glucose than their male
595 counterparts. Diet and/or sex differences in hepatic *Ppara, Pparg, Chrebp, Mt-nd1,*
596 *Fabp5* and *Gpd1* expression are also notable because these encode proteins that
597 coordinate lipid metabolism and gluconeogenesis during fasting (81-85); hence, their
598 differential expression supports the conclusion that CR in females enhances hepatic
599 gluconeogenesis, lipogenesis and lipid oxidation, whereas in males these effects of
600 CR are blunted and/or absent. This is similar to the results of Della Torre *et al,* who
601 show that fasting for 6 hours inhibits hepatic gluconeogenesis and lipogenesis in male
602 mice but not in females (22).
603
604 How is loss of visceral fat linked with these hepatic effects? Visceral adiposity is
605 strongly associated with increased hepatic fat storage (86), and decreased liver fat
606 coincides with improved insulin sensitivity during CR in rats and humans (87, 88).
607 Moreover, young female mice resist the decreases in hepatic triglycerides following
608 short-term fasting (22) and prolonged time-restricted feeding (35). Therefore, we were
609 surprised to find that CR does not affect hepatic triglyceride content in young mice of
610 either sex (Fig. 6C). This may be because our young AL controls are lean and healthy,

611 and therefore already have livers that are not excessively fatty. In contrast, many of
612 the previous studies investigated CR in animals or humans that are older and/or with
613 type 2 diabetes (35, 87, 88), thereby increasing the baseline/control hepatic fat
614 content. This would not explain why our findings differ to those of Della Torre *et al,*
615 who did study young, healthy mice (22); however, it is perhaps unsurprising that our
616 6-week CR elicits different effects to those induced during shorter-term fasting.
617
618 Our above observations are important because they show that CR can exert metabolic
619 benefits without decreasing hepatic triglyceride content. In contrast, we find that
620 effects on sphingolipids may play a role, with CR altering hepatic sphingolipid content
621 more so in young males than in young females. Here, the effect of diet and/or sex
622 depends on the sphingolipid species concerned (Fig. 6F; S8). This is reminiscent of
623 the findings of Norheim *et al*, who report sex differences in hepatic ceramide content
624 in mice fed obesogenic diets (89). They further showed that the relationship between
625 ceramides, body fat and insulin sensitivity also differs between males and females,
626 focussing on 16:0 ceramide (C16:0). Indeed, ceramides' influence on insulin sensitivity
627 depends on the ceramide species. For example, hepatic increases in C16:0 have been
628 implicated with the development of obesity-associated insulin resistance (32, 90-92).
629 This is seemingly at odds with our finding that CR increases hepatic C16:0 in young
630 males but not young females (Fig. S8). Thus, unlike in obesity, increased hepatic
631 C16:0 during CR appears to be associated with improvements in insulin sensitivity.
632
633 The rate of conversion of ceramides to DHCs has also been suggested to be a root
634 cause of insulin resistance (93, 94). This underscores the concept that the ratio
635 between each ceramide and DHC species may have a larger influence on metabolic
636 function than the absolute values of either, a concept supported by others (33). More
637 research is needed to determine how specific ceramide and DHC species, and the
638 ratios thereof, impact sex differences in the health benefits of CR. Despite these
639 remaining questions, our work extends previous observations by revealing that CR's
640 hepatic effects are sexually dimorphic and that this contributes to the sex differences
641 in glucose tolerance and insulin sensitivity.
642
643
644 ***Oestrogen as a modulator of the CR response***
645 In many cases young females' resistance to the metabolic effects of CR is associated
646 with them already being relatively metabolically healthy on an AL diet. For example,
647 young AL females have lower visceral fat mass and are more glucose tolerant than AL
648 males (Figs. 2G, 3B-C), and are similar to CR males and females in terms of fasting
649 insulin and HOMA-IR (Fig. 3D-E). We also show that, for most sphingolipid species,
650 the hepatic ceramide:DHC ratio is similar between AL females and CR mice of either
651 sex (Fig. S8C). These patterns suggest that the mechanisms through which CR
652 decreases visceral adiposity, improves glucose tolerance and modulates sphingolipid
653 metabolism are already at least partially active in young females, even on an AL diet.
654
655 This observation helps to clarify potential mechanisms for CR's age-dependent sex
656 differences. Sexual dimorphism in metabolic function typically results from sex
657 hormones, with genetic factors and/or developmental programming also playing a role.
658 For example, X-chromosome-linked genetic factors influence sex differences in
659 adiposity independently of sex hormones or other secondary sexual characteristics
660 (95, 96), while the developmental and neonatal environment causes sexually

661 dimorphic metabolic programming (97). However, it is unlikely that these
662 developmental or genetic factors explain the sex differences we observe.
663 Developmental programming is unlikely because the early life environment of our mice
664 was the same for both sexes and was devoid of any adverse or stressful stimuli.
665 Similarly, if X-linked genetic factors were responsible then the sex differences would
666 be expected to persist in aged females, but they mostly do not. This leaves sex
667 steroids as the likely mediator. Consistent with this, oestrogen is now well known to
668 influence metabolic function and the fasting response (13, 22), and many actions of
669 oestrogens overlap with those central to the effects of CR (98-101). Herein, we show
670 that the sex differences in CR's metabolic effects are absent or greatly diminished
671 when CR is initiated at 18 months of age, when C57BL/6 females are typically
672 anoestrus (34, 69). Similarly, CR does decrease fat mass in young ovariectomised
673 females (102), while oestrogen treatment protects male mice from obesity-induced
674 insulin resistance (103, 104). Therefore, we propose that young females resist the
675 metabolic effects of CR because, through endogenous oestrogen action, they already
676 have high activation of pathways that mediate CR's metabolic effects.
677
678 On a molecular level we speculate that these age and sex differences relate to
679 oestrogen receptor alpha (ERα) activity in adipose tissue and the liver, a hypothesis
680 based on numerous lines of evidence. For example, in mice ERα contributes to lower
681 visceral adiposity in females than males (105), while in males pharmacological ERα
682 activation decreases fat mass (106). Oestrogens also act via ERα to prevent insulin
683 resistance and suppress hepatic gluconeogenesis (107, 108), whereas global ERα
684 knockout mice have impaired hepatic insulin sensitivity (109). ERα has also been
685 implicated in sex differences in hepatic metabolism during fasting (22). Thus, a key
686 goal of future studies should be to determine if oestrogen and ERα contribute to CR's
687 age-dependent, sexually dimorphic metabolic effects.
688
689
690 ***Limitations and further considerations***
691 Our study represents a significant advance in knowledge by revealing that age and
692 sex have a substantial influence over the metabolic benefits of CR. This highlights the
693 need for preclinical and human CR research to address these variables as key
694 determinants of the CR response, a consideration that we show has been lacking in
695 most CR research. We show that these sex differences occur on a systemic,
696 physiological level and that they extend to cellular and molecular effects within adipose
697 tissue and the liver, observations that identify potential mechanisms for the age-
698 dependent sex differences; however, a general limitation of our study is that these
699 mechanisms remain to be directly addressed. Specific limitations are as follows.
700
701 Firstly, regarding young females' resistance to weight and fat loss, our calorimetry
702 analyses did not find any significant sex differences in energy expenditure. As
703 explained above, this may be because these analyses were done later in the CR
704 protocol when dynamic changes in fat and body mass have stabilised. Our calorimetry
705 system was also unable to reliably assess the respiratory exchange ratio of our CR
706 mice because they have increased torpor and a lower metabolic rate that is at the limit
707 of detection for our system. Therefore, future studies should analyse energy
708 homeostasis within the first two weeks of onset of CR, both in young and old mice,
709 using a more-sensitive indirect calorimetry system.
710

711 Secondly, regarding insulin sensitivity and glucose homeostasis, it would be
712 informative to use hyperinsulinaemic-euglycaemic clamps to comprehensively assess
713 systemic and hepatic insulin sensitivity and determine if, as we propose, the
714 differences in glucose homeostasis relate primarily to effects on gluconeogenesis.
715
716 Thirdly, the role of oestrogen remains to be directly tested. This could be done in mice
717 through ovariectomy studies and/or manipulation of ERα activity. We have also tried
718 to determine oestradiol concentrations in our mouse plasma samples using mass
719 spectrometry, a method required for reliable assessment of sex steroid concentrations
720 (110). However, despite extensive optimisation efforts in our world-leading mass
721 spectrometry facility, we have not yet been able to reliably measure oestradiol using
722 the relatively small plasma volumes available. Related to this, in our human studies
723 we were unable to confirm the menopausal status of the female participants. Other
724 limitations of our human studies are that they included relatively few younger
725 participants and lacked analysis of regional adiposity (e.g. visceral versus
726 subcutaneous adipose tissue). Future human research must therefore prioritise
727 assessment of menopausal status and regional adiposity if the outcomes of CR are to
728 be fully interpreted.
729
730 A final limitation regards the underlying molecular mechanisms. Pathways implicated
731 in mediating CR's effects include activation of AMPK, SIRT, IGF-1 and FGF21,
732 modulation of mitochondrial function and inhibition of mTOR (5, 18). As discussed
733 above, we speculate that ERα activation may impinge on these pathways and thereby
734 contribute to sex differences in the CR response. Thus, genetic, pharmacological
735 and/or other approaches to manipulate these candidate pathways could be used to
736 further dissect the molecular mechanisms underlying CR's age-dependent sexually
737 dimorphic metabolic effects. Such approaches are a promising basis for future
738 research.
739
740 Beyond these specific limitations, a broader question is whether other effects of CR
741 also differ between males and females in this age-dependent manner. Some research
742 suggests that CR's effects on cardiovascular function and healthy ageing differ
743 between males and females (111-113); however, given the dearth of CR studies that
744 directly assess sex differences (Fig. 1, S1) there remains a critical need to determine
745 how age and sex influence the many other outcomes of CR. This is important not only
746 for understanding fundamental biology, but also if we are to translate the benefits of
747 CR, or CR mimetics, to improve human health. For example, if younger women resist
748 many of CR's health benefits, should any CR-based intervention focus only on older
749 women, who may be more responsive?
750
751 Finally, while it will be useful to understand the extent and basis for these sex
752 differences, it is also important to consider their broader purpose. Adipose tissue
753 profoundly influences female reproductive function, with adipose-secreted factors
754 acting within the central nervous system to indicate if the body's energy stores are
755 sufficient to support reproduction (114). The hormone leptin is the key adipose-derived
756 factor that impacts the hypothalamic-pituitary-gonadal axis: if fat mass is too low, for
757 example in anorexia nervosa or lipodystrophy, then leptin levels are also insufficient,
758 resulting in decreased gonadotropin secretion and impaired reproductive capacity.
759 Normal menses resumes in anorexic individuals who regain weight and fat mass, or
760 in lipodystrophic individuals following leptin treatment (114, 115). Notably, we

761 previously demonstrated that female mice resist hypoleptinaemia during CR (21).
762 Thus, we speculate that females resist fat loss during CR to preserve reproductive
763 function. Given the tight interplay between adipose tissue and systemic metabolic
764 homeostasis, such resistance to fat loss then has consequences for CR's other
765 metabolic effects. This evolutionary pressure to overcome periods of food scarcity
766 would thereby have driven the divergence of the CR response in males and females,
767 with implications for current efforts to exploit CR therapeutically to improve human
768 health.

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AUTHOR CONTRIBUTIONS

Contributions are based on the CRediT (Contributor Roles Taxonomy) and are as follows: ***Conceptualisation***, K.J.S., B.J.T. G.G., R.J.S. A.M.J. and W.P.C.; ***Methodology***, K.J.S., B.J.T., A.A.S.T., P.D.W., R.H.S. A.M.J. and W.P.C.; ***Investigation***, K.J.S., B.J.T., R.J.S., A.L., H.J.W., X.H., D.M., C.J.A-C., P.D.W., C.F., R.H.S. A.M.J. and W.P.C.; ***Formal Analysis***, K.J.S., B.J.T., R.J.S., A.L., P.D.W., C.F., N.M.M., A.M.J. and W.P.C.; ***Resources***, A.A.S.T., G.G., N.M.M., R.H.S., A.M.J. and W.P.C.; ***Writing – Original Draft***, K.J.S., B.J.T. and W.P.C.; ***Writing – Review & Editing***, K.J.S., B.J.T., G.G., R.H.S, N.M.M W.P.C.; ***Visualisation***, K.J.S., B.J.T., and W.P.C.; ***Supervision,*** G.G. N.M.M., R.H.S., A.M.J. and W.P.C.; ***Funding Acquisition***, K.J.S, A.A.S.T., G.G., N.M.M., R.H.S., A.M.J. and W.P.C.

DECLARATION OF INTERESTS

No competing interests are declared.

801 **METHODS**

802 **Table 1: Key Resources**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CHEMICALS		
18F-Fluorodeoxyglucose	Edinburgh Clinical Research Imaging Centre (Edinburgh, UK)	N/A
Ribozol™	Amresco (USA)	N580
OLIGONUCLEOTIDES		
See Table 4	This paper	N/A
EXPERIMENTAL MODELS: Organisms/Strains		
Mouse: C57BL/6J or C57BL/6NCrl	Charles River	027
SOFTWARE		
Prism	GraphPad Software, LLC	v9.2.0
PMOD	PMOD Technologies LLC (Zurich, Switzerland)	v3.806
OTHER		
TSE Phenomaster	TSE	PhenoMaster 1.0 with software version 6.1.9
OneTouch Verio Glucometer	OneTouch	User's manual here
Gamma counter	PerkinElmer (USA)	Wizzard[2]
Preclinical PET/CT scanner	Mediso (Budapest, Hungary)	nanoScan PET/CT 122S
Phenomenex Kinetex C8 HPLC system	Phenomenex	00D-4499-AN

803

804 **Animals**

805 Studies in C57BL/6NCrl and C57BL/6JCrl mice were approved by the University of
806 Edinburgh Animal Welfare and Ethical Review Board and were done under project
807 licenses granted by the UK Home Office. Mice were originally obtained from Charles
808 River (UK) and then bred in-house. They were housed on a 12 h light/dark cycle in a
809 specific-pathogen-free facility with free access to water and food, as indicated.
810 Differences in mouse cohorts are summarised in Table 2, along with the groups being
811 compared and the experimental unit. The exact number of mice used is stated in the
812 figure legends. Sample sizes for each cohort were determined by power calculations
813 using G*Power software, with effect sizes based on pilot studies and/or previously
814 published data for fat loss and improved glucose tolerance (the primary outcomes
815 being tested) (21, 42); to increase power, data for young mice are reported across
816 multiple cohorts from separate studies, each of which revealed the same sex
817 differences in CR's metabolic effects. Mice were randomly assigned to each diet. To
818 minimise potential confounders, such as the order of treatments or measurements,
819 mice were treated or analysed based on the order of their ID number, a variable
820 randomly distributed across the experimental variables (diet and sex). Blinding was
821 not possible during the *in vivo* experiments because the experimenter(s) can see,

822 based on presence or absence of diet, which mice are AL and which are CR; however,
823 data analysis was done in a blinded manner. Mice were excluded from the final
824 analysis only if there were confounding technical issues or pathologies discovered at
825 necropsy. Ten mice from the young study were excluded from the analysis of OGTT
826 and necropsy tissue masses because of a technical issue during gavaging for the
827 OGTT, which compromised their subsequent food intake. One mouse was excluded
828 from PET/CT data analysis because it accidentally had two doses of FDG
829 administered. One aged mouse was excluded because of developing confounding
830 liver tumours. For some cohorts, specific tissues were not weighed at necropsy and
831 therefore these mice have no data for these readouts. For further details, please see
832 the Source Data file.
833
834 **Mouse CR studies**
835 Before assigning to AL or CR diets, mice were housed individually between 8-9 weeks
836 of age (for young mice) or 77-78 weeks of age (for old mice). During this time, mice
837 were fed a control diet (Research Diets, D12450B) *ad libitum* and daily food intake
838 was determined by weighing the food remaining in each cage each day. Following one
839 week of single housing, all mice were randomly assigned to control diet (Research
840 Diets D12450B, provided AL) or the CR diet (Research Diets D10012703,
841 administered at 70% of the average daily AL diet consumption). This CR diet is
842 formulated for iso-nutrition at 70% CR, ensuring that malnutrition is avoided. The
843 evening-fed CR mice (Fig. S5-S6) were fed daily between 1800-1900, while all other
844 CR mice were fed daily between 0900-1000. For the first two weeks of CR mice were
845 monitored and weighed daily to ensure they did not lose more than 30% of their initial
846 body mass. For the following 4 weeks of CR, body mass was recorded weekly. Mice
847 were assessed non-invasively for body fat, lean mass, and free fluid weekly from
848 weeks 0 to 6 using TD-NMR (Minispec LF90II; Bruker Optics, Billerica, MA, USA). At
849 necropsy, mice were humanely sacrificed via cervical dislocation and decapitation.
850 Tissues were harvested, weighed, and stored either in 4% formalin or on dry ice.
851 Tissues in formalin were fixed at 4ºC for 2-3 days before being washed and stored in
852 DPBS (Life Technologies). Tissues on dry ice were stored at -80ºC prior to
853 downstream analysis.
854

	Young	Aged	Evening-fed
Age at single housing	8 weeks	77 weeks	8 weeks
Age at start of CR	9 weeks	78 weeks	9 weeks
Time of feeding	0900-1000	0900-1000	1800-1900
Duration of CR	6 weeks	6 weeks	4 weeks
Related data	Fig. 2-6, S2-S4, S7-S8, S10	Fig. 7-8, S9-S10	Fig. S5-S6
Experimental unit	Single mouse		
Groups compared	AL vs CR (within sex); Male vs Female (within diet); and diet*sex interactions		

855 **Table 2.** Summary of CR protocol in each group of mice. Because mice are singly
856 housed, each mouse represents an independent experimental unit.
857
858 **Human weight loss study – Participants**
859 Forty-five overweight and obese males and females; age 21-61y, Body Mass Index
860 (BMI) 26-42 kg/m^2, were recruited by newspaper advertisement to participate in a

weight loss (WL) dietary intervention study. Thus, the participants were non-randomly selected individuals who were sufficiently motivated to actively respond to the request for volunteers. During recruitment, participants underwent a medical examination and their general practitioners were contacted to confirm medical suitability to participate in the study. Written, informed consent was obtained, and the study was reviewed by the NHS North of Scotland Research Ethics Service (ethics number 09/S0801/80). Participants were informed that the study was funded by an external commercial sponsor, but the company was not named, and all packaging of the food range was removed before distribution. Three participants withdrew for personal reasons therefore data for *n* = 42 is presented throughout; participant characteristics are summarised in Table 3.

Sex	**Number of subjects**	**Age** (years)	**BMI** (kg/m^2)	**Glucose** (mM)	**Diabetic**
Male	20	48.61 ± 10.7	34.7 ± 4.2	5.6 ± 0.7	0%
Female	22	44.7 ± 9.7	32.8 ± 3.7	5.2 ± 0.3	0%

Table 3. Human subject characteristics at baseline. Age, BMI and glucose are mean ± SD. *ND* = not determined.

Human weight loss study – Protocol

Participants were asked to attend the Human Nutrition Unit (HNU) at the Rowett Institute of Nutrition and Health (Aberdeen, UK) twice weekly to pick up food supplies. All required food, drinks and snacks were provided and the study dietician was present to answer any questions. Participants were provided with a diet guide whereby women were encouraged to consume no more than 6.3 MJ/d (1,500 kcal) and men 8.4 MJ/d (2,000 kcal). They were encouraged to consume 3 meals a day (breakfast, lunch and dinner) with a portion of protein at each meal, and 5 portions of fruit and vegetables, 2 portions of fats, 3 portions of milk and dairy and 4 portions of wholegrain bread, potatoes and cereals each day. Participants completed a descriptive food diary every day to record what and when they ate. Participants ate *ad libitum*, choosing when and what to eat from their supplies. They completed a detailed 7d weighed dietary record during week 4 of the WL phase of the study, to compare eating habits with a baseline 7d weighed record they completed before the study started. Energy and nutrient intakes were calculated using the Windiets programme (Univation Ltd; The Robert Gordon University, Aberdeen, UK).

Human weight loss study – Measurement of body composition

Before and after WL, at weeks 0 and 4 respectively, measurements of body composition were conducted under standardized conditions as described previously (116), with participants instructed to fast overnight (at least 10 h). Height was measured at the beginning of the study to the nearest 0.5 cm using a stadiometer (Holtain Ltd, Crymych, Dyfed, Wales). The body weight of the participants was measured at screening, during food provision days and on all test days, wearing a previously weighed dressing gown, to the nearest 100 g on a digital scale (DIGI DS-410, C.M.S. Weighing Equipment Ltd, London, UK). Fat mass (FM) and fat free mass (FFM) were measured with the use of air displacement whole-body plethysmography (BodPod® Gold - Body Composition System, Cosmed, Italy) (117).

Indirect calorimetry

Male and female AL and CR C57BL/6J mice aged ~13 weeks were housed individually in TSE PhenoMaster cages for indirect calorimetry, monitoring of physical activity, and measurement of *ad libitum* food and water consumption. Mice in each group were first housed in these cages for 24 h for acclimation and then housed for a further 72 h for data collection.

Oral glucose tolerance tests (OGTT)

Mice were given a half portion of food the night (6pm) prior to the metabolic challenge. At 12:00 pm on the day of the OGTT a basal blood sample was collected by venesection of the lateral tail vein into EDTA-coated capillary tubes (Microvette 16.444, Sarstedt, Leicester, UK) and the basal glucose levels was measured using a glucometer (OneTouch Verio IQ, LifeScan Inc., Zug, Switzerland). D-glucose (Sigma, G8270,Poole, UK) was then administered at 2 mg per g of body mass by oral gavage of a 25% (w/v) solution. At 15, 30, 60 and 120 minutes post-gavage, blood glucose was measured by a glucometer and tail vein blood was sampled in EDTA tubes, as for the basal (0 min) timepoint. Blood samples were kept on ice prior to isolation of plasma. Insulin was then measured in plasma samples by ELISA, following the manufacturer's instructions (cat. #90080, ChrystalChem, Chicago, IL, USA). Insulin was converted from ng/ml to µU/mL by multiplying the ng/mL value by 28.7174 (based on mouse insulin molar mass of 5803.69). Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and Matsuda index were calculated as previously described (25, 26)).

PET/CT scanning

Immediately before and 15- and 60-min post ^{18}F-FDG intraperitoneal injection, blood glucose was measured by tail venesection and blood was sampled directly into EDTA-microtubes (Sarstedt, Leicester, UK). At 60-minutes post ^{18}F-FDG injection, mice were anaesthetised and ^{18}F-FDG distribution was assessed by PET/CT. Following the PET/CT scan, mice were sacrificed by overdose of anaesthetic (isoflurane), death was confirmed by cervical dislocation and tissues of interest were then dissected in a lead-shielded area. Half of each of the dissected tissues were snap frozen on dry ice or placed into 10% formalin. ^{18}F-FDG uptake was then determined using a gamma counter (PerkinElmer) as previously described (29)

PET/CT analysis

PET/CT images were reconstructed and data analysed using PMOD version 3.806 (PMOD, Zurich, Switzerland). SUVs were calculated for regions of interest, namely BAT, iWAT, gWAT; heart; bone tissue (without BM) from tibiae, femurs, and humeri; To distinguish bone tissue from BM, a calibration curve was generated using HU obtained from the acquisition of a CT tissue equivalent material (TEM) phantom (CIRS, model 091) and mouse CT scans, as previously described (29).

Histology and Adipocyte size quantification

Dissected mouse tissue was fixed for 48 h and paraffin embedded by the histology core at The University of Edinburgh's Shared University Research Facilities (SuRF). Paraffin-embedded tissue sections were then sectioned at 100 µm intervals using a Leica RM2125 RTS microtome and collected onto 76 x 26 mm StarFrost 624 slides (VWR, UK). The slides were baked at 37ºC overnight before Haematoxylin and Eosin (H&E) staining. Quantification of adipocyte number and size was per formed on H&E

955 stained adipose tissue using the ImageJ plug-in Adiposoft, an automated software for
956 the analysis of adipose tissue cellularity in histological sections (118).
957
958 **RNA isolation and reverse transcription**
959 RNA was isolated from tissues using Ribozol™ solution (cat. No. N580, Amresco,
960 USA,) according to the manufacturer's protocol. RNA quantity and quality were
961 quantified using a NanoDrop spectrophotometer (Thermo Scientific,USA), and cDNA
962 was synthesised from 1 µg RNA using the Taqman® High Capacity cDNA Reverse
963 Transcriptase Kit (Applied Biosystems, USA, cat. no. N8080234) in accordance with
964 manufacturer's guidelines and as described previously (29).
965
966 **qPCR**
967 Transcript expression was analysed by qPCR in duplicate reactions using qPCRBIO
968 SyGreen Blue Mix (part number PB20.17-20; PCR Biosystems, UK). Reactions were
969 loaded into 384-well qPCR plates (part number 72.1985.202; Sarstedt, UK) and run
970 on a Light Cycler 480 (Roche). Primers (Table 4) were designed using NCBI Primer
971 Blast and validated using cDNA titrations, with melting analysis and gel
972 electrophoresis used to confirm specificity for the intended target. Transcript
973 expression was calculated based on a cDNA titration loaded on each plate and was
974 normalised to the geometric mean of *Tbp, Rn18s* and *Ppia* expression (119).
975

Transcript	***Forward primer (5'-3')***	***Reverse primer (5'-3')***
Chrebp	CCAGCCTCAAGGTGAGCAAA	CATGTCCCGCATCTGGTCA
Fabp5	TGAAAGAGCTAGGAGTAGGACTG	CTCTCGGTTTTGACCGTGATG
Gpd1	ATGGCTGGCAAGAAAGTCTG	CGTGCTGAGTGTTGATGATCT
Mt-co1	GAGAGGCCTTTGCTTCAAAAA	AGGTTGGTTCCTCGAATGTG
Mt-nd1	CACTCCTCGTCCCCATTCTA	ATGCCGTATGGACCAACAAT
Pck1	**GTTTGATGCCCAAGGCAACT**	**CCTTCCCAGTAAACACCCCC**
Ppara	CCTGAACATCGAGTGTCGAATAT	TCTTCTTCTGAATCTTGCAGCT
Pparg	GGAAAGACAACGGACAAATCAC	TACGGATCGAAACTGGCAC
Ppia	CACCGTGTTCTTCGACATCA	CAGTGCTCAGAGCTCGAAAGT
Rn18s	CGATGCTCTTAGCTGAGTGT	GGTCCAAGAATTTCACCTCT
Tbp	ACCTTATGCTCAGGGCTTGG	GCCGTAAGGCATCATTGGAC

976 **Table 4.** Sequences of primers used for qPCR
977
978 **Liver ceramide quantification**
979 Liver ceramide and dihydroceramide content was determined using LC-MS
980 (Lipidomics Core Facility; University of the Highlands and the Islands, Inverness, UK).
981 As per Folch *et al.* 1957 (120), 100 mg of frozen liver tissue was homogenised in ice-
982 cold MeOH using a mechanical tissue homogeniser. Fifty microlitres of each sample
983 was combined with 100 µL ceramide 17:0 (1 µM in MeOH) and 25 µL dihydroceramide
984 12:0 (2 µM in MeOH) (Avanti Polar Lipids, Alabaster, AL, USA) as internal standards.
985 MeOH (1.875 mL) and $CHCl_3$ (4 mL) were added. Samples were then stored on ice
986 for 1 hour with intermittent vortex mixing, centrifuged at 2,000 rpm for 5 minutes at 4°C
987 and the supernatant was collected. KCl (0.9%; 1.45 mL per sample) was added to the
988 supernatant and the samples were centrifuged at 2,000 rpm for 5 minutes at 4°C. The
989 lower phase was collected and evaporated under nitrogen and reconstituted in 1 mL
990 $CHCl_3$. Solid phase extraction was performed via a 100 mg/3 mL Isolute silica column
991 (Biotage, Uppsala, Sweden). The column was conditioned twice with 3 mL $CHCl_3$ and
992 then loaded with 1 mL of the sample. The bound sample was washed with 2 washes

of 3 mL $CHCl_3$ then eluted with 2 additions of ethylacetate $CHCl_3$ (1:1 v/v). The collected eluate was evaporated and dried under vacuum and reconstituted in 200 µL MeOH. LC-MS/MS analyses were performed in positive ion mode on a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with a HESI probe and coupled to a Thermo Accela 1250 UHPLC system. The ceramides and dihydroceramides were separated on a Kinetex C8 column (1.7 µm; 100 × 2.1 mm) (Phenomenex, Macclesfield, UK). Mobile phase A was 90% H_2O, 9.9% acetonitrile and 0.1% formic acid. Mobile phase B was 99.9% acetonitrile and 0.1% formic acid. The gradient was held at 80% mobile phase B for 1 minute then gradually increased to 100% mobile phase B over 15 minutes. Mobile phase B at 100% was maintained for 1 minute and then equilibrated to storage conditions. The flow rate used was 500 µL per minute at 40°C. Two µL of each sample was injected into the LC-MS/MS for ceramide analysis and 10 µL for dihydroceramide. Total ceramide and dihydroceramide concentrations were calculated from the summed concentrations of all the monitored molecular species. All values were normalised to wet weight of liver.

Statistical analysis

Data were analysed for normal distribution using the Shapiro-Wilk normality test. Normally distributed data were analysed by ANOVA, mixed models or t-tests, as appropriate. Where data were not normally distributed, non-parametric tests were used. When appropriate, *P* values were adjusted for multiple comparisons. Data are presented as mean ± SEM or as Violin plots, as indicated in the figure legends. All statistical analyses were performed using Prism software (GraphPad, USA). A *P*-value <0.05 (after adjustment for multiple comparisons) was considered statistically significant.

Data availability

All source data from which the figures are based is available through University of Edinburgh DataShare at https://doi.org/10.7488/ds/3277. All other relevant data are available from the authors upon reasonable request.

FIGURE TITLES AND LEGENDS

Figure 1 – Summary of sex differences in mouse and human CR research.
PubMed was searched using MeSH terms to identify research articles that studied caloric restriction *in vivo* in mice (**A,B**) or humans (**C,D**), published between 2003 and 2021. Search results were then classified into the following groups depending on the sexes included in each study: 'Male only' = male subjects used exclusively; 'Female only' = female subjects used exclusively; 'Male & Female separate' = male and female subjects used with data reported respective to each sex, allowing sex differences to be addressed; 'Male & female combined' = male and female subjects used with data from both sexes combined either in part or in full; 'N/A' = no sex data available. **(A,C)** Cumulative publications for studies within each group. The boxes between (A) and (C) highlight the dates of funders' policies highlighting the importance of sex as a biological variable. (**B,D**) Pie charts of those studies in the 'Male & Female separate' group that considered sex a biological variable, and the proportion of these that did or did not detect sex differences. Source data are provided as a Source Data file. See also Supplementary Figure 1.

Figure 2 – Female mice resist CR-induced weight loss, fat loss and adipocyte hypotrophy.
Male and female mice on a C57BL/6NCrl or C57BL/6J background were fed *ad libitum* (AL) or a 30% CR diet from 9-15 weeks of age (0-6 weeks of CR). **(A-F)** Each week mice were weighed (A,B) and body composition was determined by TD-NMR (C-F). Body mass, fat mass and lean mass are shown as absolute masses (A,C,E) or fold-change relative to baseline (B,D,F). **(G)** Masses of gWAT (gonadal WAT), iWAT (inguinal WAT), mWAT (mesenteric WAT), pWAT (perirenal WAT), pcWAT (pericardial WAT) and BAT (brown adipose tissue) were recorded at necropsy and are shown as % body mass. **(H,I)** Micrographs of H&E-stained sections of gWAT (H) were used for histomorphometric analysis of adipocyte area (I); in (H), scale bar = 100 µm. Data in (A-F) are shown as mean ±SEM of the following numbers of mice per group: *male AL*, n=42; *female AL,* n=43; *male CR,* n=44; *female CR,* n=52. Data in (G) are shown as violin plots of the following numbers of mice per group and tissue type: *male AL,* n=29 (iWAT, gWAT, mWAT, BAT), 15 (pWAT) or 14 (pcWAT); *female AL,* n=28 (iWAT, gWAT, mWAT, BAT), 14 (pWAT) or 13 (pcWAT); *male CR,* n=33 (iWAT, gWAT, mWAT, BAT), 16 (pWAT) or 15 (pcWAT); *female CR,* n=34 (iWAT, gWAT, mWAT, BAT), 15 (pWAT) or 14 (pcWAT). Data in (H-I) are shown as representative micrographs or violin plots from 5 (*male AL)* or 6 (*female AL, male CR, female CR)* mice per group. For (A-F), significant effects of diet, sex and/or time, and interactions thereof, were determined by 3-way ANOVA or a mixed-effects model. For (G), significant effects of diet and/or sex within each tissue were determined by 2-way ANOVA with Tukey's multiple comparisons tests. For (I), significant effects of diet and/or sex on adipocyte area (Ad.Ar) were determined using a mixed-effects model, while significant differences in median Ad.Ar between AL and CR mice were determined 2-way ANOVA with Šídák's multiple comparisons test. *P values* from ANOVA or mixed models are shown beneath the graphs, as indicated. For (G) and (I), significant differences between comparable groups are indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). Source data are provided as a Source Data file. See also Supplementary Figures 2-5 and 10.

Figure 3 – The effects of CR on glucose homeostasis differ between young male and female mice.

Male and female C57BL6/NCrl mice were fed AL or CR diet from 9-15 weeks of age, as described for Figure 2. **(A)** Random-fed blood glucose was recorded each week. **(B-D)** At 13 weeks of age mice underwent an oral glucose tolerance test (OGTT). (B) Blood glucose readings during the OGTT. (C) Area under the curve (AUC) during the OGTT was determined relative to 0 mmol/L (total AUC: tAUC) and relative to baseline (incremental AUC: iAUC). (D) Glucose-stimulated insulin secretion in mice during OGTT was assessed using an insulin ELISA. **(E)** HOMA-IR and Matsuda indices of mice calculated from glucose and insulin concentrations during the OGTT. Data in (A), (B), and (D) are presented as mean ± SEM. Data in (C) and (E) are presented as violin plots overlaid with individual data points. For each group and timepoint, the following numbers of mice were used: **(A)**: *male AL,* n=42 (Wk 0), 36 (Wk2, Wk 2), 34 (Wk 3), 29 (Wk 4), 26 (Wk 5), or 22 (Wk 6); *female AL,* n=43 (Wk 0), 28 (Wk 1), 35 (Wk 2), 31 (Wk 3), 27 (Wk 4), 26 (Wk 5), or 21 (Wk 6); *male CR,* n=44 (Wk 0), 40 (Wk 1), 38 (Wk 2), 35 (Wk 3), 31 (Wk 4), 29 (Wk 5), or 26 (Wk 6); *female CR,* n= 51 (Wk 0), 44 (Wk 1, Wk 2), 41 (Wk 4), 35 (Wk 4), 33 (Wk 5), or 27 (Wk 6). **(B)**: *male AL,* n=27 (T_0, T_{15}, T_{30}, T_{60}, T_{120}); *female AL,* n=26 (T_{15}, T_{30}, T_{120}), or 25 (T_0, T_{60}); *male CR,* n=27 (T_0, T_{15}, T_{30}, T_{60}, T_{120}); *female CR,* n=28 (T_0, T_{15}, T_{30}, T_{60}, T_{120}). **(C)**: *male AL,* n=27; *female AL,* n=26; *male CR,* n=27; *female CR,* n=28. **(D)**: *male AL,* n=9 (T_0, T_{15}, T_{30}, T_{60}, T_{120}); *female AL,* n=11 (T_0, T_{15}), 10 (T_{30}, T_{60}), or 8 (T_{120}); *male CR,* n=17 (T_0), 16 (T_{30}), 15 (T_{60}), 13 (T_{15}), or 11 (T_{120}); *female CR,* n=11 (T_{15}, T_{30}, T_{60},), or 10 (T_0, T_{120}). **(E)**: *male AL,* n=9; *female AL,* n=9; *male CR,* n=16; *female CR,* n=9. In (A), (B), and (D), significant effects of diet, sex and/or time, and interactions thereof, were determined using a mixed-effects model. In (C) and (E), significant effects of sex, diet, and sex-diet interaction for tAUC, iAUC, HOMA-IR and Matsuda Index were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown with each graph. For (C) and (E), statistically significant differences between comparable groups are indicated by *** ($P<0.001$) or with *P* values shown. Source data are provided as a Source Data file. See also Supplementary Figure 6.

Figure 4 – CR decreases energy expenditure in male and female mice, with a trend for greater nighttime decreases in females.

Male and female mice were fed AL or CR diets as described for Figure 2. At 12-13 or 14-15 weeks of age mice were housed individually in TSE PhenoMaster cages for 3 days for indirect calorimetry. **(A,B)** Absolute energy expenditure (watts, W) was recorded every 15 minutes throughout the 3 days. **(A)** Average energy expenditure per hour over the 24 h light (Day) and dark (Night) period, spanning days 2-3 of PhenoMaster housing. **(B)** Energy expenditure (kJ) during the Day, Night, or Day + Night (Total). **(C)** Energy expenditure normalised to lean body mass (kJ/g) during the Day, Night, or Day + Night (Total). **(D)** Linear regression of lean mass vs Total (Day + Night) energy expenditure (kJ/24 h). Data in (A-D) are from 13 (*male AL*) or 14 (*male CR, female AL, female CR)* mice per group. In (A), data are shown as mean ± SEM; significant effects of diet, sex and/or time, and interactions thereof, were determined by 3-way ANOVA, with *P* values shown beneath the graph. In (B-C), data are shown as violin plots overlaid with individual data points; within each time period (Day, Night, or Total), significant effects of sex, diet, and diet*sex interaction were assessed using 2-way ANOVA, with *P* values shown beneath each graph. Statistically significant differences between comparable groups were further assessed by Tukey's multiple

comparisons tests and are indicated by ** (*P*<0.01) or *** (*P*<0.001). For linear regression in (D), ANCOVA was used to test if the relationship between lean mass and total energy expenditure differs significantly across all of the individual diet-sex groups (*i. All mice*); between AL and CR mice, irrespective of sex (*ii. AL vs CR (M+F)*); and between males and females fed AL diet (*iii*) or CR diet (*iv*) only. ANCOVA *P* values for differences in slope and intercept are reported beneath the graph.

Figure 5 – The effects of CR on glucose uptake do not differ between male and female mice. Male and female C57BL6/NCrl mice were fed AL or CR diet from 9-15 weeks of age, as described for Figure 2. At 15 weeks of age, glucose uptake was assessed by PET/CT with ^{18}F-fluorodeoxyglucose (^{18}F-FDG). **(A-C)** ^{18}F-FDG uptake in the indicated tissues was determined as SUVs from PET/CT scans (A) or as % injected dose per gram of tissue (%ID/g) from gamma counting of dissected whole tissues (C). **(B)** PET/CT images confirm that ^{18}F-FDG uptake in interscapular BAT is greater in females than in males, regardless of diet. Data are shown as violin plots (A,C) or representative images (B) of 7 (*male AL, female AL, male CR*) or 6 (*female CR*) mice per group. For each tissue in (A) and (C), significant effects of sex, diet, and sex-diet interaction were assessed by 2-way ANOVA; overall *P* values for each variable, and their interactions, are shown beneath each graph. Significant differences between each group, as determined using Tukey's multiple comparisons test, are indicated by * (*P* <0.05). Source data are provided as a Source Data file. See also Supplementary Figure 7.

Figure 6 – CR exerts sexually dimorphic effects on hepatic gene expression and sphingolipid content.
Male and female C57BL6/NCrl mice were fed AL or CR diet from 9-15 weeks of age, as described for Figure 2. Livers were sampled at necropsy (15 weeks) and used to assess mRNA transcripts, triglyceride content, and ceramide and dihydroceramide content. **(A)** Expression of the indicated transcripts was determined by qPCR. Transcript expression was normalised to the geometric mean of *Tbp, Rn18s* and *Ppia* expression and is presented relative to the highest group average. **(B)** Principal component analysis of transcript expression in (A). **(C)** Hepatic triglyceride content. **(D,E)** LC-MS analysis of total, 22:0 and 23:0 ceramides (D), dihydroceramides (E, left side) and the ceramide:DHC ratio (E, right side). **(F)** Principal component analysis of ceramide and dihydroceramide content based on data in (D-E) and Supplementary Figure 8. Data in (A), (C), (D), and (E) are presented as truncated violin plots overlaid with individual data points. Data represent the following numbers of mice per group. **(A-B)**: *male AL,* n=6; *female AL,* n=6; *male CR,* n=6; *female CR,* n=6. **(C)**: *male AL,* n=9; *female AL,* n=8; *male CR,* n=9; *female CR,* n=8. **(D-F):** 6 mice per group. For (A), (C), (D) and (E), significant effects of sex, diet, and sex-diet interaction were assessed using 2-way ANOVA; overall *P* values for each variable, and their interactions, are shown beneath each graph. Significant differences between comparable groups were assess using Tukey's multiple comparison test and are indicated by * (*P*<0.05), ** (*P*<0.01) or *** (*P*<0.001). Source data are provided as a Source Data file. See also Supplementary Figure 8.

Figure 7 – Sex differences in CR-induced weight loss and fat loss are absent when CR is initiated in aged mice.

Male and female C57BL6/NCrl mice were single housed and fed AL or a CR diet (70% of daily AL intake) from 78-84 weeks of age. **(A-F)** Each week mice were weighed (A,B) and body composition was determined by TD-NMR (C-F). Body mass, fat mass and lean mass are shown as absolute masses (A,C,E) or fold-change relative to baseline (B,D,F). **(G)** Masses of gWAT, iWAT, mWAT, pWAT, pcWAT and BAT were recorded at necropsy and are shown as % body mass. Data in (A-F) are shown as mean ±SEM. Data in (G) are shown as violin plots overlaid with individual data points. For each group and timepoint, the following numbers of mice were used: **(A,B)**: *male AL*, n=9 (Wk 0, Wk 2, Wk3, Wk 4), 8 (Wk 1), or 6 (Wk 5, Wk 6); *female AL,* n=13 (Wk 0, Wk 2), 12 (Wk 4, Wk 5), 11 (Wk 6), 9 (Wk 1, or 8 (Wk 3); *male CR,* n=10 (Wk 0, Wk 2-4), 9 (Wk 1), 7 (Wk 5), or 6 (Wk 6); *female CR,* n=14 (Wk 0, Wk 4, Wk 5), 13 (Wk 2, Wk 6), 10 (Wk 3), or 9 (Wk 1). **(C-F)**: *male AL,* n=9 (Wk 0, Wk 2), 8 (Wk 3, Wk 4, Wk 6), or 7 (Wk 1, Wk 5); *female AL,* n=13 (Wk 0, Wk 2), 9 (Wk 4, Wk 6), 8 (Wk 5), 6 (Wk 1), or 5 (Wk 3); *male CR,* n=10 (Wk 0, Wk 2), 9 (Wk3-6), or 8 (Wk 1); *female CR,* n=14 (Wk 1, Wk 2), 11 (Wk 4-6), 7 (Wk 3), or 6 (Wk 1). **(G)**: *male AL,* n=9; *female AL,* n=12 (iWAT, mWAT) or 11 (gWAT, pWAT); *male CR,* n=10 (iWAT, pWAT), or 9 (gWAT, mWAT); *female CR,* n=14 (mWAT, pWAT), 13 (iWAT), or 12 (gWAT). For (A-F), significant effects of diet, sex and/or time, and interactions thereof, were determined by 3-way ANOVA or a mixed-effects model. For (G), significant effects of sex, diet, and sex-diet interaction were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown beneath each graph. For (G), significant differences between comparable groups are indicated by ** ($P<0.01$) or *** ($P<0.001$). Source data are provided as a Source Data file. See also Supplementary Figures 9 and 10.

Figure 8 – Sex differences in CR's effects on glucose homeostasis are largely absent when CR is initiated in aged mice.

Male and female C57BL6/NCrl mice were fed AL or CR diet from 78-84 weeks of age, as described for Figure 7. **(A)** Random-fed blood glucose was recorded each week. **(B-D)** At 82 weeks of age mice underwent OGTTs. Blood glucose was recorded at each timepoint (B) and the tAUC and iAUC was determined (C). (D) Glucose-stimulated insulin secretion in mice during OGTT was assessed using an insulin ELISA. **(E)** HOMA-IR and Matsuda indices of mice calculated from glucose and insulin concentrations during the OGTT. Data in (A), (B), and (D) are presented as mean ± SEM. Data in (C) and (E) are presented as violin plots overlaid with individual data points. For each group and timepoint the following numbers of mice were used: **(A)**: *male AL,* n=9 (Wk 0, Wk 2-4, Wk 6), 8 (Wk 1), or 5 (Wk 5); *female AL,* n=12 (Wk 0, Wk2, Wk4), 8 (Wk 1, Wk 3), or 11 (Wk 5, Wk 6); *male CR,* n=10 (Wk 0, Wk 2-4, Wk 6), 9 (Wk 1), or 7 (Wk 5); *female CR,* n= 14 (Wk 0, Wk 2, Wk 4-6), 9 (Wk 1) or 10 (Wk 3). **(B-E)**: *male AL,* n=9 (B-C) or 5 (D-E); *female AL,* n=12; *male CR,* n=10; *female CR,* n=14. In (A), (B), and (D), significant effects of diet, sex and/or time, and interactions thereof, were determined using a mixed-effects model or 3-way ANOVA. In (C) and (E), significant effects of sex, diet, and sex-diet interaction for tAUC, iAUC, HOMA-IR and Matsuda Index were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown with each graph. For (C) and (E), statistically significant differences

between comparable groups are indicated by * (P<0.05) or *** (P<0.001), or with P values shown. Source data are provided as a Source Data file.

Figure 9 – Effects of CR on body mass and body composition in humans are sex- and age-dependent.

Twenty male and twenty-two female volunteers participated in a weight loss study involving a 4-week dietary intervention. **(A-F)** Body mass was recorded each week (A,B)**.** Fat mass (FM) and fat-free mass (FFM) were measured by air-displacement whole-body plethysmography at weeks 0 and 4 (C-F). Body mass, fat mass and fat-free mass are shown as absolute masses (A,C,E) or fold-change relative to baseline (B,D,F). Data are presented as mean ± SEM**.** For (A-C) and (E), significant effects of time, sex, and time*sex interaction were assessed using 2-way ANOVA. In (B), (D) and (F), significant differences between males and females at each time point were determined by Šídák's multiple comparisons test (B) or unpaired T-test (D,F) and are indicated by * (P<0.05) or ** (P<0.01). **(G-I)** Simple linear regression of age vs fold-change (*week 4 vs week 0)* in body mass (G), fat mass (H) and fat-free mass (I). For each sex, significant associations between age and outcome (fold-change) are indicated beneath each graph as '*P,* Slope'. ANCOVA was further used to test if the age-outcome relationship differs significantly between males and females. ANCOVA results are reported beneath each graph as '*P,* Slope' and '*P,* Intercept' for males vs females (M vs F). In (G), similar slopes but different intercepts show that sex significantly influences weight loss, but the influence of age does not differ between the sexes. In (H,I) the slopes differ significantly, indicating that the age-outcome relationship differs between the sexes. **(J-L)** Fold-change (week 4 vs week 0) in body mass (J), fat mass (K) and fat-free mass (L) for males vs females separated into younger (<45 years) and older (>45 years) groups. Data are presented as violin plots overlaid with individual data points. Significant effects of age, sex, and age*sex interaction were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall P values for each variable, and their interactions, are shown beneath each graph. Significant differences between comparable groups are indicated by * (P<0.05) or *** (P<0.001). Source data are provided as a Source Data file. See also Table 3 and Supplementary Figures 10 and 11.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1, Related to Figure 1 – Methods used and additional outcomes relating to the comprehensive review of the CR literature.
The NIH PubMed database was searched using MeSH terms to identify research articles that studied CR *in vivo* in mice or humans, published between 2003 and 2021. **(A)** Method of database search presented as a flow diagram, including search terms, reasons for exclusion, and number of results in each category. **(B)** Percentage of mouse or human publications for each year, with publications classified into each group as described for Figure 1. Source data for (B) are provided as a Source Data file.

Supplementary Figure 2, Related to Figure 2 – The effect of CR on percent fat and lean mass differs between male and female mice.
Male and female mice were fed AL or CR diets and body composition was assessed weekly, as described for Figure 2. **(A,B)** Fat mass (A) and lean mass (B) were determined by TD-NMR and calculated as % body mass. Data are shown as mean ±SEM of the following numbers of mice per group: *male AL*, n=42; *female AL,* n=43; *male CR,* n=44; *female CR,* n=52. Significant effects of diet, sex and/or duration were determined by 3-way ANOVA (A) or a mixed-effects model (B); *P* values are shown beneath each graph. Source data are provided as a Source Data file.

Supplementary Figure 3, Related to Figure 2 – CR and sex effects on the masses of adipose and non-adipose tissues.
Male and female mice were fed AL or CR diets, as described for Figure 2. After six weeks of CR (15 weeks of age) tissues were sampled and weighed. **(A-C)** Violin plots, overlaid with individual data points, showing the absolute mass (A,B) or % body mass (C) of each tissue; Gastroc = gastrocnemius muscle. For (A), the numbers of mice per group and tissue type are as reported for Figure 2G. For (B) and (C), the following numbers of mice were used for each group and tissue type: *male AL,* n=29 (liver, spleen), 23 (pancreas, kidneys, heart, thymus), 22 (adrenals) or 16 (gastroc.); *female AL,* n=33 (liver, spleen), 27 (pancreas, kidneys, heart, thymus, adrenals) or 16 (gastroc.); *male CR,* n=28 (liver, spleen), 22 (pancreas, kidneys, heart, thymus, adrenals) or 14 (gastroc.); *female CR,* n=34 (liver, spleen), 28 (pancreas, kidneys, heart, thymus, adrenals) or 15 (gastroc.). For each tissue, significant effects of diet and/or sex were determined by 2-way ANOVA with Tukey's multiple comparisons tests and are reported as described for Figure 2. Source data are provided as a Source Data file.

Supplementary Figure 4, Related to Figure 2 – CR decreases mWAT adipocyte size in male but not in female mice.
Male and female mice were fed AL or CR diets as described for Figure 2. After six weeks of CR (15 weeks of age) mWAT was sampled and processed for histological analysis. **(A,B)** Micrographs of H&E-stained sections of mWAT (A) were used for histomorphometric analysis of adipocyte area (B); in (A), scale bar = 100 µm. Data in are shown as representative micrographs (A) or violin plots (B) from 5 (*male AL, male CR)* or 6 (*female AL, female CR)* mice per group. For (B), significant effects of diet

and/or sex on adipocyte area (Ad.Ar) were determined by 3-way ANOVA, with *P* values shown beneath the graph. Significant differences in median Ad.Ar between AL and CR mice were further determined 2-way ANOVA with Šídák's multiple comparisons test; * = *P*<0.05. Source data are provided as a Source Data file.

Supplementary Figure 5, Related to Figure 2 – Sex differences in the effects of CR on body mass and composition persist when CR mice are fed in the evening.
Male and female C57BL6/NCrl mice were single housed and fed AL or CR diet (70% of daily AL intake) from 9-13 weeks of age, with CR mice receiving their daily diet ration between 18:00-19:00. **(A-F)** Each week mice were weighed (A,B) and body composition was determined by TD-NMR (C-F). Body mass, fat mass and lean mass are shown as absolute masses (A,C,E) or fold-change relative to baseline (B,D,F). **(G,H)** Masses of the indicated tissues were recorded at necropsy and are shown as % body mass. Data in (A-H) represent 5 (*male AL, female AL*) or 6 (*male CR, female CR*) mice per group and are shown as mean ±SEM (A-F) or as truncated volcano plots overlaid with individual data points (G,H). For (A-F), significant effects of diet, sex or time, and interactions thereof, were determined by 3-way ANOVA. For (G-H), significant effects of sex, diet, and sex-diet interaction were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown beneath each graph. For (G-H), statistically significant differences between comparable groups are indicated by ** (*P*<0.01). Source data are provided as a Source Data file.

Supplementary Figure 6, Related to Figure 3 – Sex differences in the effects of CR on glucose homeostasis persist when CR mice are fed in the evening.
Male and female C57BL6/NCrl mice were fed AL or CR diet from 9-15 weeks of age, as described for Supplementary Figure 5. **(A,B)** At 13 weeks of age mice underwent OGTTs. Blood glucose was recorded at each timepoint (A) and the tAUC and iAUC was determined (B). Data represent 5 (*male AL, female AL*) or 6 (*male CR, female CR*) mice per group and are shown as mean ±SEM (A) or as truncated volcano plots overlaid with individual data points (B). In (A), significant effects of diet, sex or time, and interactions thereof, were determined by 3-way ANOVA. In (B), significant effects of sex, diet, and sex-diet interaction were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown beneath each graph. For (B), statistically significant differences between comparable groups are indicated by ** (*P*<0.01). Source data are provided as a Source Data file.

Supplementary Figure 7, Related to Figure 5 – The effects of CR on glucose uptake in peripheral tissues.
Male and female C57BL6/NCrl mice were fed AL or CR diet and glucose uptake was assessed by ^{18}F-FDG PET/CT, as described for Figure 5. **(A,B)** ^{18}F-FDG uptake in the indicated tissues was determined by PMOD analysis of PET/CT scans (A) or gamma counting of dissected whole tissues (B). Data in (A) and (B) are presented as truncated violin plots, overlaid with individual data points, from 7 (*male AL, female AL, male CR*) or 6 (*female CR*) mice per group; in (B), data for spleen are from 6 mice per group

only. Statistical significance was determined as described for Figure 5. Source data are provided as a Source Data file.

Supplementary Figure 8, Related to Figure 6 – CR exerts sexually dimorphic effects on hepatic sphingolipid content.

Male and female mice were fed AL or CR diets as described for Figure 2. **(A-C)** At necropsy, livers were sampled for analysis of ceramide and dihydroceramide content by LC-MS. Data show the concentration of each species of ceramide (A), dihydroceramide (B), or dihydroceramide:ceramide ratio (C) and are presented as truncated violin plots showing all points. The following numbers of mice were used per group and species: *male AL*, n=6 (all except: DHC 20:1 & 26:0, Cer:DHC 20:1 & 26:0), 1 (DHC & Cer:DHC 20:1), or 0 (DHC & Cer:DHC 26:0); *female AL,* n=6; *male CR,* n=6 (all except DHC & Cer:DHC 26:0), or 1 (DHC & Cer:DHC 26:0); *female CR,* n=6. For each species, significant effects of sex, diet, and sex-diet interaction were assessed using 2-way ANOVA; overall *P* values for each variable, and their interactions, are shown beneath each graph. Significant differences between comparable groups were assess using Tukey's multiple comparison test and are indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). Source data are provided as a Source Data file.

Supplementary Figure 9, Related to Figure 7 – CR and sex effects on the masses of adipose and non-adipose tissues in aged mice.

Male and female mice were fed AL or CR diets from 78-84 weeks of age, as described for Figure 7. After six weeks of CR (84 weeks of age) tissues were sampled and weighed. **(A-C)** Violin plots, overlaid with individual data points, showing the absolute mass (A,B) or % body mass (C) of each tissue; Gastroc = gastrocnemius muscle. The numbers of mice per group and tissue type are as reported for Figure 7G. For each tissue, significant effects of diet and/or sex were determined by 2-way ANOVA with Tukey's multiple comparisons test and are reported as described for Figure 7. Source data are provided as a Source Data file.

Supplementary Figure 10, Related to Figures 2, 7 and 9 – CR-induced fat loss in mice is age- and sex-dependent.

Male and female mice were fed AL or CR diets from 9-15 or 78-84 weeks of age and fat mass was determined each week, as described for Figures 2 and 7. For each mouse (CR and AL diets) the ratio of absolute fat masses (g) was calculated for week 4 vs week 0 (i.e. 13 vs 9 weeks of age for young mice; 82 vs 78 weeks for aged mice); week 4 was selected for consistency with the 4-week duration of our human CR study (Fig. 9). For the CR mice, the fold-change in fat mass, relative to AL mice, was then determined as follows: i) the mean week 4:week 0 ratio was calculated for AL mice in each age group; ii) within each sex and age group, the week 4:week 0 ratio for each CR mouse was calculated relative to the mean week 4:week 0 ratio for the corresponding AL group. The rationale for showing these "fold changes of fold changes" is that the AL mice gain fat mass over the course of the study and therefore we must determine how much the fat mass of each CR mouse has changed compared to where we would expect it to be if it had continued on AL diet. Data in are shown as violin plots of the following numbers of mice per group: *young female,* n=31; *young male,* n=26; *old female,* n=11; *old male,* n=9. Significant effects of diet and/or sex were

determined by 2-way ANOVA with Tukey's multiple comparisons test and are reported as described for Figure S3. Source data are provided as a Source Data file.

Supplementary Figure 11, Related to Figure 9 – Body fat percentage in human CR participants.

Twenty male and twenty-two female volunteers participated in a weight loss study involving a 4-week dietary intervention, as described for Figure 9. Total % fat mass at week 0 is shown for males vs females separated into younger (<45 years) and older (>45 years) groups. Data are presented as violin plots overlaid with individual data points. Significant effects of age, sex, and age*sex interaction were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown beneath the graph. Significant differences between comparable groups are indicated by ** ($P<0.01$) or *** ($P<0.001$). See also Table 3. Source data are provided as a Source Data file.

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Figure 1

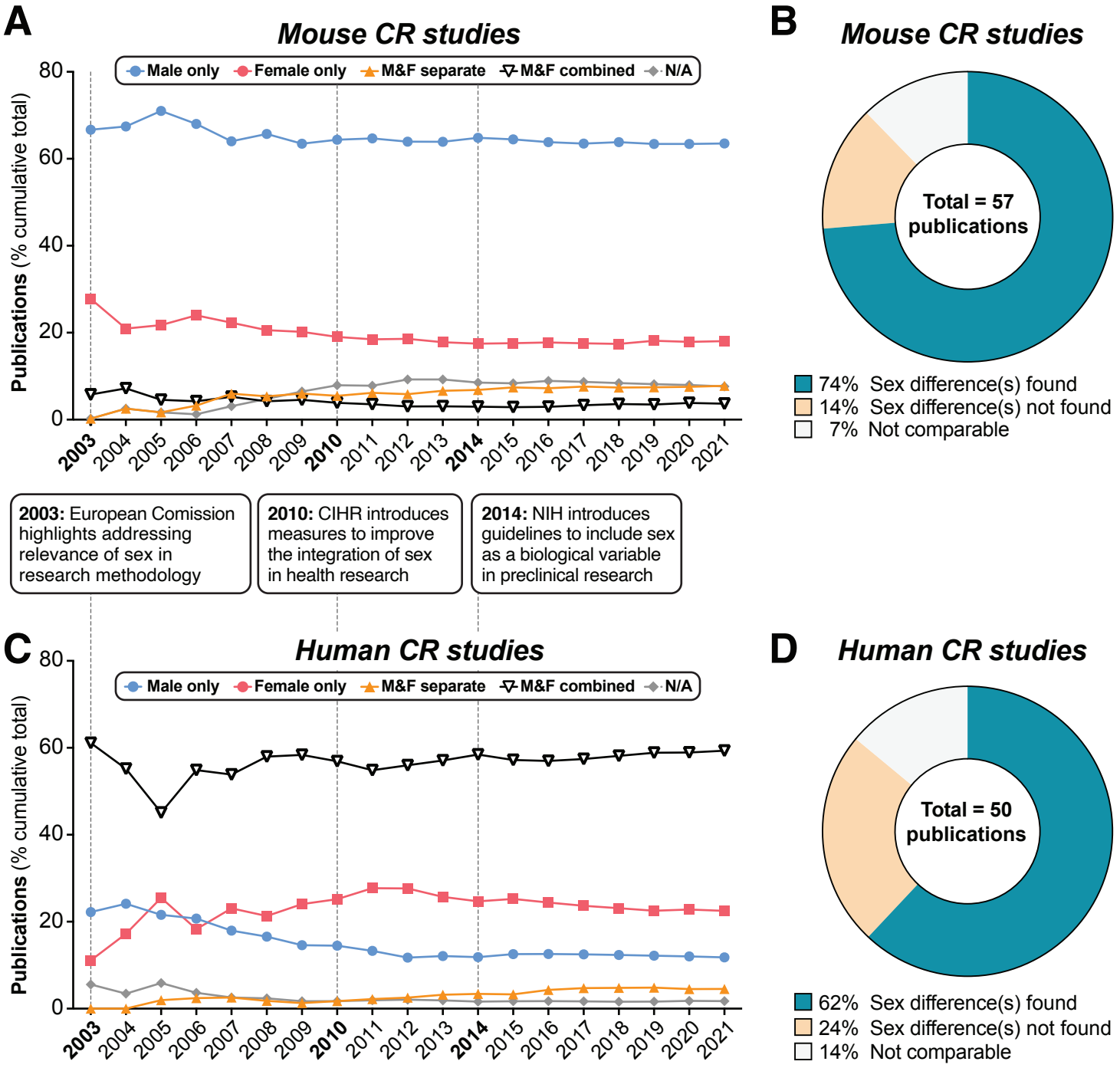

A
Mouse CR studies
Male only
Female only
M&F separate
M&F combined
N/A
Publications (% cumulative total)
80
60
40
20
0
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
2003: European Comission highlights addressing relevance of sex in research methodology
2010: CIHR introduces measures to improve the integration of sex in health research
2014: NIH introduces guidelines to include sex as a biological variable in preclinical research
B
Mouse CR studies
Total = 57 publications
74% Sex difference(s) found
14% Sex difference(s) not found
7% Not comparable
C
Human CR studies
Male only
Female only
M&F separate
M&F combined
N/A
Publications (% cumulative total)
80
60
40
20
0
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
2021
D
Human CR studies
Total = 50 publications
62% Sex difference(s) found
24% Sex difference(s) not found
14% Not comparable

Figure 1 – Summary of sex differences in mouse and human CR research.
PubMed was searched using keywords to identify research articles that studied caloric restriction *in vivo* in mice (**A,B**) or humans (**C,D**), published between 2003 and 2021. Search results were then classified into the following groups depending on the sexes included in each study: 'Male only' = male subjects used exclusively; 'Female only' = female subjects used exclusively; 'Male & Female separate' = male and female subjects used with data reported respective to each sex, allowing sex differences to be addressed; 'Male & female combined' = male and female subjects used with data from both sexes combined either in part or in full; 'N/A' = no sex data available. **(A,C)** Cumulative publications for studies within each group. The boxes between (A) and (C) highlight the dates of funders' policies highlighting the importance of sex as a biological variable. (**B,D**) Pie charts of those studies in the 'Male & Female separate' group that considered sex a biological variable, and the proportion of these that did or did not detect sex differences. Source data are provided as a Source Data file. See also Supplementary Figure 1.

Figure 2

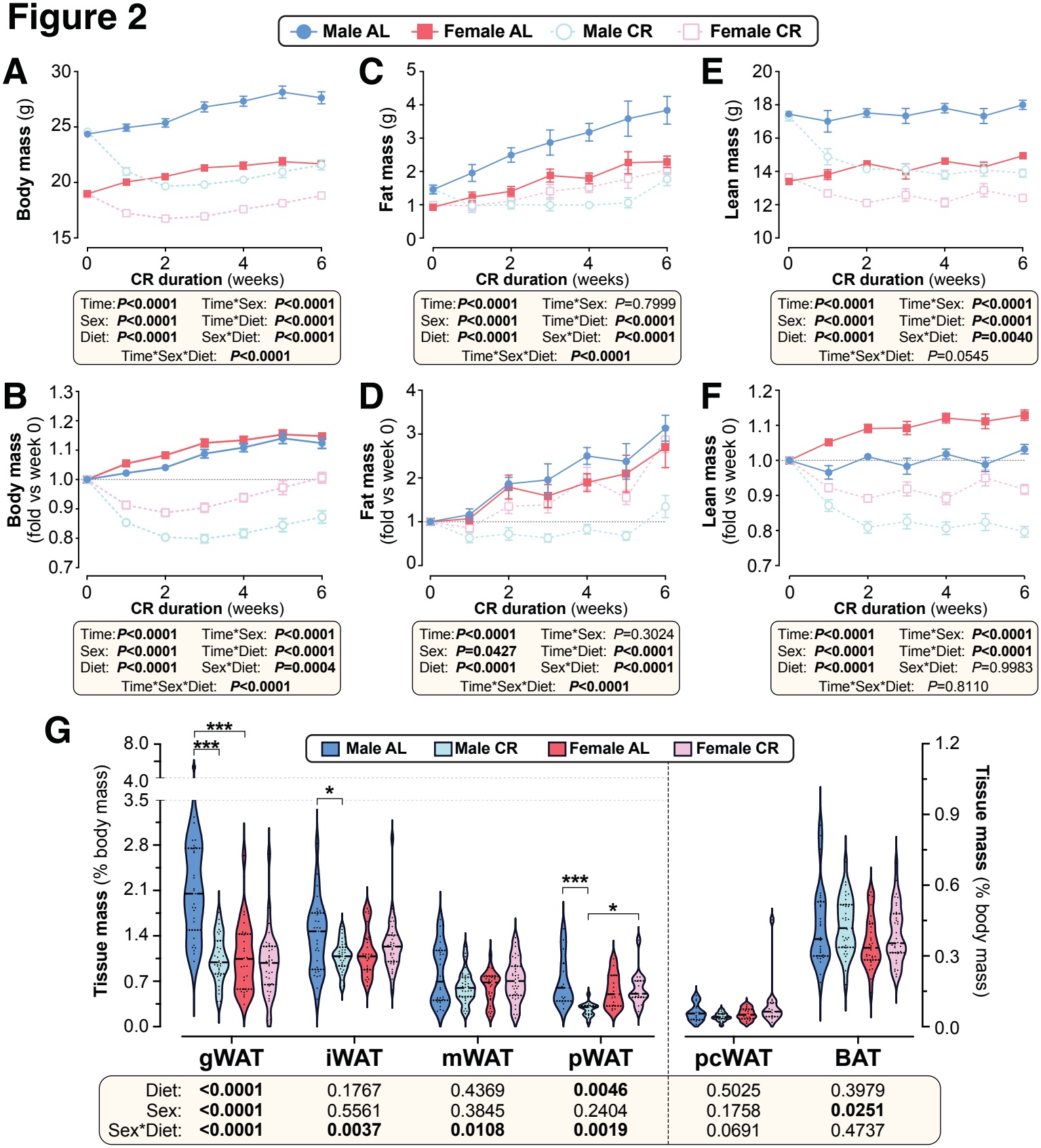

Male AL
Female AL
Male CR
Female CR
A
Body mass (g)
CR duration (weeks)
Time: P<0.0001 Time*Sex: P<0.0001
Sex: P<0.0001 Time*Diet: P<0.0001
Diet: P<0.0001 Sex*Diet: P<0.0001
Time*Sex*Diet: P<0.0001
C
Fat mass (g)
CR duration (weeks)
Time: P<0.0001 Time*Sex: P=0.7999
Sex: P<0.0001 Time*Diet: P<0.0001
Diet: P<0.0001 Sex*Diet: P<0.0001
Time*Sex*Diet: P<0.0001
E
Lean mass (g)
CR duration (weeks)
Time: P<0.0001 Time*Sex: P<0.0001
Sex: P<0.0001 Time*Diet: P<0.0001
Diet: P<0.0001 Sex*Diet: P=0.0040
Time*Sex*Diet: P=0.0545
B
Body mass (fold vs week 0)
CR duration (weeks)
Time: P<0.0001 Time*Sex: P<0.0001
Sex: P<0.0001 Time*Diet: P<0.0001
Diet: P<0.0001 Sex*Diet: P=0.0004
Time*Sex*Diet: P<0.0001
D
Fat mass (fold vs week 0)
CR duration (weeks)
Time: P<0.0001 Time*Sex: P=0.3024
Sex: P=0.0427 Time*Diet: P<0.0001
Diet: P<0.0001 Sex*Diet: P<0.0001
Time*Sex*Diet: P<0.0001
F
Lean mass (fold vs week 0)
CR duration (weeks)
Time: P<0.0001 Time*Sex: P<0.0001
Sex: P<0.0001 Time*Diet: P<0.0001
Diet: P<0.0001 Sex*Diet: P=0.9983
Time*Sex*Diet: P=0.8110
G
Male AL
Male CR
Female AL
Female CR
Tissue mass (% body mass)
gWAT
iWAT
mWAT
pWAT
pcWAT
BAT
Diet: <0.0001 0.1767 0.4369 0.0046 0.5025 0.3979
Sex: <0.0001 0.5561 0.3845 0.2404 0.1758 0.0251
Sex*Diet: <0.0001 0.0037 0.0108 0.0019 0.0691 0.4737
H
Male AL
Female AL
Male CR
Female CR
100 µm
I
Adipocyte area (µm2)
Male AL
Male CR
Female AL
Female CR
Ad.Ar*Sex*Diet: P <0.0001
Ad.Ar: P <0.0001 Ad.Ar*Sex: P =0.19
Sex: P >0.99 Ad.Ar*Diet: P <0.0001
Diet: P >0.99 Sex*Diet: P >0.99

Figure 2 – Female mice resist CR-induced weight loss, fat loss and adipocyte hypotrophy.
Male and female mice on a C57BL/6NCrl or C57BL/6J background were fed *ad libitum* (AL) or a 30% CR diet from 9-15 weeks of age (0-6 weeks of CR). **(A-F)** Each week mice were weighed (A,B) and body composition was determined by TD-NMR (C-F). Body mass, fat mass and lean mass are shown as absolute masses (A,C,E) or fold-change relative to baseline (B,D,F). **(G)** Masses of gWAT (gonadal WAT), iWAT (inguinal WAT), mWAT (mesenteric WAT), pWAT (perirenal WAT), pcWAT (pericardial WAT) and BAT (brown adipose tissue) were recorded at necropsy and are shown as % body mass. **(H,I)** Micrographs of H&E-stained sections of gWAT (H) were used for histomorphometric analysis of adipocyte area (I); in (H), scale bar = 100 µm. Data in (A-F) are shown as mean ±SEM of the following numbers of mice per group: *male AL*, n=42; *female AL,* n=43; *male CR,* n=44; *female CR,* n=52. Data in (G) are shown as violin plots of the following numbers of mice per group and tissue type: *male AL,* n=29 (iWAT, gWAT, mWAT, BAT), 15 (pWAT) or 14 (pcWAT); *female AL,* n=28 (iWAT, gWAT, mWAT, BAT), 14 (pWAT) or 13 (pcWAT); *male CR,* n=33 (iWAT, gWAT, mWAT, BAT), 16 (pWAT) or 15 (pcWAT); *female CR,* n=34 (iWAT, gWAT, mWAT, BAT), 15 (pWAT) or 14 (pcWAT). Data in (H-I) are shown as representative micrographs or violin plots from 5 (*male AL)* or 6 (*female AL, male CR, female CR)* mice per group. For (A-F), significant effects of diet, sex and/or time, and interactions thereof, were determined by 3-way ANOVA or a mixed-effects model. For (G), significant effects of diet and/or sex within each tissue were determined by 2-way ANOVA with Tukey's multiple comparisons tests. For (I), significant effects of diet and/or sex on adipocyte area (Ad.Ar) were determined using a mixed-effects model, while significant differences in median Ad.Ar between AL and CR mice were determined 2-way ANOVA with Šídák's multiple comparisons test. *P values* from ANOVA or mixed models are shown beneath the graphs, as indicated. For (G) and (I), significant differences between comparable groups are indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). Source data are provided as a Source Data file. See also Supplementary Figures 2-5.

Figure 3

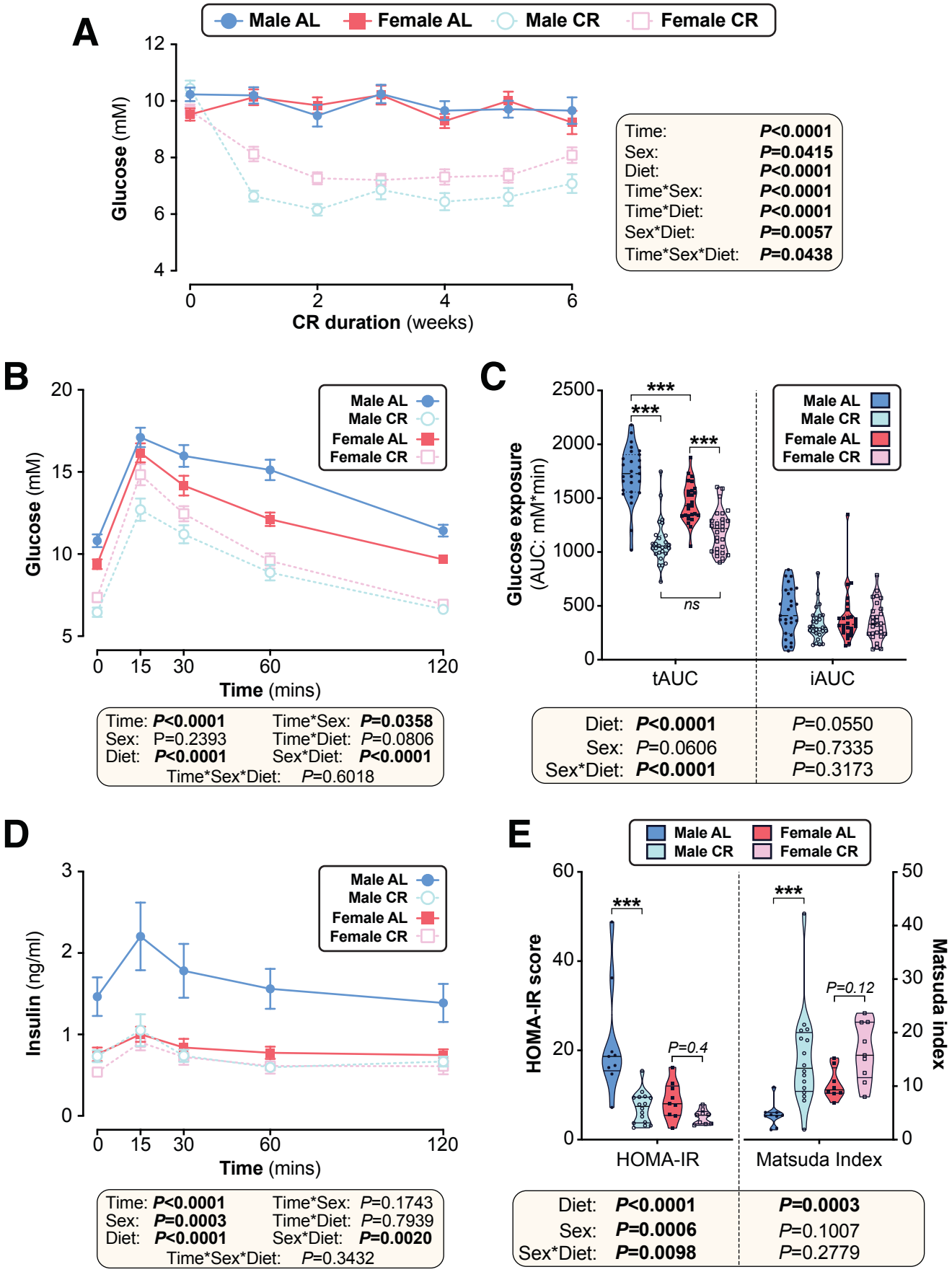
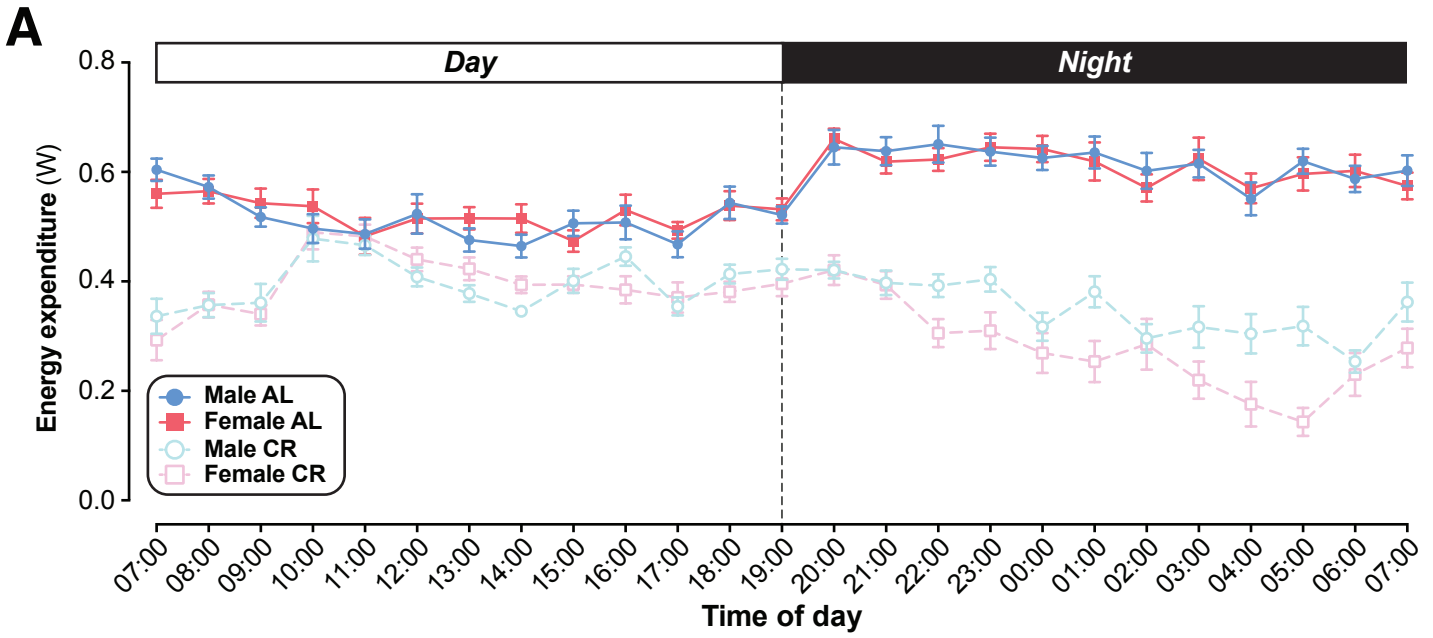

A
Male AL
Female AL
Male CR
Female CR
Glucose (mM)
CR duration (weeks)
Time: P<0.0001
Sex: P=0.0415
Diet: P<0.0001
Time*Sex: P<0.0001
Time*Diet: P<0.0001
Sex*Diet: P=0.0057
Time*Sex*Diet: P=0.0438
B
Glucose (mM)
Time (mins)
Time: P<0.0001
Sex: P=0.2393
Diet: P<0.0001
Time*Sex: P=0.0358
Time*Diet: P=0.0806
Sex*Diet: P<0.0001
Time*Sex*Diet: P=0.6018
C
Glucose exposure (AUC: mM*min)
***
ns
tAUC
iAUC
Diet: P<0.0001 P=0.0550
Sex: P=0.0606 P=0.7335
Sex*Diet: P<0.0001 P=0.3173
D
Insulin (ng/ml)
Time (mins)
Time: P<0.0001
Sex: P=0.0003
Diet: P<0.0001
Time*Sex: P=0.1743
Time*Diet: P=0.7939
Sex*Diet: P=0.0020
Time*Sex*Diet: P=0.3432
E
HOMA-IR score
Matsuda index
P=0.4
P=0.12
HOMA-IR
Matsuda Index
Diet: P<0.0001 P=0.0003
Sex: P=0.0006 P=0.1007
Sex*Diet: P=0.0098 P=0.2779

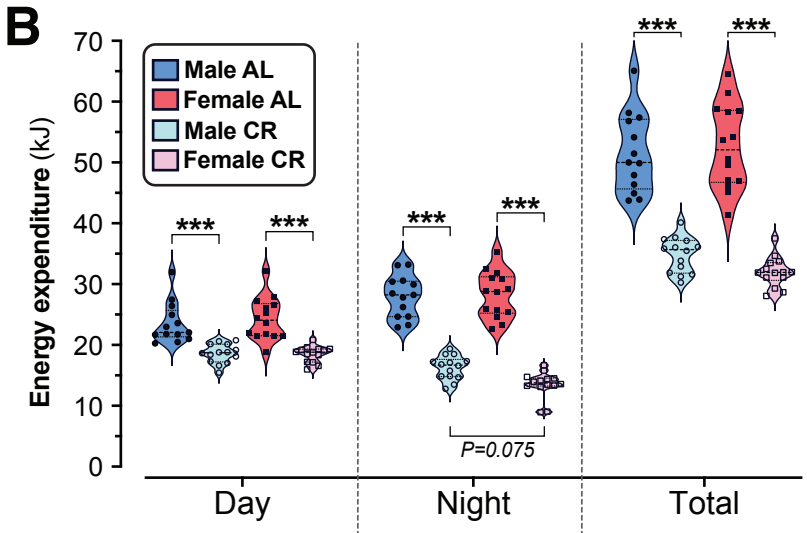
Figure 3 – The effects of CR on glucose homeostasis differ between young male and female mice.

Male and female C57BL6/NCrl mice were fed AL or CR diet from 9-15 weeks of age, as described for Figure 2. **(A)** Random-fed blood glucose was recorded each week. **(B-D)** At 13 weeks of age mice underwent an oral glucose tolerance test (OGTT). (B) Blood glucose readings during the OGTT. (C) Area under the curve (AUC) during the OGTT was determined relative to 0 mmol/L (total AUC: tAUC) and relative to baseline (incremental AUC: iAUC). (D) Glucose-stimulated insulin secretion in mice during OGTT was assessed using an insulin ELISA. **(E)** HOMA-IR and Matsuda indices of mice calculated from glucose and insulin concentrations during the OGTT. Data in (A), (B), and (D) are presented as mean ± SEM. Data in (C) and (E) are presented as violin plots overlaid with individual data points. For each group and timepoint, the following numbers of mice were used: **(A)**: *male AL,* n=42 (Wk 0), 36 (Wk2, Wk 2), 34 (Wk 3), 29 (Wk 4), 26 (Wk 5), or 22 (Wk 6); *female AL,* n=43 (Wk 0), 28 (Wk 1), 35 (Wk 2), 31 (Wk 3), 27 (Wk 4), 26 (Wk 5), or 21 (Wk 6); *male CR,* n=44 (Wk 0), 40 (Wk 1), 38 (Wk 2), 35 (Wk 3), 31 (Wk 4), 29 (Wk 5), or 26 (Wk 6); *female CR,* n= 51 (Wk 0), 44 (Wk 1, Wk 2), 41 (Wk 4), 35 (Wk 4), 33 (Wk 5), or 27 (Wk 6). **(B)**: *male AL,* n=27 (T_0, T_{15}, T_{30}, T_{60}, T_{120}); *female AL,* n=26 (T_{15}, T_{30}, T_{120}), or 25 (T_0, T_{60}); *male CR,* n=27 (T_0, T_{15}, T_{30}, T_{60}, T_{120}); *female CR,* n=28 (T_0, T_{15}, T_{30}, T_{60}, T_{120}). **(C)**: *male AL,* n=27; *female AL,* n=26; *male CR,* n=27; *female CR,* n=28. **(D)**: *male AL,* n=9 (T_0, T_{15}, T_{30}, T_{60}, T_{120}); *female AL,* n=11 (T_0, T_{15}), 10 (T_{30}, T_{60}), or 8 (T_{120}); *male CR,* n=17 (T_0), 16 (T_{30}), 15 (T_{60}), 13 (T_{15}), or 11 (T_{120}); *female CR,* n=11 (T_{15}, T_{30}, T_{60},), or 10 (T_0, T_{120}). **(E)**: *male AL,* n=9; *female AL,* n=9; *male CR,* n=16; *female CR,* n=9. In (A), (B), and (D), significant effects of diet, sex and/or time, and interactions thereof, were determined using a mixed-effects model. In (C) and (E), significant effects of sex, diet, and sex*diet interaction for tAUC, iAUC, HOMA-IR and Matsuda Index were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown with each graph. For (C) and (E), statistically significant differences between comparable groups are indicated by *** ($P<0.001$) or with *P* values shown. Source data are provided as a Source Data file. See also Supplementary Figures 6-7.

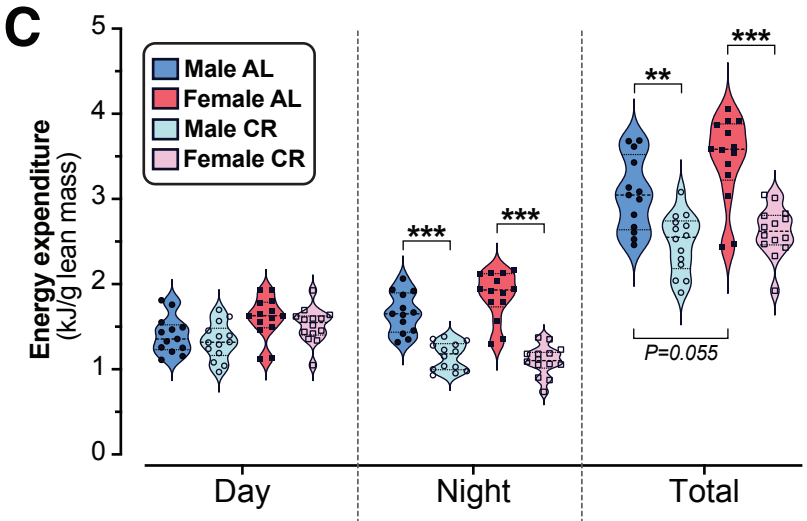
Figure 4



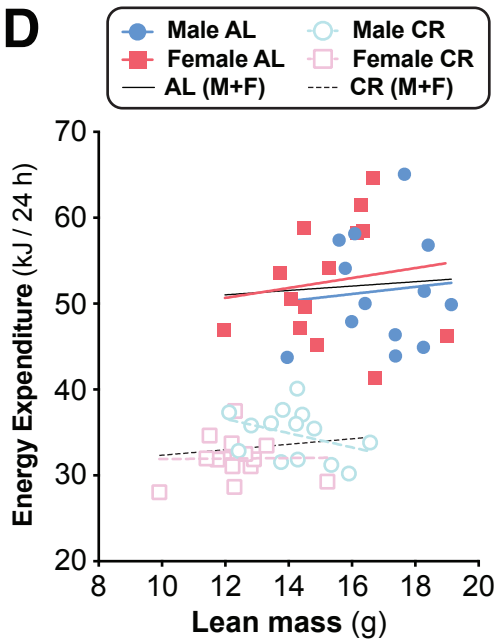
Time: ***P*<0.0001**	Sex: *P*=0.3705	Diet: ***P*<0.0001**	Time*Sex: ***P*<0.0001**	
Time*Diet: ***P*<0.0001**	Sex*Diet: *P*=0.1103	Time*Sex*Diet: *P*=0.1557		



	Day	Night	Total
Sex:	0.6289	0.1309	0.5283
Diet:	**<0.0001**	**<0.0001**	**<0.0001**
Sex*Diet:	0.5680	0.0574	0.1627



	Day	Night	Total
Sex:	**0.0018**	0.2155	**0.0161**
Diet:	0.1963	**<0.0001**	**<0.0001**
Sex*Diet:	0.9202	**0.0333**	0.2177

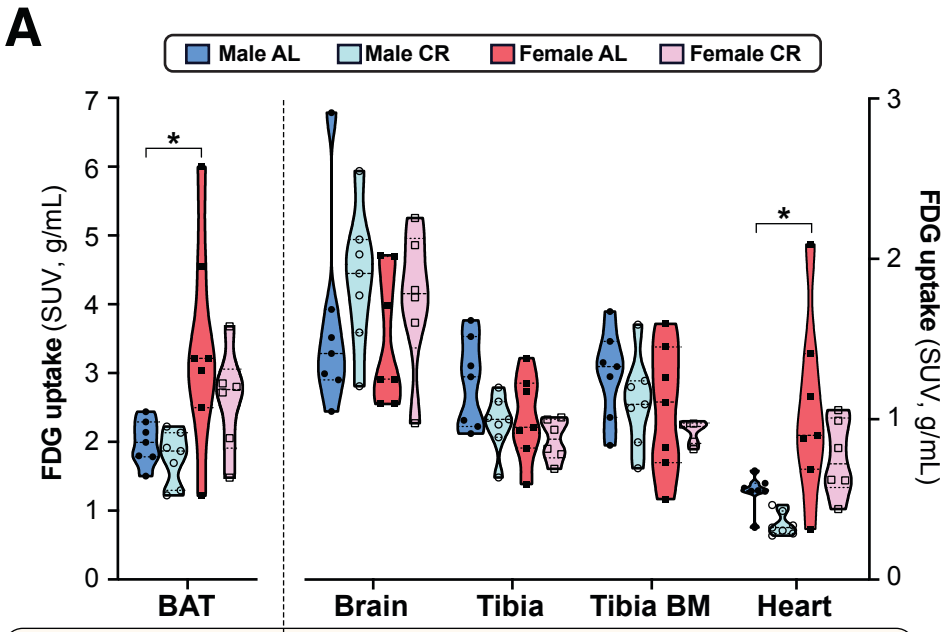


	P, Slopes	*P*, Intercepts
i. All groups:	0.790	**<0.0001**
ii. AL vs CR (M+F):	0.942	**<0.0001**
iii. M vs F (AL only):	0.928	0.527
iv. M vs F (CR only):	0.089	**0.024**

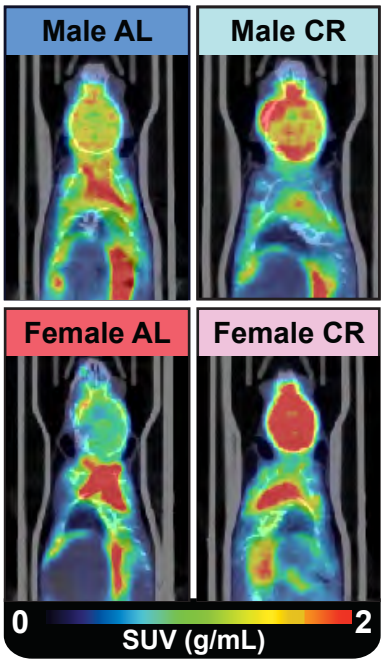
Figure 4 – CR decreases energy expenditure in male and female mice, with a trend for greater nighttime decreases in females.

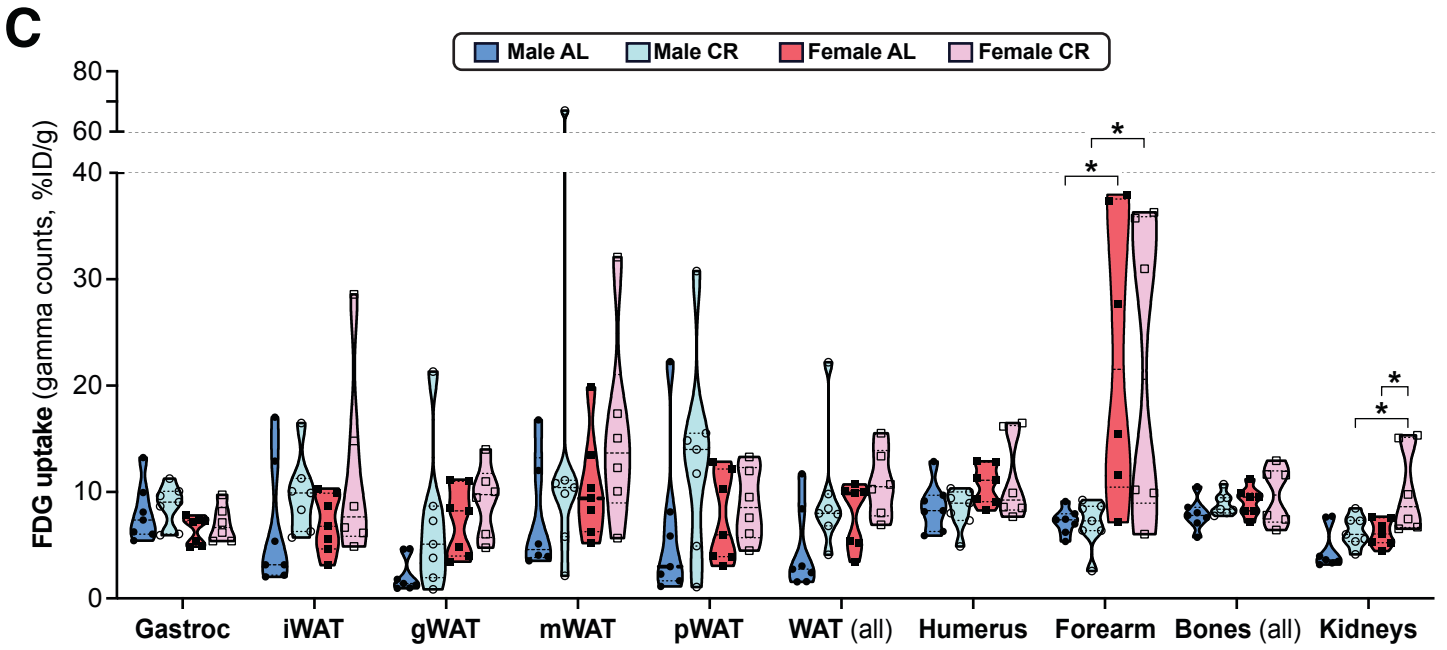
Male and female mice were fed AL or CR diets as described for Figure 2. At 12-13 or 14-15 weeks of age mice were housed individually in TSE PhenoMaster cages for 3 days for indirect calorimetry. **(A,B)** Absolute energy expenditure (watts, W) was recorded every 15 minutes throughout the 3 days. **(A)** Average energy expenditure per hour over the 24 h light (Day) and dark (Night) period, spanning days 2-3 of PhenoMaster housing. **(B)** Energy expenditure (kJ) during the Day, Night, or Day + Night (Total). **(C)** Energy expenditure normalised to lean body mass (kJ/g) during the Day, Night, or Day + Night (Total). **(D)** Linear regression of lean mass vs Total (Day + Night) energy expenditure (kJ/24 h). Data in (A-D) are from 13 (*male AL*) or 14 (*male CR, female AL, female CR)* mice per group. In (A), data are shown as mean ± SEM; significant effects of diet, sex and/or time, and interactions thereof, were determined by 3-way ANOVA, with *P* values shown beneath the graph. In (B-C), data are shown as violin plots overlaid with individual data points; within each time period (Day, Night, or Total), significant effects of sex, diet, and diet*sex interaction were assessed using 2-way ANOVA, with *P* values shown beneath each graph. Statistically significant differences between comparable groups were further assessed by Tukey's multiple comparisons tests and are indicated by ** ($P<0.01$) or *** ($P<0.001$). For linear regression in (D), ANCOVA was used to test if the relationship between lean mass and total energy expenditure differs significantly across all of the individual diet-sex groups (*i. All mice*); between AL and CR mice, irrespective of sex (*ii. AL vs CR (M+F)*); and between males and females fed AL diet (*iii*) or CR diet (*iv*) only. ANCOVA *P* values for differences in slope and intercept are reported beneath the graph.

Figure 5



	BAT	Brain	Tibia	Tibia BM	Heart
Diet:	0.1512	0.1585	**0.0342**	0.1733	0.0517
Sex:	**0.0035**	0.5617	0.0829	0.0928	**0.0010**
Sex*Diet:	0.4190	0.9282	0.5143	0.8955	0.6854

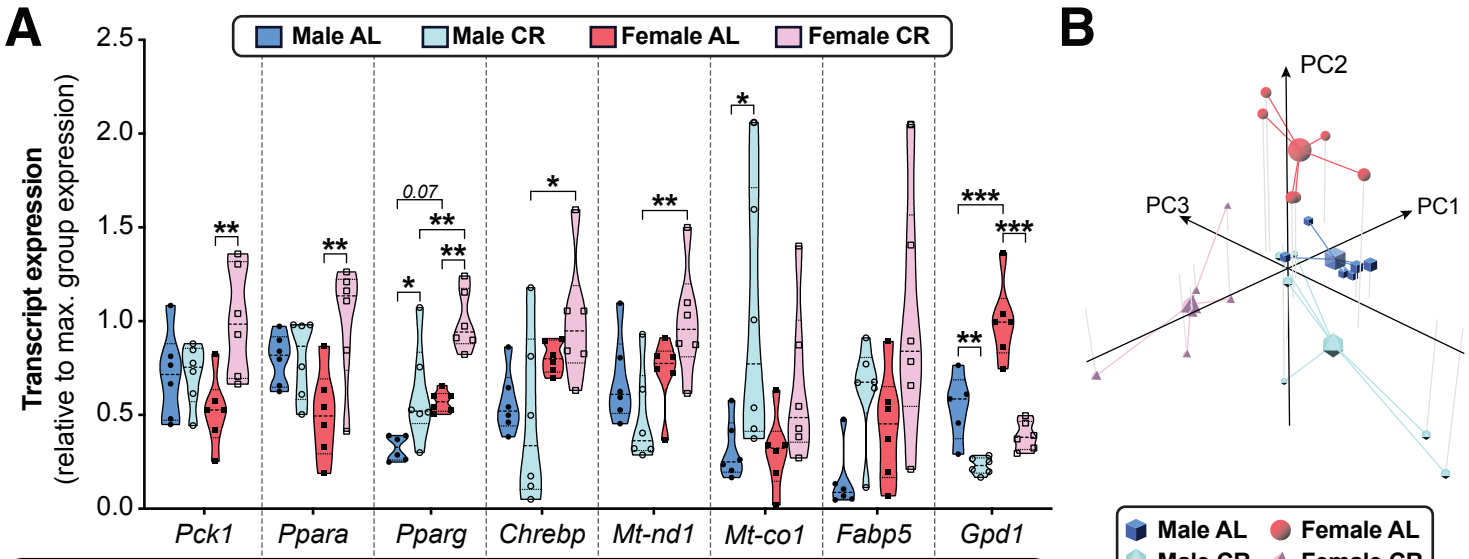




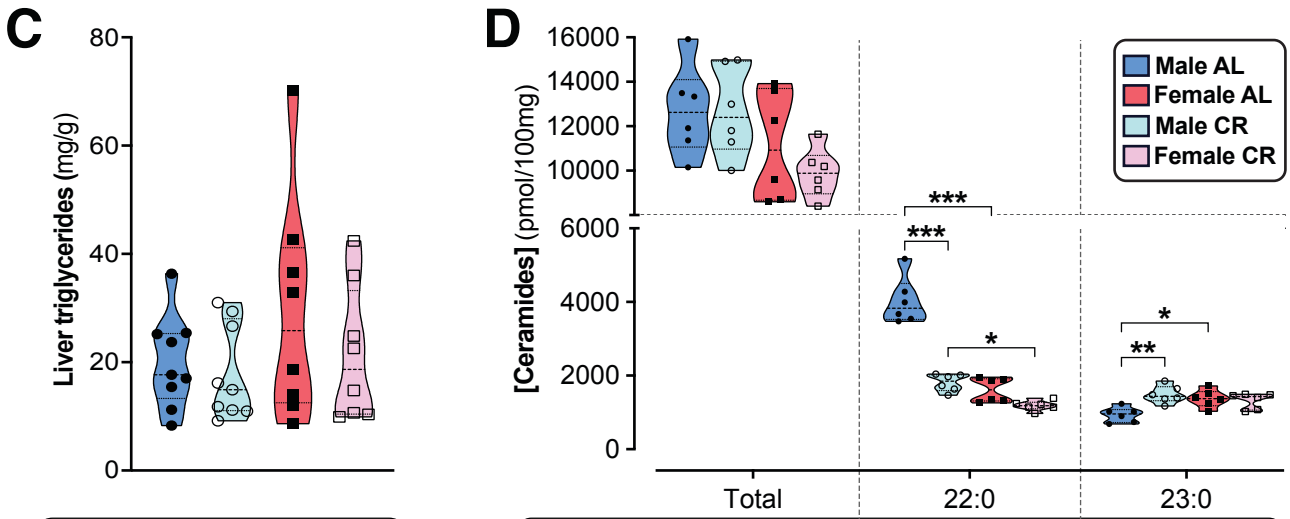
	Gastroc	iWAT	gWAT	mWAT	pWAT	WAT (all)	Humerus	Forearm	Bones (all)	Kidneys
Diet:	0.4443	0.0889	0.0553	0.1807	0.1220	**0.0200**	0.8700	0.8362	0.2682	**0.0058**
Sex:	**0.0447**	0.5962	**0.0373**	0.8837	0.5290	0.1747	**0.0232**	**0.0005**	0.1470	**0.0085**
Sex*Diet:	0.9959	0.7461	0.3961	0.6896	0.2926	0.5137	0.7253	0.8818	0.7469	0.2069

Figure 5 – The effects of CR on glucose uptake do not differ between male and female mice. Male and female C57BL6/NCrl mice were fed AL or CR diet from 9-15 weeks of age, as described for Figure 2. At 15 weeks of age, glucose uptake was assessed by PET/CT with ^{18}F-fluorodeoxyglucose (^{18}F-FDG). **(A-C)** ^{18}F-FDG uptake in the indicated tissues was determined as SUVs from PET/CT scans (A) or as % injected dose per gram of tissue (%ID/g) from gamma counting of dissected whole tissues (C). **(B)** PET/CT images confirm that ^{18}F-FDG uptake in interscapular BAT is greater in females than in males, regardless of diet. Data are shown as violin plots (A,C) or representative images (B) of 7 (*male AL, female AL, male CR*) or 6 (*female CR*) mice per group. For each tissue in (A) and (C), significant effects of sex, diet, and sex*diet interaction were assessed by 2-way ANOVA; overall *P* values for each variable, and their interactions, are shown beneath each graph. Significant differences between each group, as determined using Tukey's multiple comparisons test, are indicated by * (P <0.05). Source data are provided as a Source Data file. See also Supplementary Figure 7.

Figure 6

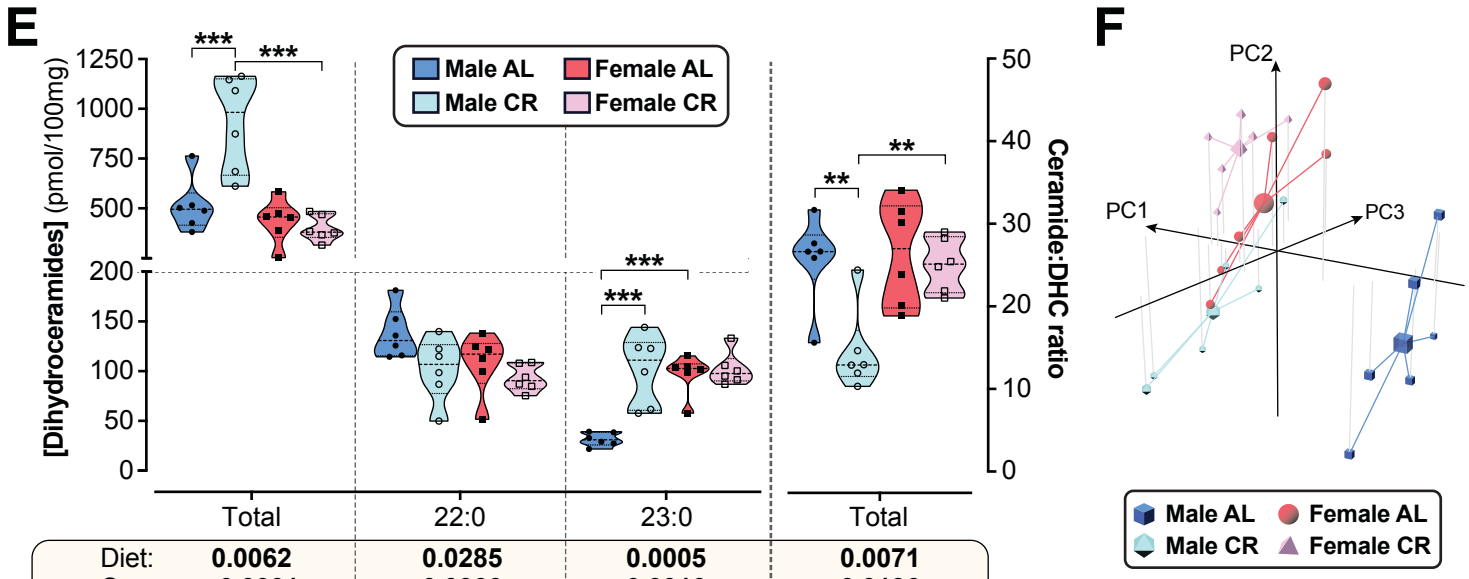


	Pck1	Ppara	Pparg	Chrebp	Mt-nd1	Mt-co1	Fabp5	Gpd1
Diet:	**0.0155**	**0.0171**	**<0.0001**	0.6800	0.7258	**0.0074**	**0.0035**	**<0.0001**
Sex:	0.6089	0.6168	**0.0001**	**0.0045**	**0.0112**	0.3174	**0.0496**	**<0.0001**
Sex*Diet:	**0.0180**	**0.0182**	0.2992	0.2470	**0.0292**	0.3348	0.8097	**0.0211**



Diet:	0.2738
Sex:	0.1647
Sex*Diet:	0.5224

	Total	22:0	23:0
Diet:	0.4408	**<0.0001**	**0.0122**
Sex:	**0.0130**	**<0.0001**	0.1435
Sex*Diet:	0.4620	**<0.0001**	**0.0033**

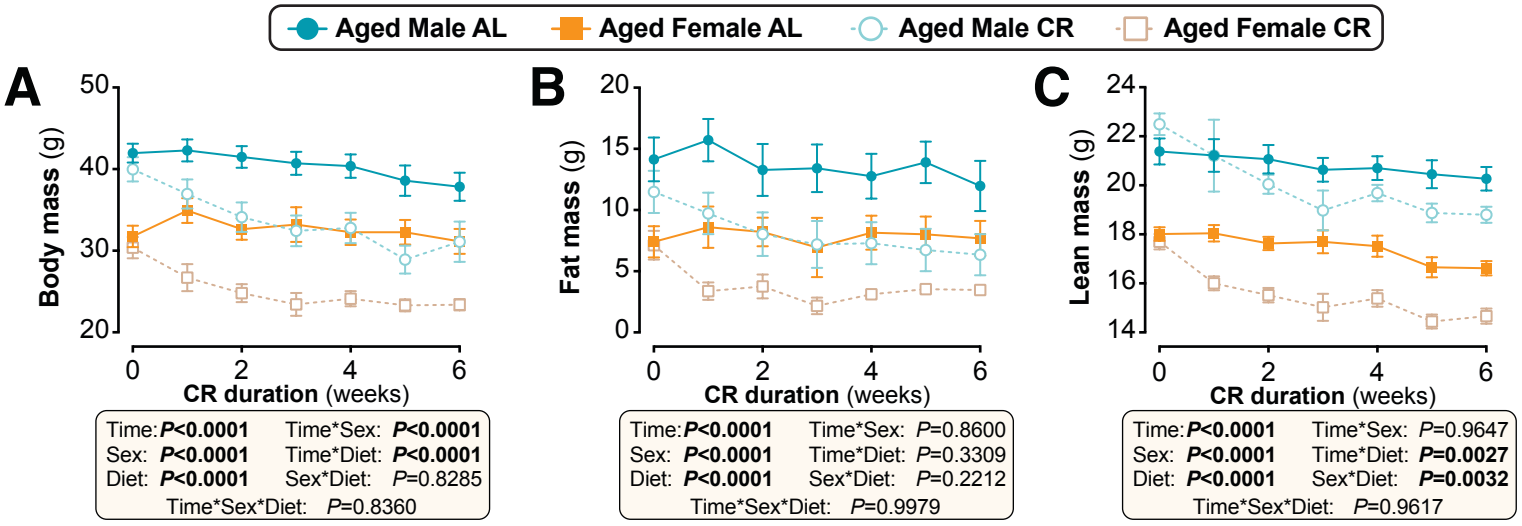


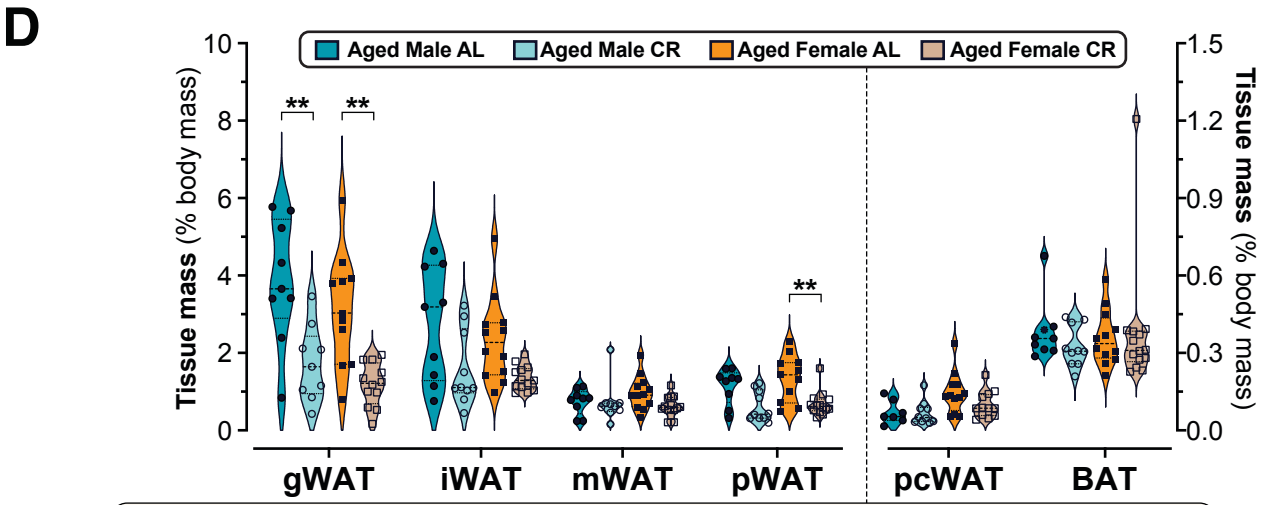
	Total	22:0	23:0	Total
Diet:	**0.0062**	**0.0285**	**0.0005**	**0.0071**
Sex:	**<0.0001**	0.0866	**0.0016**	**0.0136**
Sex*Diet:	**0.0016**	0.3517	**0.0019**	**0.0294**

Figure 6 – CR exerts sexually dimorphic effects on hepatic gene expression and sphingolipid content.

Male and female C57BL6/NCrl mice were fed AL or CR diet from 9-15 weeks of age, as described for Figure 2. Livers were sampled at necropsy (15 weeks) and used to assess mRNA transcripts, triglyceride content, and ceramide and dihydroceramide content. **(A)** Expression of the indicated transcripts was determined by qPCR. Transcript expression was normalised to the geometric mean of *Tbp, Rn18s* and *Ppia* expression and is presented relative to the highest group average. **(B)** Principle component analysis of transcript expression in (A). **(C)** Hepatic triglyceride content. **(D,E)** LC-MS analysis of total, 22:0 and 23:0 ceramides (D), dihydroceramides (E, left side) and the ceramide:DHC ratio (E, right side). **(F)** Principle component analysis of ceramide and dihydroceramide content based on data in (D-E) and Supplementary Figure 8. Data in (A), (C), (D), and (E) are presented as truncated violin plots overlaid with individual data points. Data represent the following numbers of mice per group. **(A-B)**: *male AL,* n=6; *female AL,* n=6; *male CR,* n=6; *female CR,* n=6. **(C)**: *male AL,* n=9; *female AL,* n=8; *male CR,* n=9; *female CR,* n=8. **(D-F):** 6 mice per group. For (A), (C), (D) and (E), significant effects of sex, diet, and sex*diet interaction were assessed using 2-way ANOVA; overall *P* values for each variable, and their interactions, are shown beneath each graph. Significant differences between comparable groups were assess using Tukey's multiple comparison test and are indicated by * ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). Source data are provided as a Source Data file. See also Supplementary Figure 8.

Figure 7





	gWAT	iWAT	mWAT	pWAT	pcWAT	BAT
Diet:	**<0.0001**	**0.0010**	0.1239	**<0.0001**	0.2085	0.6892
Sex:	0.0999	0.2925	0.6107	0.3291	**0.0081**	0.7631
Sex*Diet:	0.8188	0.7561	0.1590	0.5891	0.2591	0.4565

Figure 7 – Sex differences in CR-induced weight loss and fat loss are absent when CR is initiated in aged mice.

Male and female C57BL6/NCrl mice were single housed and fed AL or a CR diet (70% of daily AL intake) from 78-84 weeks of age. **(A-F)** Each week mice were weighed (A,B) and body composition was determined by TD-NMR (C-F). Body mass, fat mass and lean mass are shown as absolute masses (A,C,E) or fold-change relative to baseline (B,D,F). **(G)** Masses of gWAT, iWAT, mWAT, pWAT, pcWAT and BAT were recorded at necropsy and are shown as % body mass. Data in (A-F) are shown as mean ±SEM. Data in (G) are shown as violin plots overlaid with individual data points. For each group and timepoint, the following numbers of mice were used: **(A,B)**: *male AL*, n=9 (Wk 0, Wk 2, Wk3, Wk 4), 8 (Wk 1), or 6 (Wk 5, Wk 6); *female AL,* n=13 (Wk 0, Wk 2), 12 (Wk 4, Wk 5), 11 (Wk 6), 9 (Wk 1, or 8 (Wk 3); *male CR,* n=10 (Wk 0, Wk 2-4), 9 (Wk 1), 7 (Wk 5), or 6 (Wk 6); *female CR,* n=14 (Wk 0, Wk 4, Wk 5), 13 (Wk 2, Wk 6), 10 (Wk 3), or 9 (Wk 1). **(C-F)**: *male AL,* n=9 (Wk 0, Wk 2), 8 (Wk 3, Wk 4, Wk 6), or 7 (Wk 1, Wk 5); *female AL,* n=13 (Wk 0, Wk 2), 9 (Wk 4, Wk 6), 8 (Wk 5), 6 (Wk 1), or 5 (Wk 3); *male CR,* n=10 (Wk 0, Wk 2), 9 (Wk3-6), or 8 (Wk 1); *female CR,* n=14 (Wk 1, Wk 2), 11 (Wk 4-6), 7 (Wk 3), or 6 (Wk 1). **(G)**: *male AL,* n=9; *female AL,* n=12 (iWAT, mWAT) or 11 (gWAT, pWAT); *male CR,* n=10 (iWAT, pWAT), or 9 (gWAT, mWAT); *female CR,* n=14 (mWAT, pWAT), 13 (iWAT), or 12 (gWAT). For (A-F), significant effects of diet, sex and/or time, and interactions thereof, were determined by 3-way ANOVA or a mixed-effects model. For (G), significant effects of sex, diet, and sex*diet interaction were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown beneath each graph. For (G), significant differences between comparable groups are indicated by ** ($P<0.01$) or *** ($P<0.001$). Source data are provided as a Source Data file. See also Supplementary Figure 9.

Figure 8

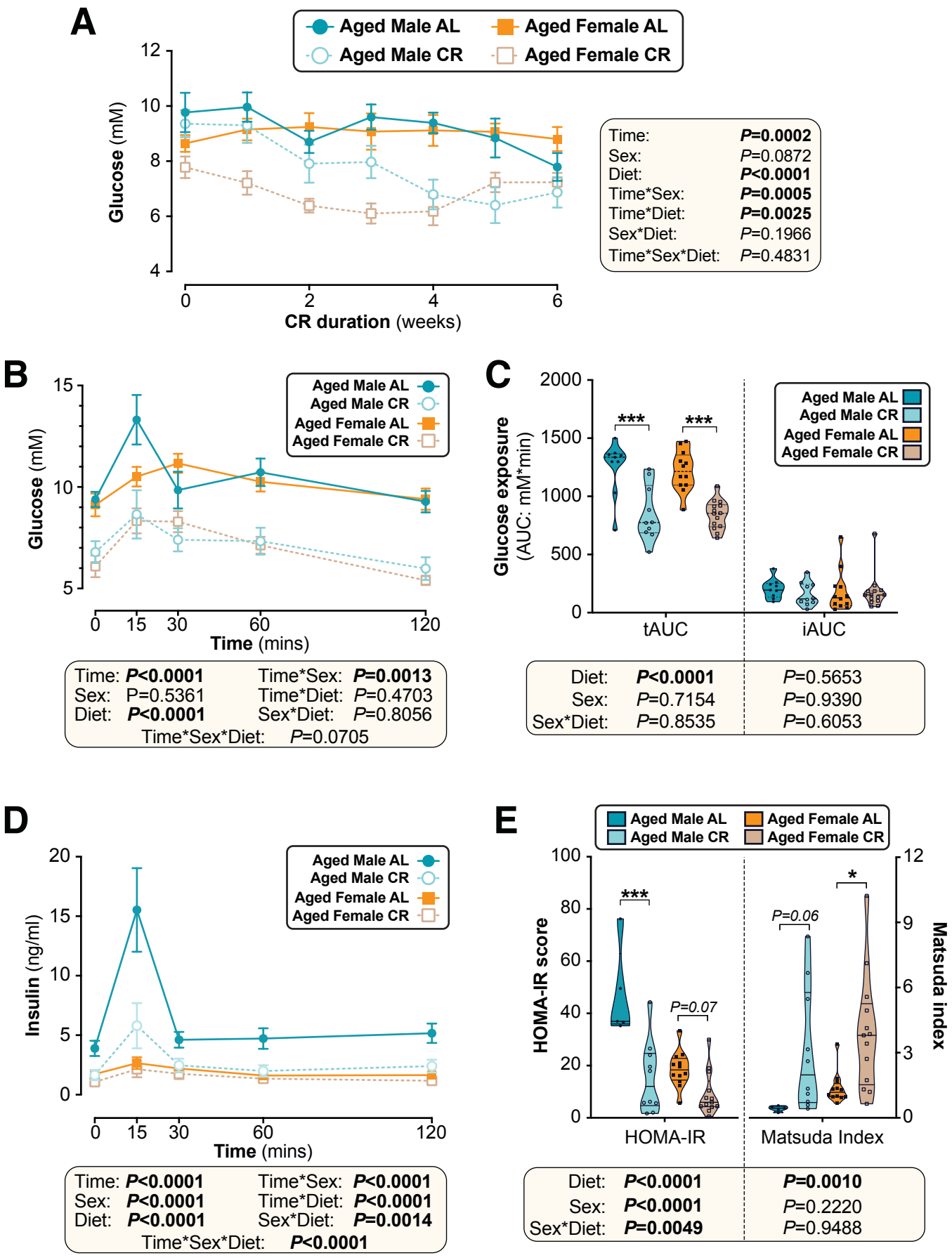

A
Aged Male AL
Aged Female AL
Aged Male CR
Aged Female CR
Glucose (mM)
CR duration (weeks)
Time: P=0.0002
Sex: P=0.0872
Diet: P<0.0001
Time*Sex: P=0.0005
Time*Diet: P=0.0025
Sex*Diet: P=0.1966
Time*Sex*Diet: P=0.4831
B
Glucose (mM)
Time (mins)
Time: P<0.0001
Sex: P=0.5361
Diet: P<0.0001
Time*Sex: P=0.0013
Time*Diet: P=0.4703
Sex*Diet: P=0.8056
Time*Sex*Diet: P=0.0705
C
Glucose exposure (AUC: mM*min)
tAUC
iAUC
Diet: P<0.0001 P=0.5653
Sex: P=0.7154 P=0.9390
Sex*Diet: P=0.8535 P=0.6053
D
Insulin (ng/ml)
Time (mins)
Time: P<0.0001
Sex: P<0.0001
Diet: P<0.0001
Time*Sex: P<0.0001
Time*Diet: P<0.0001
Sex*Diet: P=0.0014
Time*Sex*Diet: P<0.0001
E
HOMA-IR score
Matsuda index
HOMA-IR
Matsuda Index
P=0.07
P=0.06
Diet: P<0.0001 P=0.0010
Sex: P<0.0001 P=0.2220
Sex*Diet: P=0.0049 P=0.9488

Figure 8 – Sex differences in CR's effects on glucose homeostasis are largely absent when CR is initiated in aged mice.

Male and female C57BL6/NCrl mice were fed AL or CR diet from 78-84 weeks of age, as described for Figure 7. **(A)** Random-fed blood glucose was recorded each week. **(B-D)** At 82 weeks of age mice underwent OGTTs. Blood glucose was recorded at each timepoint (B) and the tAUC and iAUC was determined (C). (D) Glucose-stimulated insulin secretion in mice during OGTT was assessed using an insulin ELISA. **(E)** HOMA-IR and Matsuda indices of mice calculated from glucose and insulin concentrations during the OGTT. Data in (A), (B), and (D) are presented as mean ± SEM. Data in (C) and (E) are presented as violin plots overlaid with individual data points. For each group and timepoint the following numbers of mice were used: **(A)**: *male AL,* n=9 (Wk 0, Wk 2-4, Wk 6), 8 (Wk 1), or 5 (Wk 5); *female AL,* n=12 (Wk 0, Wk2, Wk4), 8 (Wk 1, Wk 3), or 11 (Wk 5, Wk 6); *male CR,* n=10 (Wk 0, Wk 2-4, Wk 6), 9 (Wk 1), or 7 (Wk 5); *female CR,* n= 14 (Wk 0, Wk 2, Wk 4-6), 9 (Wk 1) or 10 (Wk 3). **(B-E)**: *male AL,* n=9 (B-C) or 5 (D-E); *female AL,* n=12; *male CR,* n=10; *female CR,* n=14. In (A), (B), and (D), significant effects of diet, sex and/or time, and interactions thereof, were determined using a mixed-effects model or 3-way ANOVA. In (C) and (E), significant effects of sex, diet, and sex*diet interaction for tAUC, iAUC, HOMA-IR and Matsuda Index were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall *P* values for each variable, and their interactions, are shown with each graph. For (C) and (E), statistically significant differences between comparable groups are indicated by * ($P<0.05$) or *** ($P<0.001$), or with *P* values shown. Source data are provided as a Source Data file.

Figure 9

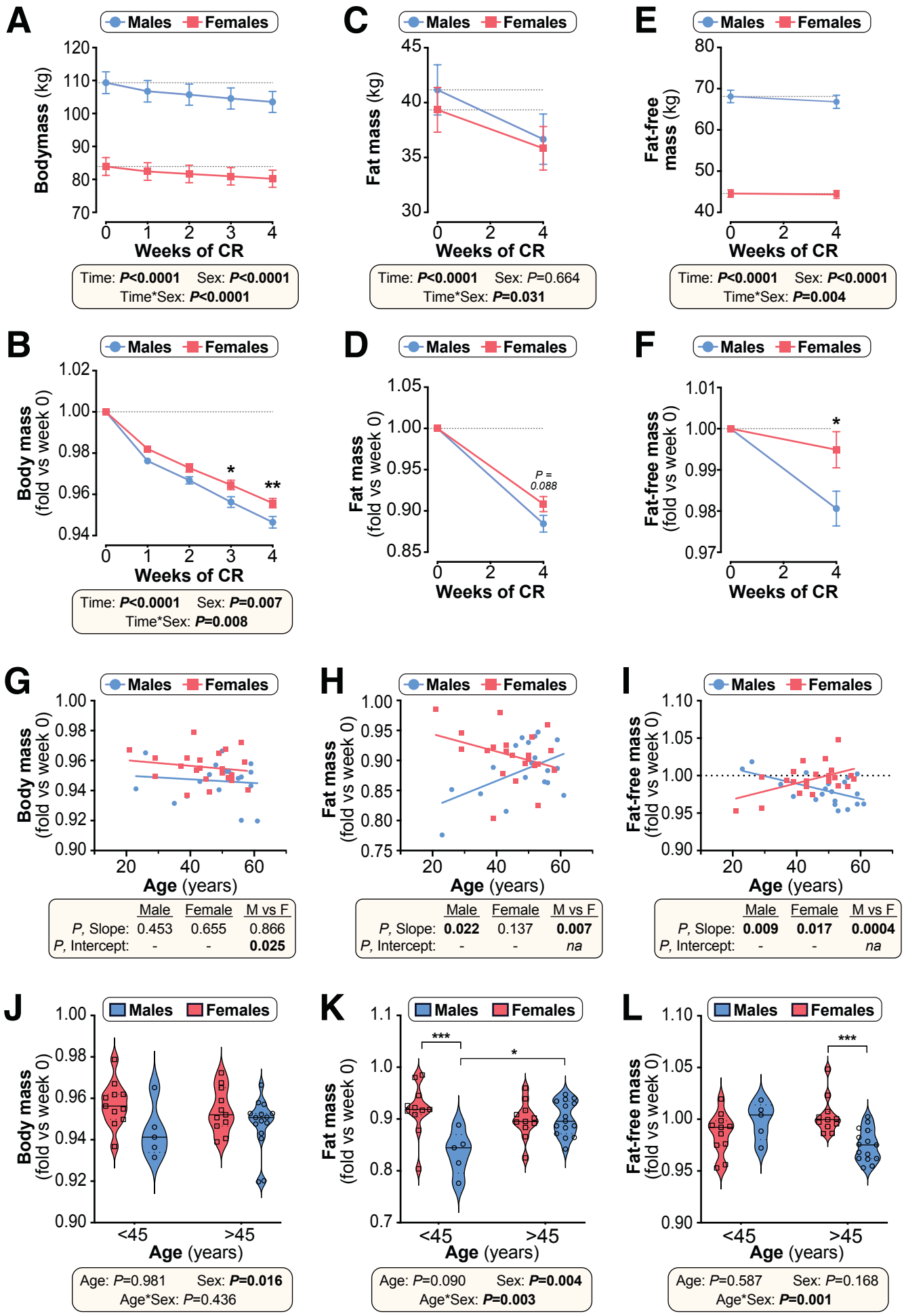

A
Males
Females
Bodymass (kg)
Weeks of CR
Time: P<0.0001 Sex: P<0.0001
Time*Sex: P<0.0001
C
Fat mass (kg)
Time: P<0.0001 Sex: P=0.664
Time*Sex: P=0.031
E
Fat-free mass (kg)
Time: P<0.0001 Sex: P<0.0001
Time*Sex: P=0.004
B
Body mass (fold vs week 0)
Time: P<0.0001 Sex: P=0.007
Time*Sex: P=0.008
D
Fat mass (fold vs week 0)
P = 0.088
F
Fat-free mass (fold vs week 0)
G
Age (years)
Male Female M vs F
P, Slope: 0.453 0.655 0.866
P, Intercept: - - 0.025
H
Male Female M vs F
P, Slope: 0.022 0.137 0.007
P, Intercept: - - na
I
Male Female M vs F
P, Slope: 0.009 0.017 0.0004
P, Intercept: - - na
J
<45
>45
Age: P=0.981 Sex: P=0.016
Age*Sex: P=0.436
K
Age: P=0.090 Sex: P=0.004
Age*Sex: P=0.003
L
Age: P=0.587 Sex: P=0.168
Age*Sex: P=0.001

Figure 9 – Effects of CR on body mass and body composition in humans are sex- and age-dependent.

Twenty male and twenty-two female volunteers participated in a weight loss study involving a 4-week dietary intervention. **(A-F)** Body mass was recorded each week (A,B)**.** Fat mass (FM) and fat-free mass (FFM) were measured by air-displacement whole-body plethysmography at weeks 0 and 4 (C-F). Body mass, fat mass and fat-free mass are shown as absolute masses (A,C,E) or fold-change relative to baseline (B,D,F). Data are presented as mean ± SEM**.** For (A-C) and (E), significant effects of time, sex, and time*sex interaction were assessed using 2-way ANOVA. In (B), (D) and (F), significant differences between males and females at each time point were determined by Šídák's multiple comparisons test (B) or unpaired T-test (D,F) and are indicated by * (P<0.05) or ** (P<0.01). **(G-I)** Simple linear regression of age vs fold-change (*week 4 vs week 0)* in body mass (G), fat mass (H) and fat-free mass (I). For each sex, significant associations between age and outcome (fold-change) are indicated beneath each graph as '*P,* Slope'. ANCOVA was further used to test if the age-outcome relationship differs significantly between males and females. ANCOVA results are reported beneath each graph as '*P,* Slope' and '*P,* Intercept' for males vs females (M vs F). In (G), similar slopes but different intercepts show that sex significantly influences weight loss, but the influence of age does not differ between the sexes. In (H,I) the slopes differ significantly, indicating that the age-outcome relationship differs between the sexes. **(J-L)** Fold-change (week 4 vs week 0) in body mass (J), fat mass (K) and fat-free mass (L) for males vs females separated into younger (<45 years) and older (>45 years) groups. Data are presented as violin plots overlaid with individual data points. Significant effects of age, sex, and age*sex interaction were assessed using 2-way ANOVA with Tukey's multiple comparisons test. Overall P values for each variable, and their interactions, are shown beneath each graph. Significant differences between comparable groups are indicated by * (P<0.05) or *** (P<0.001). See also Table 3. Source data are provided as a Source Data file.