THE UNIVERSITY of EDINBURGH

Edinburgh Research Explorer

Expansion of bone marrow adipose tissue during caloric restriction is associated with increased circulating glucocorticoids and not with hypoleptinemia.

Citation for published version:
Cawthorn, W, Scheller, EL, Parlee, SD, Pham, HA, Learman, BS, Redshaw, C, Sulston, R, Burr, A, Das, A, Simon, BR, Mori, H, Bree, AJ, Schell, B, Krishnan, V & MacDougald, OA 2016, 'Expansion of bone marrow adipose tissue during caloric restriction is associated with increased circulating glucocorticoids and not with hypoleptinemia.' Endocrinology, vol. 157, no. 2, 26696121, pp. 508-521. DOI: 10.1210/en.2015-1477

Digital Object Identifier (DOI):
10.1210/en.2015-1477

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Endocrinology

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 05. Apr. 2019

Expansion of bone marrow adipose tissue during caloric restriction is associated with increased circulating glucocorticoids and not with hypoleptinemia.

William P. Cawthorn[1,4,5,#], Erica L. Scheller[1], Sebastian D. Parlee[1], H. An Pham[1], Brian S. Learman[1], Catherine M.H. Redshaw[5], Richard J. Sulston[5], Aaron A. Burr[1], Arun K. Das[2], Becky R. Simon[3], Hiroyuki Mori[1], Adam J. Bree[1], Benjamin Schell[1], Venkatesh Krishnan[4], and Ormond A. MacDougald[1,2,3,#]

[1]Department of Molecular & Integrative Physiology, [2]Department of Internal Medicine, and [3]Program in Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI, USA. [4]Musculoskeletal Research, Lilly Research Laboratories, Indianapolis, Indiana, USA. [5]BHF/University Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh, UK.

Abbreviated title: Marrow fat expansion during caloric restriction

Key terms: Bone marrow adipose tissue, caloric restriction, leptin, glucocorticoids, adiponectin

Word count: 6,321

Number of figures and tables: 7

[#]***Corresponding authors*** *(please address reprint requests to William P. Cawthorn)*

Ormond A. MacDougald, Ph.D

Brehm Diabetes Center

1000 Wall St, Room 6313

University of Michigan

Ann Arbor, MI 48105

Tel: (734) 647-4880

Fax: (734) 232-8175

Email: macdouga@umich.edu

William P. Cawthorn, Ph.D

University/BHF Centre for Cardiovascular Science

The Queen's Medical Research Institute, University of Edinburgh

47 Little France Crescent

Edinburgh, EH16 4TJ

Tel: (+44) 131-242-6691

Email: W.Cawthorn@ed.ac.uk

Funding: This work was supported by grants from the National Institutes of Health (R24 DK092759 to O.A.M; K99-DE024178 to E.L.S; 5-T32-HD007505 to A.A.B; S10-RR026475-01 to the University of Michigan School of Dentistry microCT Core; and P30 DK089503 to the Michigan Nutrition Obesity Research Center, who oversaw NMR analysis of mouse body composition). W.P.C. is supported by a Career Development Award (MR/M021394/1) from the Medical Research Council (UK) and by a Chancellor's Fellowship from the University of Edinburgh, and previously was supported by a Lilly Innovation Fellowship Award and by a Postdoctoral Research Fellowship from the Royal Commission for the Exhibition of 1851 (UK). H. M. was supported by a mentor-based postdoctoral fellowship from the American Diabetes Association.

DISCLOSURE SUMMARY: S.D.P., E.L.S., H.A.P., B.S.L., C.M.H.R., R.J.S., A.A.B., A.K.D., B.R.S., H.M., A.J.B., and B.S. have nothing to declare. W.P.C. held a postdoctoral fellowship funded by Eli Lilly and Company. V.K. is employed by Eli Lilly and Company. O.A.M. has received research funding from Eli Lilly and Company.

ABSTRACT

Bone marrow adipose tissue (MAT) accounts for up to 70% of bone marrow volume in healthy adults and increases further in clinical conditions of altered skeletal or metabolic function. Perhaps most strikingly, and in stark contrast to white adipose tissue (WAT), MAT has been found to increase during caloric restriction (CR) in humans and many other species. Hypoleptinemia may drive MAT expansion during CR but this has not been demonstrated conclusively. Indeed, MAT formation and function are poorly understood; hence, the physiological and pathological roles of MAT remain elusive. We recently revealed that MAT contributes to hyperadiponectinemia and systemic adaptations to CR. To further these observations, we have now performed CR studies in rabbits to determine if CR affects adiponectin production by MAT. Moderate or extensive CR decreased bone mass, WAT mass, and circulating leptin, but, surprisingly, did not cause hyperadiponectinemia or MAT expansion. Although this unexpected finding limited our subsequent MAT characterization, it demonstrates that during CR, bone loss can occur independently of MAT expansion; increased MAT may be required for hyperadiponectinemia; and hypoleptinemia is not sufficient for MAT expansion. We further investigated this relationship in mice. In females, CR increased MAT without decreasing circulating leptin, suggesting that hypoleptinemia is also not necessary for MAT expansion. Finally, circulating glucocorticoids increased during CR in mice but not rabbits, suggesting that glucocorticoids might drive MAT expansion during CR. These observations provide insights into the causes and consequences of CR-associated MAT expansion, knowledge with potential relevance to health and disease.

INTRODUCTION

Adipocytes are a major component of human bone marrow (BM), comprising up to 70% of BM volume and accounting for over 10% of total adipose mass in lean, healthy adults (1, 2). Such marrow adipose tissue (MAT) further increases in diverse clinical conditions, including aging-associated bone loss and osteoporosis (3); estrogen deficiency (4, 5); type I diabetes (6, 7); and during treatment with pharmacological agents such as glucocorticoids, thiazolidinediones, or fibroblast growth factor-21 (FGF21) (8-12). Perhaps most strikingly, MAT is not catabolized during acute starvation (13, 14) but instead increases during anorexia nervosa and other conditions of prolonged caloric restriction (CR) (15-18). This is in stark contrast to white adipose tissue (WAT), underscoring the notion that MAT and WAT are developmentally and functionally distinct. However, both MAT formation and function are poorly understood, and therefore the impact of MAT on human physiology and disease remains to be established.

Understanding why MAT increases during CR might yield insights into MAT's physiological and pathological functions. Many changes that occur during CR are physiological adaptations that improve the ability to survive and/or recover from starvation (19). Such beneficial adaptations are likely to have been strongly selected for during mammalian evolution (20), and therefore the fact that MAT expands during CR suggests that MAT might serve an important physiological function. Alternatively, CR-associated MAT expansion might be a neutral, inconsequential phenomenon, or a pathological response that negatively impacts human health (18). Whatever the case, improved knowledge of the causes and consequences of MAT expansion during CR might shed light on the role of MAT in human physiology and disease.

Several hypotheses have been proposed to explain why MAT increases during CR (18). For example, CR and/or fasting are associated with decreased circulating leptin, estradiol, and insulin-like growth factor-1 (IGF1), and increased circulating FGF21, ghrelin, and cortisol or corticosterone (15, 21-25). Each of these changes has also been linked to increased BM adiposity in other contexts (5, 11, 12, 26-29), and therefore each of these factors has been proposed as a mediator of CR-associated MAT

expansion. The strongest argument has been made for hypoleptinemia (18). *In vitro* studies suggest that leptin directly inhibits adipogenic differentiation of BM stromal cells (30, 31), whereas MAT is increased in leptin-deficient *ob/ob* mice (26). Moreover, central or peripheral leptin administration leads to decreased BM adiposity in *ob/ob* mice (26, 32), type 1 diabetic mice (33) and Sprague-Dawley rats (34). Leptin may promote MAT loss by acting centrally to increase activity of the sympathetic nervous system, a mechanism through which leptin also promotes bone resorption (35). Finally, serum leptin concentrations inversely associate with vertebral BM adiposity in a cohort of healthy and anorexic humans (36). Thus, several lines of evidence support the possibility that hypoleptinemia drives MAT expansion during CR. However, why CR leads to MAT expansion remains to be established.

In addition to MAT formation, the function of MAT during CR has also begun to be addressed. We recently found that, during CR, MAT contributes to increased circulating levels of adiponectin (2), a hormone associated with enhanced insulin sensitivity and fat oxidation, anti-atherogenic and anti-cancer effects. Moreover, we found that in mice with impaired MAT expansion, skeletal muscle adaptations to CR are suppressed (2). These observations support the concept that MAT is an endocrine organ and suggest that MAT exerts systemic effects to impact adaptive responses to CR (2). However, numerous questions remain. For example, does CR alter MAT's expression or secretion of adiponectin, or other endocrine properties of MAT? And does MAT produce other endocrine factors that contribute to systemic effects of CR?

To address these questions, we investigated the effect of moderate or extensive CR in rabbits, a species that allows isolation of relatively large amounts of intact MAT for downstream analysis (2). Surprisingly, these CR regimens did not lead to MAT expansion or increased circulating adiponectin, despite marked bone loss and hypoleptinemia. Conversely, in female mice we found that CR promotes MAT expansion without altering circulating leptin. Our rabbit studies further suggest site-dependent differences in BM adipocyte size and responsiveness to varying degrees of CR. Finally, we found that CR is associated with increased circulating glucocorticoids in mice, but not in rabbits, suggesting that glucocorticoid excess might contribute to MAT expansion during CR. Together, these observations shed

118 new light on the potential mechanisms of MAT formation; the site-dependent nature of MAT

119 characteristics; and the interplay between MAT expansion, bone loss, and circulating adiponectin.

MATERIALS AND METHODS

Animals and animal care

New Zealand White rabbits were purchased from Harlan Laboratories (Haslett, MI, USA) or were generously provided by Dr. Yuqiang Chen (University of Michigan Medical School, Ann Arbor, MI, USA). Body mass and random-fed blood glucose were recorded weekly. For euthanasia, rabbits were first sedated by intramuscular injection of ketamine (40 mg/kg) and xylazine (5 mg/kg) before euthanizing by intravenous injection of pentobarbital (65 mg/kg) via the marginal ear vein. C57BL/6J mice were bred in-house as described previously (2). Body fat, lean mass, and free fluid were measured in conscious mice using an NMR analyzer (Minispec LF90II; Bruker Optics, Billerica, MA, USA). The University of Michigan Committee on the Use and Care of Animals approved all animal experiments, with daily care of mice and rabbits overseen by the Unit for Laboratory Animal Medicine (ULAM).

Caloric restriction

For moderate CR in rabbits (Fig. 1-2; Supplemental Fig. 1-2), 15-week-old male rabbits (3.14 ± 0.19 kg, mean ± SD) were randomly assigned into control (n=5) and CR (n=6) diet groups. Each group was fed a high-fiber diet (LabDiet 5326). Control rabbits received 100 g/day (31.91 ±0.19 g/kg body mass/day; mean ±SD) whereas CR rabbits received 70 g/day (23.00 ±0.35 g/kg body mass/day; mean ±SD), consistent with 30% CR that was found previously to drive MAT expansion in mice (2, 15). For extensive CR in rabbits (Fig 3-4; Supplemental Fig. 3), young males (1.04 ± 0.09 kg, mean ±SD) were fed *ad libitum* from 5 to 6 weeks of age to establish baseline food intake. Rabbits were then randomly assigned to control (n=6) or CR (n=6) diet groups. From 6-13 weeks of age, control rabbits were fed *ad libitum* (68.26 ± 4.82 g/kg body mass/day; mean ±SD) whereas CR rabbits were fed 40-50 g/day depending on body mass (30.65 ± 4.92 g/kg body mass/day; mean ±SD); this is consistent with the 50-70% reduction of *ad libitum* food intake used in recent rabbit CR studies (37, 38). For comparisons between the moderate and extensive CR cohorts, the following differences are worth considering: firstly, the moderate CR

animals were older, and therefore heavier; secondly, control-fed rabbits in the extensive CR cohort were fed *ad libitum,* while those in the moderate CR cohort were fed 100 g/day (consistent with ULAM guidelines). These differences likely explain why percent fat pad mass is greater in the extensive CR controls than in the moderate CR control rabbits (Fig. 1B vs Fig. 3B). To avoid malnutrition associated with micronutrient deficiency, both groups were fed throughout with a complete diet for growing rabbits (LabDiet 5321). The CR protocol for C57BL/6J mice was described previously (2).

Blood collection and serum hormone analysis

Blood was sampled from the marginal ear artery of rabbits or the lateral tail vein of mice using Microvette CB 300 capillary collection tubes (Sarstedt, Newton, NC, USA). To obtain serum, blood samples were allowed to clot on ice for two hours before centrifuging at 3,800 RCF for 5 min at 4 ºC. Serum leptin was determined using an ELISA kit (catalog no. MOB00) from R&D Systems Inc. (Minneapolis, MD, USA). Total and HMW serum adiponectin were determined using an ELISA kit (catalog no. 47-ADPHU-E01) from Alpco (Salem, NH, USA). ELISA kits to determine concentrations of corticosterone (catalog no. ADI-900-097) and cortisol (catalog no. ADI-900-071) were from Enzo Life Sciences, Inc (Exeter, UK).

Analysis of bone morphology by µCT

Femoral heads of rabbits were surgically isolated and embedded in 1% agarose and scanned using a µCT system (µCT100 Scanco Medical, Bassersdorf, Switzerland). Agarose-embedded femoral heads of rabbits were placed in a 48 mm diameter tube prior to scanning the femoral neck using the following settings: voxel size 36 µm, 70 kVp, 114 µA, 0.5 mm AL filter, and integration time 500 ms. Rabbit trabeculae were analyzed by contouring the inner trabecular compartment using the manufacturer's software (Analysis #15: trabecular, threshold 220), starting 20 slices away from the growth plate and contouring every 10 slices for a total of 30 slices. Density measurements were calibrated to the manufacturer's hydroxyapatite phantom. Analysis was performed using the manufacturer's evaluation software.

Real-time quantitative PCR (qPCR)

Total RNA was isolated from tissues using RNA STAT60 reagent (Tel-Test Inc, Friendswood, TX, USA) according to the manufacturer's instructions. Reverse transcription, primer design and qPCR were performed as described previously (39). Primers for rabbit *Lep, Adipoq, Pparg, Cebpa, Ppia* and *Tbp* were described previously (2). Sequences for other primers (5`-3`) are as follows: *Ero1l,* (F) TTGGCTAGAAGGCCTGTGTG, (R) GCCTTCTCCCTCGGTCAAAA; *Erp44*, (F) CTCCAGCAGATTGCCCTGTT, (R) GGGTGGACTGCTTGCTACAT; *Rab11fip1*, (F) CAGTACGGCAGAAGCTCCAA, (R) CCGAGGGGCTGTATTTCTTCA.

Immunoblot analysis

To detect total adiponectin in sera, samples were reduced and denatured by mixing with 4X SDS loading buffer, incubating at 95 ºC for 5 min, and cooling on ice for 1 min before separating by SDS-PAGE, as described previously (40). To isolate total protein, tissues were first pulverized in liquid nitrogen using a pestle and mortar. Pulverized tissues were then mixed with lysis buffer (1% SDS, 12.7 mM EDTA, 60 mM Tris-HCl; pH 6.8) heated to 95 ºC, and homogenized by sequential passaging through 21-gauge and 26-gauge needles. Lysates were then centrifuged at 20,000 RCF for 15 min at 4 ºC, lipid layers were discarded and supernatants transferred to fresh tubes and stored at -80 ºC. Protein concentration in tissue lysates was estimated using the BCA protein assay (Thermo Scientific, Waltham, MA, USA). SDS-PAGE and immunoblotting of tissue lysates was done as described previously (39). Mouse monoclonal anti-adiponectin (MA1-054) and rat monoclonal anti-α-tubulin (MA1-80017) were from Thermo Scientific. Mouse monoclonal anti-perilipin A (#4854) was from Vala Sciences (San Diego, CA, USA).

Isolation of BM, MAT, or RM

To visualize the spatial distribution of MAT and red marrow (RM) *in situ*, humeri, tibiae, and femurs were longitudinally bisected using a Dremel rotary tool with a #409 cutoff wheel (Robert Bosch Tool

196 Corporation, Addison, IL, USA); a constant drip of sterile USP-grade water was used during cutting to
197 prevent overheating. MAT or RM was then removed using a stainless steel spatula. To isolate MAT from
198 radii or ulnae, epiphyses were removed by lateral incisions with the Dremel tool, thereby allowing access
199 to the marrow cavity. BM was then extruded by first tracing the perimeter of the marrow cavity with a 2-
200 inch, 21-gauge needle, and subsequently scraping the BM out using a stainless steel spatula.
201
202 ***Triacylglycerol content of rabbit femoral BM***
203 One femur of each rabbit was bisected and whole BM removed. BM plugs were then frozen on dry ice
204 before cryopulverization in liquid nitrogen using a pestle and mortar. Total lipid was then extracted from
205 ~56 mg of each sample using the Folch method (41) as follows: *1)*, in a glass vial, mix sample with 1 mL
206 methanol and dissolve by sonication for 4 x 5 min, vortexing between each sonication; *2),* mix 0.5 mL of
207 tissue/methanol homogenate with 1 mL chloroform and vortex for 30 seconds; *3),* add 0.5 mL of 0.1N
208 HCl and vortex the vial to mix; *4),* centrifuge for 10 min at 500 RCF; *5)* remove the top layer and the
209 protein/debris interphase carefully by aspiration under mild vacuum; *6),* wash the lower organic layer by
210 adding 0.5mL of 50% methanol and vortex to mix; *7),* centrifuge for 3 min at 12,000 RCF; *8),* discard
211 upper layer using vacuum aspirator; *9),* repeat steps 6-8; *10),* transfer 0.3 mL of each sample to a new
212 glass vial and evaporate solvent using nitrogen gas; *11),* re-suspend the remaining lipid in 100 µL
213 chloroform. These lipid samples were then separated by thin layer chromatography on a silica gel plate;
214 the triacylglycerol bands were then identified and excised. This portion was then extracted from the silica
215 gel and resuspended in 500 µL of 68% ethanol (357 µL of 95% ethanol, plus 143 µL of isotonic saline).
216 The triacylglycerol concentration in 20 µL of each sample was then determined using the Triglyceride
217 Determination Kit (Sigma Aldrich) based on the manufacturer's instructions.
218
219 ***Histology and analysis of adipocyte morphology***
220 Intact pieces of WAT, MAT, and RM were fixed in 10% neutral-buffered formalin, paraffin-embedded,
221 sectioned, and stained with H&E prior to determination of adipocyte size distribution, as described

previously (42). Analysis of MAT and RM was done without reference to the directionality or orientation within the bone.

Osmium tetroxide staining

Mouse tibiae were stained with osmium tetroxide (Electron Microscopy Sciences #19170) and MAT was then visualized by μCT, as described previously (2). MAT volume in distinct tibial regions was then quantified and is presented relative to total tibial marrow volume, as described previously (43). Osmium tetroxide staining could not be used for quantitation of MAT volume in rabbit bones, because the osmium tetroxide is unable to stain beyond the periphery of these large tissues, especially those that are densely packed with fat (E. Scheller, unpublished observations).

Statistical Analysis

Data are represented as box and whisker plots overlaid with individual values, or as mean ± standard deviation (for data where box and whisker plots would be too cluttered). For box and whisker plots, the box extends from the 25^{th} to 75^{th} percentiles, with the central line representing the median and the whiskers showing the minimum and maximum values. Statistical analysis was done using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA). Significant differences in body mass, tissue mass, circulating leptin, circulating adiponectin, circulating corticosterone, transcript expression, femoral head bone characteristics, BM triacylglycerol, bone length, body composition, and MAT volume were assessed using two-sample t-tests. For moderate CR rabbits, differences in circulating cortisol were assessed using two-sample t-tests; however, for extensive CR rabbits, cortisol concentrations were non-normally distributed, and therefore significant differences were assessed using the Mann-Whitney U test (Fig. 6D). Significant differences in adipocyte size were assessed by ANOVA with a Tukey post-test for multiple comparisons. The D'Agostino test confirmed that adipocyte areas were normally distributed across all tissues for control and CR rabbits. A p-value of < 0.05 was considered statistically significant.

RESULTS

Circulating adiponectin does not increase during moderate CR in rabbits

We previously used rabbits to characterize expression and secretion of adiponectin in MAT, because, unlike mice or rats, this species allows isolation of relatively large and intact MAT samples (2). Thus, to investigate further if CR affects adiponectin production by MAT, we pursued CR studies in rabbits. We fed rabbits in the CR group 30% less food than their control counterparts, consistent with the extent of CR used in our previous mouse studies (2). Herein, this 30% CR regimen is described as 'moderate' CR. As expected, moderate CR was associated with decreased body mass, circulating leptin, and mass of WAT in inguinal (iWAT), gonadal (gWAT), and perirenal (pWAT) depots (Fig. 1A-C; Supplemental Fig. 1). CR-associated bone loss was also apparent, with μCT of femoral heads revealing that CR-fed rabbits had significantly decreased bone volume fraction (BVF), connectivity density (Conn. Dens), and bone mineral content (BMC) compared to controls (Table 1). To next investigate if CR impacts adiponectin production by MAT, and how this compares to effects in WAT, we first analyzed expression of adiponectin mRNA (*Adipoq*). As a positive control, we also measured expression of leptin (*Lep*), which is known to be decreased in WAT during fasting or CR (44-47). As shown in Fig. 1D, CR led to significantly decreased *Lep* expression in iWAT, whereas adiponectin (*Adipoq*) expression was not significantly altered by CR. Similar effects were observed in gWAT and pWAT (data not shown). These findings are consistent with observations in rodents and humans (44-50). In tibial MAT, CR was also associated with decreased expression of *Lep* but not *Adipoq* (Fig. 1E). To begin assessing potential effects on adiponectin secretion, we next analyzed expression of factors known to regulate this process. Ero1-Lα is an ER chaperone that promotes adiponectin secretion, whereas ERp44 and Rab11-FIP1 inhibit adiponectin secretion (51-53). We found that CR did not affect *Ero1l, Erp44,* or *Rab11fip1* expression in iWAT (Fig. 1D). Expression of *Ero1l* and *Erp44* in tibial MAT was similarly unaffected, whereas *Rab11fip1* expression was significantly higher in MAT of CR-fed rabbits (Fig. 1E). These observations are consistent with the concept that adiponectin secretion from WAT does not increase during CR (54),

and suggest that this is also true for tibial MAT. However, there is often a disparity between adiponectin transcript expression and circulating adiponectin levels (48, 50, 55). Thus, we next used immunoblotting to assess total adiponectin in serum, expecting that, as in rodents and humans (48, 56), this would increase with moderate CR. Counter to these expectations, total serum adiponectin did not differ between control and CR-fed rabbits (Fig. 1F-G). Thus, moderate CR in rabbits exerts expected effects on body mass, fat mass, bone mass, circulating leptin, and expression of leptin and adiponectin in WAT; however, moderate CR in rabbits is not associated with hyperadiponectinemia.

MAT expansion does not occur during moderate CR in rabbits

Our recent work reveals that in mice, CR-associated hyperadiponectinemia is blunted when MAT expansion is impaired (2). Therefore, the lack of hyperadiponectinemia observed above suggests that MAT expansion might be limited or absent during moderate CR in rabbits. To address this possibility, we bisected bones of these animals and characterized BM adiposity. The BM of each group appeared grossly similar, with no differences in the amount of fatty yellow marrow in humeri and femurs (Fig. 2A-B), or in radii, ulnae, and tibiae (data not shown). To further assess MAT content, we analyzed whole femoral BM for expression of transcripts and proteins typical of BM adipocytes, as well as total triacylglycerol content; unlike the above qPCR analysis of tibial MAT (Fig. 1E), these analyses sought to determine adipocyte content in more heterogeneous femoral BM samples. Control and moderate CR-fed rabbits had similar BM expression of *Pparg* and *Lep* transcripts (Fig. 2C), while expression of Perilipin A protein was also similar (Fig. 2D). In contrast, moderate CR was associated with a trend for decreased *Cepba* expression and significantly lower *Adipoq* expression (Fig. 2C). Similarly, total femoral BM triacylglycerol content tended to be lower with moderate CR (Fig. 2E). These observations suggest that moderate CR does not lead to MAT expansion.

To fully determine the impact of CR on BM adiposity throughout the skeleton, we analyzed adipocyte size distribution in MAT and red marrow (RM) obtained from different skeletal sites (Fig. 2F-G; Supplemental Fig. 2). As shown in Supplemental Fig. 2, CR did not affect adipocyte size in any MAT

or RM depot analyzed, except in ulnae, where CR led to a small but significant increase in adipocyte size (Supplemental Fig. 2H). This suggests depot-specific variation in MAT responsiveness to CR. Indeed, in CR-fed rabbits the adipocytes in distal MAT depots (tibia, radius, ulna) tended to be larger than those in proximal MAT (femur, humerus) (Fig. 2F). For further comparison, we also analyzed adipocyte sizes in WAT. In contrast to RM or MAT, CR led to markedly decreased adipocyte size in iWAT, gWAT, and pWAT (Fig. 2G; Supplemental Fig. 2I). WAT adipocytes were also significantly larger than BM adipocytes in control-fed rabbits, but not after CR (Fig. 2G). Thus, adipocyte size and responsiveness to CR differs both between BM and WAT, and also across different MAT depots.

Together, these analyses of BM triacylglycerol content, adipocyte marker expression, and adipocyte size demonstrate that in rabbits, moderate CR does not lead to MAT expansion.

Extensive CR in rabbits leads to BM adipocyte hypotrophy, suggesting loss of MAT

The lack of hyperadiponectinemia and MAT expansion during these moderate CR studies was unexpected. Given that effects of CR are dependent upon the degree of restriction (47, 57, 58), one possibility is that that the extent of moderate CR was insufficient to drive hyperadiponectinemia or MAT expansion. Another possible explanation relates to the fact that our above studies were in skeletally mature rabbits, whereas previous research into CR-associated MAT expansion in mice has been done in young, growing animals (2, 15). To address these possibilities, we next investigated the effects of more extensive CR in a cohort of young, growing rabbits. As found above for moderate CR (Fig. 1), extensive CR was associated with significantly decreased body mass, fat pad mass, and circulating leptin (Fig. 3A-C); however, each of these changes was more pronounced than those that occurred during moderate CR (Fig. 1A-E). Bone length was also markedly decreased with extensive CR (Fig. 3D), consistent with suppressed skeletal development observed in previous rabbit CR studies (59). Thus, extensive CR was associated with expected effects on body mass, peripheral adiposity, circulating leptin, and skeletal

biology. However, as found for moderate CR, extensive CR did not affect total circulating adiponectin (Fig. 3E-F).

We next assessed BM adiposity. Upon bisecting bones for MAT isolation we were struck by the very dark appearance of BM in the extensive CR-fed rabbits (Fig. 4A-B), suggesting decreased BM adiposity in these animals. Indeed, we were unable to isolate intact pieces of MAT from extensive CR-fed rabbits, which prevented us from analyzing MAT adiponectin production in these animals. However, we could isolate small pieces of less pure MAT from the radius and ulna, and from distal regions of the humerus, femur, and tibia. Thus, to further assess effects of extensive CR on BM adiposity we analyzed adipocyte size distribution in these MAT samples, and in RM obtained from humeri, femurs, and tibiae. For comparison we also analyzed adipocyte sizes in WAT. We found that extensive CR led to significantly reduced adipocyte size in each WAT depot; in humerus MAT and RM; and in femoral and tibial RM (Fig. 4C; Supplemental Fig. 3). There was also a trend for decreased adipocyte size in radial MAT ($P = 0.068$) and tibial MAT ($P = 0.077$). Such hypotrophy suggests lipolytic breakdown of BM adipocytes and/or impaired MAT development, possibilities that remain to be formally tested; however, these possibilities are highly likely based on current understanding of adipose tissue biology, and are consistent with the conclusion that extensive CR leads to MAT depletion. One notable exception was the ulna, where CR did not affect adipocyte size (Supplemental Fig. 3H). As noted for moderate CR (Fig. 2G), in extensive CR rabbits the distal MAT depots (tibia, radius, ulna) tended to have larger adipocytes than more proximal depots (femur, humerus) (Fig. 4C). Differences in adipocyte size were even more pronounced between BM and WAT, with gWAT, iWAT, and pWAT of control-fed rabbits having significantly larger adipocytes than any of the RM or MAT depots (Fig. 4C). Thus, as found for the moderate CR cohort, in control rabbits BM adipocytes are smaller than WAT adipocytes, and the response of BM adipocytes to extensive CR varies across the different skeletal sites. Ultimately, both moderate CR and extensive CR led to decreased circulating leptin without resulting in MAT expansion.

347 *MAT expansion is not associated with hypoleptinemia during CR in female mice*

348 The above findings demonstrate that hypoleptinemia *per se* is not sufficient to cause MAT expansion, at
349 least in rabbits. To determine the relevance of this finding to other species, we next investigated the
350 relationship between CR, leptin, and MAT expansion in mice. We fed male and female C57BL/6J mice a
351 control or 30% CR diet from 9-15 weeks of age and analyzed MAT, total adiposity, lean mass, and
352 circulating leptin. As expected, in both sexes CR was associated with decreased body mass (Supplemental
353 Fig. 4A-B), hyperadiponectinemia (Supplemental Fig. 4C, F), and significant MAT expansion, as
354 assessed by analysis of osmium tetroxide-stained tibiae (Fig. 5A-B). MAT expansion occurred
355 predominantly in the proximal tibia, from the growth plate to tibia-fibula junction, consistent with this
356 region containing 'regulated' MAT (rMAT) that is more responsive to external stimuli than the
357 'constitutive' MAT (cMAT) in the distal tibia (43). However, other effects of CR differed between males
358 and females. Thus, in males CR led to decreases in total adiposity and the masses of iWAT, gWAT and
359 liver, both in terms of absolute mass (Fig. 5C-D) and percent body mass (Supplemental Fig. 4D-E).
360 Consistent with this decreased adiposity, circulating leptin was markedly lower in CR-fed males
361 compared to their control counterparts (Fig. 5E). In contrast, in females CR did not decrease the absolute
362 masses of iWAT, gWAT, or total body fat, despite decreased body mass (Fig. 5F-G; Supplemental Fig.
363 4B). As such, CR in females was associated with significantly increased body fat percentage and percent
364 iWAT mass, while percent lean mass was decreased (Supplemental Fig. 4G-H). Thus, unlike in male
365 mice, CR in female mice led to loss of lean mass without decreasing WAT mass or total adiposity.
366 Consistent with this maintenance of fat mass, circulating leptin did not differ between CR-fed females
367 and their control counterparts (Fig. 5H). These observations demonstrate that in female mice, CR-
368 associated MAT expansion is not associated with hypoleptinemia, suggesting that the latter is not required
369 for MAT expansion.
370
371

372 *MAT expansion during CR is associated with increased circulating glucocorticoids*

373 The above observations show that MAT expansion occurs during CR in mice but not in rabbits, and that
374 this is not associated with hypoleptinemia. Therefore, we next investigated if there is another endocrine
375 basis for this differential response. One well-established effect of CR is to increase levels of circulating
376 glucocorticoids, such as cortisol in humans and corticosterone in rodents (25, 60). In contrast, one study
377 finds that glucocorticoids are not increased during CR in rabbits (61). This supports the possibility that
378 differential MAT expansion during CR in mice and rabbits might relate to divergent effects on circulating
379 glucocorticoids. Therefore, we next analyzed circulating glucocorticoids in our cohorts of mice and
380 rabbits with or without CR. Consistent with previous studies, serum concentrations of corticosterone, the
381 major circulating glucocorticoid in rodents, were increased during CR in male and female mice (Fig. 6A-
382 B). In contrast, circulating cortisol and corticosterone were unaltered during moderate or extensive CR in
383 rabbits (Fig. 6C-F). These observations support the possibility that increases in circulating glucocorticoids
384 are a stimulus for CR-associated MAT expansion.

DISCUSSION

In the present study our original aim was to determine how CR affects adiponectin production by MAT. However, in pursuing this goal we found, unexpectedly, that CR in rabbits does not lead to increased circulating glucocorticoids, MAT expansion, or hyperadiponectinemia. Conversely, in female mice CR-associated MAT expansion occurs without decreased circulating leptin, whereas in both males and females CR is associated with increased serum corticosterone. Together, these observations provide insights into the mechanisms of MAT expansion, the impact of MAT on bone remodeling, and the potential function of MAT as an endocrine organ.

Unexpected effects of CR in rabbits

The lack of MAT expansion during moderate or extensive CR in rabbits was unexpected, because increased BM adiposity during CR has been observed repeatedly in other species (18). However, while previous rabbit studies report maintenance of BM adipocyte size even after 10-21 days' starvation (13, 14), there are no reports of *increased* MAT during CR in rabbits. Instead, MAT loss has been noted in rabbits when food deprivation extends beyond 25 days (62, 63), which is far shorter than the 7-week timeframe of our CR studies. Thus, extensive CR can lead to MAT loss in rabbits. Similarly, in anorexia nervosa MAT expansion occurs in patients with more minimal weight loss, whereas BM lipid content and adipocyte size decrease in patients with the greatest weight loss (17). In severely anorexic patients, BM becomes serous-like, with atrophy of adipocytes and hematopoietic cells (64). MAT loss following extensive CR has also been reported in other species (65). One limitation of our extensive CR studies is that we did not sample total BM from any bones; hence, unlike for the moderate CR rabbits, we could not analyze other markers of MAT content, such as total triacylglycerol. It is also plausible that extensive CR causes BM adipocyte hypotrophy not because of MAT loss, but as a result of constraints imposed by decreased bone size (Fig. 3D); however, this is perhaps unlikely given that CR also increases BM volume via bone loss (2), which may compensate for decreases in bone size. Ultimately, the marked BM adipocyte hypotrophy in extensive CR rabbits, together with the darker appearance of BM in this group,

supports the conclusion of MAT loss in this context. Together with the preclinical and clinical studies described above, our findings demonstrate that MAT depletion can occur when the degree and duration of CR are sufficient.

Although CR can cause MAT loss, we find that CR-associated adipocyte hypotrophy is far greater in WAT than in MAT. Thus, MAT adipocytes might be more resistant to lipolysis than adipocytes in WAT. Moreover, it is notable that, in moderate and extensive CR, adipocytes in WAT and MAT reach a similar size. This suggests that MAT adipocyte size might represent a minimum threshold for adipocytes, both in MAT and WAT, which is defended in catabolic states.

Sexually dimorphic effects of CR

In addition to the lack of MAT expansion in rabbits, our finding that CR does not decrease WAT mass or circulating leptin in female mice was initially unexpected; however, our observations are consistent with previous CR studies in female C57BL/6 mice. For example, Varady *et al* observe increased scWAT and unaltered leptin after 25% CR from 7-11 weeks of age (66); Fenton *et al* report no change in leptin following 30% CR from 6-16 weeks of age (67); and Li *et al* find increased adiposity after 5% CR from 13-16 or 15-19 weeks of age (68). Our finding that CR decreases adiposity and circulating leptin in male C57BL/6J mice is also consistent with previous studies (15, 69, 70), which suggests that responses to CR in C57BL/6J mice are sex-specific. Similarly, Shi *et al* showed that CR in FVBN mice leads to hypoleptinemia and decreased scWAT mass in males but not females (71). Such sexually dimorphic effects of CR have also been noted in many other species (72-74). In humans, several CR studies report greater loss of total or visceral fat mass in men than in women (75-78); however, this has not been found in all studies (79, 80), and therefore the relevance of such sexual dimorphism to humans remains to be established. Given the extensive interest in potential benefits of CR to human health (81), this issue clearly warrants further investigation.

Endocrine factors as potential mediators of CR-associated MAT expansion

Our observations in rabbits and female mice suggest that hypoleptinemia *per se* is neither necessary nor sufficient for MAT expansion during CR, which contradicts the hypothesis that hypoleptinemia is a key driver of CR-associated MAT expansion (18). This hypothesis is indirectly supported by observations that leptin-deficient mice have increased MAT, which decreases following exogenous leptin administration (26, 32-34, 82). This leptin supplementation approach could also be used during CR to directly test if hypoleptinemia is required for MAT expansion. Here, it would be important to ensure that such leptin supplementation does not increase circulating leptin to supraphysiological concentrations. Indeed, exogenous leptin administration also leads to profound MAT loss in animals that are not leptin-deficient (34, 82, 83). In such states of leptin sufficiency, effects of exogenous leptin typically depend upon administration of high doses that elevate circulating leptin to supraphysiological concentrations (84). In contrast, our results demonstrate that hypoleptinemia alone, within a normal physiological range, is not sufficient for CR-associated MAT expansion in rabbits, while lack of hypoleptinemia, without resorting to exogenous leptin treatment, does not prevent MAT expansion during CR in female mice. Moreover, estrogen deficiency is associated with increases in MAT and circulating leptin (4, 85), demonstrating that hypoleptinemia is not required for MAT expansion in other contexts. Nevertheless, it remains possible that our observations in rabbits and female mice are species-, sex-, age-, and/or protocol-specific. For example, we cannot exclude the possibility that hypoleptinemia contributes to MAT expansion in male C57BL/6J mice, or that moderate CR would promote MAT expansion in younger rabbits. Therefore, there is clear utility in pursuing leptin supplementation experiments to directly test if hypoleptinemia is required for MAT expansion during CR.

The basis for our observed species-specific differences in MAT expansion remains to be firmly established. As described in the introduction, several endocrine changes have been proposed to contribute to MAT expansion during CR, including decreased IGF1 and increases in FGF21 or glucocorticoids. Circulating FGF21 increases during short-term fasting (23, 24), but recent studies demonstrate no differences during longer-term CR (86). This argues against a role of FGF21 excess in driving MAT

463 expansion during CR. In contrast, decreased IGF1 is well established during anorexia nervosa in humans
464 (60) and CR in rodents (58), supporting the possibility that this contributes to MAT expansion; however,
465 IGF1 also decreases during CR in rabbits (87), and therefore this is unlikely to account for the differences
466 in MAT expansion observed between rabbits and mice. Moreover, CR in non-anorexic humans was
467 recently shown to decrease bone mass without affecting circulating IGF1 (88), demonstrating that
468 decreased IGF1 is not necessary for effects of CR on bone.

469 Our present findings suggest that the species-specific differences in MAT expansion may relate to
470 effects on circulating glucocorticoids. CR-induced increases in circulating glucocorticoids are well
471 established in rodents and humans, but only one prior study reports the lack of this response in rabbits
472 (61). Our observations therefore build on this work by further demonstrating that, unlike in other species,
473 circulating cortisol and corticosterone do not increase during CR in rabbits. Given that glucocorticoids
474 increase BM adiposity (11), our findings support the possibility that increased circulating glucocorticoids
475 are necessary for CR-associated MAT expansion. We are currently investigating this hypothesis further.
476

477 *Depot-specific differences in MAT characteristics*

478 Two previous studies report that adipocytes in pWAT are larger than those in femoral RM (14,
479 89), consistent with our present findings that BM adipocytes are smaller than those in WAT. However,
480 our study is the first to comprehensively compare BM adipocyte sizes across different skeletal sites. We
481 found that adipocytes in distal regions of the skeleton (tibia, radius, ulna) tend to be larger than those in
482 MAT from more proximal depots (femur, humerus). Responsiveness to CR also varies across these sites:
483 unlike adipocytes in more proximal BM regions, adipocytes in ulnal MAT undergo hypertrophy during
484 moderate CR and resist hypotrophy during extensive CR. This is consistent with an early study of
485 starvation in rabbits, which shows that BM adiposity decreases more in proximal bones (e.g. humeri
486 ,femurs) than in distal regions (e.g. tibiae, radii, ulnae) (63). This is also consistent with our recent
487 research that identifies regionally distinct MAT subtypes with different characteristics: constitutive MAT
488 (cMAT) exists at distal sites and is relatively refractory to external stimuli, whereas regulated MAT

(rMAT) is interspersed within RM at proximal skeletal sites and is more responsive to external stimuli, such as cold exposure (43). Our present analyses confirm and extend these observations by revealing that CR-associated MAT expansion predominantly occurs within rMAT, while increases in cMAT are far less pronounced (Fig. 5A-B). Determining if these site-specific differences extend to other MAT properties might provide fundamental insights into MAT formation and function.

Relationship between MAT expansion and bone loss

Our results shed further light on the relationship between MAT and bone. Given that MAT is increased in osteoporosis (3), BM adipocytes have been postulated to directly inhibit bone formation and/or promote bone resorption (90-93). Indeed, increased MAT volume is now considered a clinical risk factor for fracture (94). However, this concept is coming under scrutiny following numerous recent studies (95). For example, across several inbred mouse strains there is no correlation between BM adipocyte numbers and femoral bone mineral density (96), while blocking MAT expansion does not prevent bone loss during type 1 diabetes or ovariectomy (33, 97, 98). Our observations in rabbits further demonstrate that MAT expansion is not necessary for bone loss during CR. Such knowledge has direct clinical relevance to diseases such as anorexia nervosa, which is associated with bone loss and life-long increases in fracture risk (3, 99). It remains possible that altered MAT characteristics, independent of MAT expansion, contribute to bone loss in osteoporosis, type 1 diabetes, CR, or other conditions. However, our present study provides further evidence that MAT expansion *per se* does not promote bone loss.

Potential endocrine functions of MAT

Our previous research supports the concept that MAT is an endocrine organ that contributes to hyperadiponectinemia during CR (2). This conclusion is partly based on the observation that increased MAT is required for full increases in circulating adiponectin during CR, at least in mice. Herein, we find that neither MAT nor circulating adiponectin increases during CR in rabbits, while in mice the increases

in MAT and circulating adiponectin are greater in females than in males. These observations further support the concept that CR-associated hyperadiponectinemia is linked to MAT expansion. Our rabbit studies also reveal that MAT leptin expression decreases during CR, a phenomenon well established in WAT (44, 47). In contrast, we find that CR does not increase adiponectin expression in MAT or WAT. Our data also support the possibility that secretion of adiponectin is not increased, at least based on the expression of known regulators of adiponectin secretion. Similar observations have been made for WAT of rodents and humans (47-50, 54); however, given the lack of hyperadiponectinemia and MAT expansion during CR in rabbits, the relevance of these observations to MAT of humans and other species remains unclear. This uncertainty, as well as the inability to isolate sufficient MAT from extensive CR rabbits, limited our ability to more comprehensively investigate the endocrine properties of MAT. Thus, establishing how CR affects MAT's potential endocrine functions will require further studies in other species. Such research will be crucial if we are to better understand MAT's nascent role as an endocrine organ, as well as the impact of MAT on human health and disease.

ACKNOWLEDGEMENTS

We are grateful to Greg Whitlock for providing the Dremel™ tool for rabbit bone bisection, and to Janet Cawthorn, Abigail Moon, and Dennis Simon for assistance with the rabbit CR studies.

REFERENCES

1. C. Hindorf, G. Glatting, C. Chiesa, O. Linden and G. Flux. EANM Dosimetry Committee guidelines for bone marrow and whole-body dosimetry. Eur J Nucl Med Mol Imaging 2010; 37:1238-1250.
2. W. P. Cawthorn, E. L. Scheller, B. S. Learman, S. D. Parlee, B. R. Simon, H. Mori, X. Ning, A. J. Bree, B. Schell, D. T. Broome, S. S. Soliman, J. L. DelProposto, C. N. Lumeng, A. Mitra, S. V. Pandit, K. A. Gallagher, J. D. Miller, V. Krishnan, S. K. Hui, M. A. Bredella, P. K. Fazeli, A. Klibanski, M. C. Horowitz, C. J. Rosen and O. A. MacDougald. Bone Marrow Adipose Tissue Is an Endocrine Organ that Contributes to Increased Circulating Adiponectin during Caloric Restriction. Cell Metabolism 2014; 20:368-375.
3. P. K. Fazeli, M. C. Horowitz, O. A. Macdougald, E. L. Scheller, M. S. Rodeheffer, C. J. Rosen and A. Klibanski. Marrow fat and bone--new perspectives. J Clin Endocrinol Metab 2013; 98:935-945.
4. R. B. Martin, B. D. Chow and P. A. Lucas. Bone marrow fat content in relation to bone remodeling and serum chemistry in intact and ovariectomized dogs. Calcified tissue international 1990; 46:189-194.
5. F. A. Syed, M. J. Oursler, T. E. Hefferanm, J. M. Peterson, B. L. Riggs and S. Khosla. Effects of estrogen therapy on bone marrow adipocytes in postmenopausal osteoporotic women. Osteoporosis International 2008; 19:1323-1330.
6. S. Botolin, M. C. Faugere, H. Malluche, M. Orth, R. Meyer and L. R. McCabe. Increased bone adiposity and peroxisomal proliferator-activated receptor-gamma2 expression in type I diabetic mice. Endocrinology 2005; 146:3622-3631.
7. S. Botolin and L. R. McCabe. Bone loss and increased bone adiposity in spontaneous and pharmacologically induced diabetic mice. Endocrinology 2007; 148:198-205.
8. A. A. Ali, R. S. Weinstein, S. A. Stewart, A. M. Parfitt, S. C. Manolagas and R. L. Jilka. Rosiglitazone causes bone loss in mice by suppressing osteoblast differentiation and bone formation. Endocrinology 2005; 146:1226-1235.
9. L. Tornvig, L. I. Mosekilde, J. Justesen, E. Falk and M. Kassem. Troglitazone treatment increases bone marrow adipose tissue volume but does not affect trabecular bone volume in mice. Calcif Tissue Int 2001; 69:46-50.
10. C. L. Ackert-Bicknell, K. R. Shockley, L. G. Horton, B. Lecka-Czernik, G. A. Churchill and C. J. Rosen. Strain-specific effects of rosiglitazone on bone mass, body composition, and serum insulin-like growth factor-I. Endocrinology 2009; 150:1330-1340.
11. B. C. Vande Berg, J. Malghem, F. E. Lecouvet, J. P. Devogelaer, B. Maldague and F. A. Houssiau. Fat conversion of femoral marrow in glucocorticoid-treated patients: a cross-sectional and longitudinal study with magnetic resonance imaging. Arthritis Rheum 1999; 42:1405-1411.
12. W. Wei, P. A. Dutchak, X. Wang, X. Ding, X. Wang, A. L. Bookout, R. Goetz, M. Mohammadi, R. D. Gerard, P. C. Dechow, D. J. Mangelsdorf, S. A. Kliewer and Y. Wan. Fibroblast growth factor 21 promotes bone loss by potentiating the effects of peroxisome proliferator-activated receptor gamma. Proc Natl Acad Sci U S A 2012; 109:3143-3148.
13. M. Tavassoli. Differential response of bone marrow and extramedullary adipose cells to starvation. Experientia 1974; 30:424-425.
14. A. Bathija, S. Davis and S. Trubowitz. Bone marrow adipose tissue: response to acute starvation. Am J Hematol 1979; 6:191-198.
15. M. J. Devlin, A. M. Cloutier, N. A. Thomas, D. A. Panus, S. Lotinun, I. Pinz, R. Baron, C. J. Rosen and M. L. Bouxsein. Caloric restriction leads to high marrow adiposity and low bone mass in growing mice. J Bone Miner Res 2010; 25:2078-2088.
16. M. A. Bredella, P. K. Fazeli, K. K. Miller, M. Misra, M. Torriani, B. J. Thomas, R. H. Ghomi, C. J. Rosen and A. Klibanski. Increased bone marrow fat in anorexia nervosa. J Clin Endocrinol Metab 2009; 94:2129-2136.

17. E. Abella, E. Feliu, I. Granada, F. Milla, A. Oriol, J. M. Ribera, L. Sanchez-Planell, L. I. Berga, J. C. Reverter and C. Rozman. Bone marrow changes in anorexia nervosa are correlated with the amount of weight loss and not with other clinical findings. Am J Clin Pathol 2002; 118:582-588.
18. M. J. Devlin. Why does starvation make bones fat? Am J Hum Biol 2011; 23:577-585.
19. M. D. McCue. Starvation physiology: reviewing the different strategies animals use to survive a common challenge. Comp Biochem Physiol A Mol Integr Physiol 2010; 156:1-18.
20. T. L. Kirkwood, P. Kapahi and D. P. Shanley. Evolution, stress, and longevity. J Anat 2000; 197 Pt 4:587-590.
21. J. Nedvidkova, I. Krykorkova, V. Bartak, H. Papezova, P. W. Gold, S. Alesci and K. Pacak. Loss of meal-induced decrease in plasma ghrelin levels in patients with anorexia nervosa. J Clin Endocrinol Metab 2003; 88:1678-1682.
22. M. Misra, K. K. Miller, J. Cord, R. Prabhakaran, D. B. Herzog, M. Goldstein, D. K. Katzman and A. Klibanski. Relationships between serum adipokines, insulin levels, and bone density in girls with anorexia nervosa. J Clin Endocrinol Metab 2007; 92:2046-2052.
23. M. K. Badman, P. Pissios, A. R. Kennedy, G. Koukos, J. S. Flier and E. Maratos-Flier. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell metabolism 2007; 5:426-437.
24. T. Inagaki, P. Dutchak, G. Zhao, X. Ding, L. Gautron, V. Parameswara, Y. Li, R. Goetz, M. Mohammadi, V. Esser, J. K. Elmquist, R. D. Gerard, S. C. Burgess, R. E. Hammer, D. J. Mangelsdorf and S. A. Kliewer. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell metabolism 2007; 5:415-425.
25. E. A. Levay, A. H. Tammer, J. Penman, S. Kent and A. G. Paolini. Calorie restriction at increasing levels leads to augmented concentrations of corticosterone and decreasing concentrations of testosterone in rats. Nutrition research 2010; 30:366-373.
26. M. W. Hamrick, C. Pennington, D. Newton, D. Xie and C. Isales. Leptin deficiency produces contrasting phenotypes in bones of the limb and spine. Bone 2004; 34:376-383.
27. N. M. Thompson, D. A. Gill, R. Davies, N. Loveridge, P. A. Houston, I. C. Robinson and T. Wells. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor. Endocrinology 2004; 145:234-242.
28. M. A. Bredella, M. Torriani, R. H. Ghomi, B. J. Thomas, D. J. Brick, A. V. Gerweck, C. J. Rosen, A. Klibanski and K. K. Miller. Vertebral Bone Marrow Fat Is Positively Associated With Visceral Fat and Inversely Associated With IGF-1 in Obese Women. Obesity (Silver Spring) 2011;
29. G. W. Li, Z. Xu, Q. W. Chen, S. X. Chang, Y. N. Tian and J. Z. Fan. The temporal characterization of marrow lipids and adipocytes in a rabbit model of glucocorticoid-induced osteoporosis. Skeletal Radiol 2013; 42:1235-1244.
30. T. Thomas, F. Gori, S. Khosla, M. D. Jensen, B. Burguera and B. L. Riggs. Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. Endocrinology 1999; 140:1630-1638.
31. E. L. Scheller, J. Song, M. I. Dishowitz, F. N. Soki, K. D. Hankenson and P. H. Krebsbach. Leptin functions peripherally to regulate differentiation of mesenchymal progenitor cells. Stem Cells 2009; 28:1071-1080.
32. M. W. Hamrick, M. A. Della-Fera, Y. H. Choi, C. Pennington, D. Hartzell and C. A. Baile. Leptin treatment induces loss of bone marrow adipocytes and increases bone formation in leptin-deficient ob/ob mice. J Bone Miner Res 2005; 20:994-1001.
33. K. J. Motyl and L. R. McCabe. Leptin treatment prevents type I diabetic marrow adiposity but not bone loss in mice. J Cell Physiol 2009; 218:376-384.
34. M. W. Hamrick, M. A. Della Fera, Y. H. Choi, D. Hartzell, C. Pennington and C. A. Baile. Injections of leptin into rat ventromedial hypothalamus increase adipocyte apoptosis in peripheral fat and in bone marrow. Cell Tissue Res 2007; 327:133-141.

35. S. Takeda, F. Elefteriou, R. Levasseur, X. Liu, L. Zhao, K. L. Parker, D. Armstrong, P. Ducy and G. Karsenty. Leptin regulates bone formation via the sympathetic nervous system. Cell 2002; 111:305-317.
36. P. K. Fazeli, M. A. Bredella, M. Misra, E. Meenaghan, C. J. Rosen, D. R. Clemmons, A. Breggia, K. K. Miller and A. Klibanski. Preadipocyte factor-1 is associated with marrow adiposity and bone mineral density in women with anorexia nervosa. J Clin Endocrinol Metab 2010; 95:407-413.
37. S. van Harten and L. A. Cardoso. Feed restriction and genetic selection on the expression and activity of metabolism regulatory enzymes in rabbits. Animal 2010; 4:1873-1883.
38. A. M. Almeida, S. van Harten, A. Campos, A. V. Coelho and L. A. Cardoso. The effect of weight loss on protein profiles of gastrocnemius muscle in rabbits: a study using 1D electrophoresis and peptide mass fingerprinting. J Anim Physiol Anim Nutr (Berl) 2010; 94:174-185.
39. W. P. Cawthorn, A. J. Bree, Y. Yao, B. Du, N. Hemati, G. Martinez-Santibanez and O. A. MacDougald. Wnt6, Wnt10a and Wnt10b inhibit adipogenesis and stimulate osteoblastogenesis through a beta-catenin-dependent mechanism. Bone 2012; 50:477-489.
40. B. Du, W. P. Cawthorn, A. Su, C. R. Doucette, Y. Yao, N. Hemati, S. Kampert, C. McCoin, D. T. Broome, C. J. Rosen, G. Yang and O. A. MacDougald. The transcription factor paired-related homeobox 1 (Prrx1) inhibits adipogenesis by activating transforming growth factor-beta (TGFbeta) signaling. J Biol Chem 2013; 288:3036-3047.
41. J. Folch, M. Lees and G. H. Sloane Stanley. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957; 226:497-509.
42. S. D. Parlee, S. I. Lentz, H. Mori and O. A. MacDougald. Quantifying size and number of adipocytes in adipose tissue. Methods in Enzymology 2014; 537:93-122.
43. E. L. Scheller, C. R. Doucette, B. S. Learman, W. P. Cawthorn, S. Khandaker, B. Schell, B. Wu, S. Y. Ding, M. A. Bredella, P. K. Fazeli, B. Khoury, K. J. Jepsen, P. F. Pilch, A. Klibanski, C. J. Rosen and O. A. MacDougald. Region-specific variation in the properties of skeletal adipocytes reveals regulated and constitutive marrow adipose tissues. Nat Commun 2015; 6:7808.
44. O. A. MacDougald, C. S. Hwang, H. Fan and M. D. Lane. Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. Proc Natl Acad Sci U S A 1995; 92:9034-9037.
45. R. Saladin, P. De Vos, M. Guerre-Millo, A. Leturque, J. Girard, B. Staels and J. Auwerx. Transient increase in obese gene expression after food intake or insulin administration. Nature 1995; 377:527-529.
46. Y. Zhang, M. Matheny, S. Zolotukhin, N. Tumer and P. J. Scarpace. Regulation of adiponectin and leptin gene expression in white and brown adipose tissues: influence of beta3-adrenergic agonists, retinoic acid, leptin and fasting. Biochimica et Biophysica Acta 2002; 1584:115-122.
47. E. Arvidsson, N. Viguerie, I. Andersson, C. Verdich, D. Langin and P. Arner. Effects of different hypocaloric diets on protein secretion from adipose tissue of obese women. Diabetes 2004; 53:1966-1971.
48. T. P. Combs, A. H. Berg, M. W. Rajala, S. Klebanov, P. Iyengar, J. C. Jimenez-Chillaron, M. E. Patti, S. L. Klein, R. S. Weinstein and P. E. Scherer. Sexual differentiation, pregnancy, calorie restriction, and aging affect the adipocyte-specific secretory protein adiponectin. Diabetes 2003; 52:268-276.
49. M. Garaulet, N. Viguerie, S. Porubsky, E. Klimcakova, K. Clement, D. Langin and V. Stich. Adiponectin gene expression and plasma values in obese women during very-low-calorie diet. Relationship with cardiovascular risk factors and insulin resistance. The Journal of clinical endocrinology and metabolism 2004; 89:756-760.
50. C. J. Behre, A. Gummesson, M. Jernas, T. C. Lystig, B. Fagerberg, B. Carlsson and L. M. Carlsson. Dissociation between adipose tissue expression and serum levels of adiponectin during and after diet-induced weight loss in obese subjects with and without the metabolic syndrome. Metabolism: clinical and experimental 2007; 56:1022-1028.

51. L. Qiang, H. Wang and S. R. Farmer. Adiponectin secretion is regulated by SIRT1 and the endoplasmic reticulum oxidoreductase Ero1-L alpha. Molecular and cellular biology 2007; 27:4698-4707.
52. Z. V. Wang, T. D. Schraw, J. Y. Kim, T. Khan, M. W. Rajala, A. Follenzi and P. E. Scherer. Secretion of the adipocyte-specific secretory protein adiponectin critically depends on thiol-mediated protein retention. Molecular and cellular biology 2007; 27:3716-3731.
53. B. P. Carson, J. M. Del Bas, J. M. Moreno-Navarrete, J. M. Fernandez-Real and S. Mora. The rab11 effector protein FIP1 regulates adiponectin trafficking and secretion. PLoS One 2013; 8:e74687.
54. Z. Kovacova, M. Vitkova, M. Kovacikova, E. Klimcakova, M. Bajzova, Z. Hnevkovska, L. Rossmeislova, V. Stich, D. Langin and J. Polak. Secretion of adiponectin multimeric complexes from adipose tissue explants is not modified by very low calorie diet. Eur J Endocrinol 2009; 160:585-592.
55. M. Yoneda, T. Iwasaki, K. Fujita, H. Kirikoshi, M. Inamori, Y. Nozaki, S. Maeyama, K. Wada, S. Saito, Y. Terauchi and A. Nakajima. Hypoadiponectinemia plays a crucial role in the development of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus independent of visceral adipose tissue. Alcoholism, Clinical and Experimental Research 2007; 31:S15-21.
56. M. Zhu, J. Miura, L. X. Lu, M. Bernier, R. DeCabo, M. A. Lane, G. S. Roth and D. K. Ingram. Circulating adiponectin levels increase in rats on caloric restriction: the potential for insulin sensitization. Experimental gerontology 2004; 39:1049-1059.
57. K. A. Varady, L. Tussing, S. Bhutani and C. L. Braunschweig. Degree of weight loss required to improve adipokine concentrations and decrease fat cell size in severely obese women. Metabolism 2009; 58:1096-1101.
58. L. M. Nogueira, J. A. Lavigne, G. V. Chandramouli, H. Lui, J. C. Barrett and S. D. Hursting. Dose-dependent effects of calorie restriction on gene expression, metabolism, and tumor progression are partially mediated by insulin-like growth factor-1. Cancer medicine 2012; 1:275-288.
59. S. Judex, G. R. Wohl, R. B. Wolff, W. Leng, A. M. Gillis and R. F. Zernicke. Dietary fish oil supplementation adversely affects cortical bone morphology and biomechanics in growing rabbits. Calcif Tissue Int 2000; 66:443-448.
60. E. A. Lawson and A. Klibanski. Endocrine abnormalities in anorexia nervosa. Nature Clinical Practice Endocrinology & Metabolism 2008; 4:407-414.
61. F. Moradi, M. R. V. Mahdavi, A. Ahmadiani, M. Rogani, A. R. Delshad, S. Mojarab and T. Altiraihi. Social instability, food deprivation and food inequality can promote accumulation of lipofuscin and induced apoptosis in hepatocytes. World Applied Sciences Journal 2012; 20:310-318.
62. A. A. Dietz and B. Steinberg. Chemistry of bone marrow. VIII. Composition of rabbit bone marrow in inanition. Arch Biochem Biophys 1953; 45:10-20.
63. P. Cohen and F. H. Gardner. Effect of Massive Triamcinolone Administration in Blunting the Erythropoietic Response to Phenylhydrazine Hemolysis. J Lab Clin Med 1965; 65:88-101.
64. B. C. Vande Berg, J. Malghem, F. E. Lecouvet, M. Lambert and B. E. Maldague. Distribution of serouslike bone marrow changes in the lower limbs of patients with anorexia nervosa: predominant involvement of the distal extremities. AJR Am J Roentgenol 1996; 166:621-625.
65. C. Thouzeau, C. Duchamp and Y. Handrich. Energy metabolism and body temperature of barn owls fasting in the cold. Physiol Biochem Zool 1999; 72:170-178.
66. K. A. Varady, C. A. Allister, D. J. Roohk and M. K. Hellerstein. Improvements in body fat distribution and circulating adiponectin by alternate-day fasting versus calorie restriction. The Journal of Nutritional Biochemistry 2010; 21:188-195.
67. J. I. Fenton, N. P. Nunez, S. Yakar, S. N. Perkins, N. G. Hord and S. D. Hursting. Diet-induced adiposity alters the serum profile of inflammation in C57BL/6N mice as measured by antibody array. Diabetes Obes Metab 2009; 11:343-354.
68. X. Li, M. B. Cope, M. S. Johnson, D. L. Smith, Jr. and T. R. Nagy. Mild calorie restriction induces fat accumulation in female C57BL/6J mice. Obesity 2010; 18:456-462.

69. S. Hempenstall, L. Picchio, S. E. Mitchell, J. R. Speakman and C. Selman. The impact of acute caloric restriction on the metabolic phenotype in male C57BL/6 and DBA/2 mice. Mechanisms of Ageing and Development 2010; 131:111-118.
70. E. Kurki, J. Shi, E. Martonen, P. Finckenberg and E. Mervaala. Distinct effects of calorie restriction on adipose tissue cytokine and angiogenesis profiles in obese and lean mice. Nutrition and Metabolism 2012; 9:64.
71. H. Shi, A. D. Strader, S. C. Woods and R. J. Seeley. Sexually dimorphic responses to fat loss after caloric restriction or surgical lipectomy. American journal of physiology. Endocrinology and metabolism 2007; 293:E316-326.
72. T. Magwere, T. Chapman and L. Partridge. Sex differences in the effect of dietary restriction on life span and mortality rates in female and male Drosophila melanogaster. J Gerontol A Biol Sci Med Sci 2004; 59:3-9.
73. B. Martin, M. Pearson, L. Kebejian, E. Golden, A. Keselman, M. Bender, O. Carlson, J. Egan, B. Ladenheim, J. L. Cadet, K. G. Becker, W. Wood, K. Duffy, P. Vinayakumar, S. Maudsley and M. P. Mattson. Sex-dependent metabolic, neuroendocrine, and cognitive responses to dietary energy restriction and excess. Endocrinology 2007; 148:4318-4333.
74. C. Miersch and F. Doring. Sex differences in body composition, fat storage, and gene expression profile in Caenorhabditis elegans in response to dietary restriction. Physiol Genomics 2013; 45:539-551.
75. R. Leenen, K. van der Kooy, A. Droop, J. C. Seidell, P. Deurenberg, J. A. Weststrate and J. G. Hautvast. Visceral fat loss measured by magnetic resonance imaging in relation to changes in serum lipid levels of obese men and women. Arterioscler Thromb 1993; 13:487-494.
76. A. Wirth and B. Steinmetz. Gender differences in changes in subcutaneous and intra-abdominal fat during weight reduction: an ultrasound study. Obes Res 1998; 6:393-399.
77. B. Parker, M. Noakes, N. Luscombe and P. Clifton. Effect of a high-protein, high-monounsaturated fat weight loss diet on glycemic control and lipid levels in type 2 diabetes. Diabetes Care 2002; 25:425-430.
78. E. M. Evans, M. C. Mojtahedi, M. P. Thorpe, R. J. Valentine, P. M. Kris-Etherton and D. K. Layman. Effects of protein intake and gender on body composition changes: a randomized clinical weight loss trial. Nutr Metab (Lond) 2012; 9:55.
79. I. Janssen and R. Ross. Effects of sex on the change in visceral, subcutaneous adipose tissue and skeletal muscle in response to weight loss. Int J Obes Relat Metab Disord 1999; 23:1035-1046.
80. D. L. Ballor and E. T. Poehlman. Exercise-training enhances fat-free mass preservation during diet-induced weight loss: a meta-analytical finding. Int J Obes Relat Metab Disord 1994; 18:35-40.
81. L. Fontana, L. Partridge and V. D. Longo. Extending healthy life span--from yeast to humans. Science 2010; 328:321-326.
82. S. M. Bartell, S. Rayalam, S. Ambati, D. R. Gaddam, D. L. Hartzell, M. Hamrick, J. X. She, M. A. Della-Fera and C. A. Baile. Central (ICV) leptin injection increases bone formation, bone mineral density, muscle mass, serum IGF-1, and the expression of osteogenic genes in leptin-deficient ob/ob mice. Journal of Bone and Mineral Research 2011; 26:1710-1720.
83. S. Ambati, Q. Li, S. Rayalam, D. L. Hartzell, M. A. Della-Fera, M. W. Hamrick and C. A. Baile. Central leptin versus ghrelin: effects on bone marrow adiposity and gene expression. Endocrine 2010; 37:115-123.
84. M. Rosenbaum and R. L. Leibel. 20 years of leptin: role of leptin in energy homeostasis in humans. J Endocrinol 2014; 223:T83-96.
85. D. A. Ainslie, M. J. Morris, G. Wittert, H. Turnbull, J. Proietto and A. W. Thorburn. Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y. Int J Obes Relat Metab Disord 2001; 25:1680-1688.
86. N. Sharma, C. M. Castorena and G. D. Cartee. Greater insulin sensitivity in calorie restricted rats occurs with unaltered circulating levels of several important myokines and cytokines. Nutr Metab (Lond) 2012; 9:90.

87. N. M. Daoud, K. F. Mahrous and O. H. Ezzo. Feed restriction as a biostimulant of the production of oocyte, their quality and GDF-9 gene expression in rabbit oocytes. Anim Reprod Sci 2012; 136:121-127.
88. D. T. Villareal, L. Fontana, S. K. Das, L. Redman, S. R. Smith, E. Saltzman, C. Bales, J. Rochon, C. Pieper, M. Huang, M. Lewis, A. V. Schwartz and C. S. Group. Effect of Two-Year Caloric Restriction on Bone Metabolism and Bone Mineral Density in Non-Obese Younger Adults: A Randomized Clinical Trial. J Bone Miner Res 2015;
89. S. Trubowitz and A. Bathija. Cell size and plamitate-1-14c turnover of rabbit marrow fat. Blood 1977; 49:599-605.
90. J. M. Gimble, C. E. Robinson, X. Wu and K. A. Kelly. The function of adipocytes in the bone marrow stroma: an update. Bone 1996; 19:421-428.
91. A. C. Maurin, P. M. Chavassieux, L. Frappart, P. D. Delmas, C. M. Serre and P. J. Meunier. Influence of mature adipocytes on osteoblast proliferation in human primary cocultures. Bone 2000; 26:485-489.
92. M. E. Nuttall and J. M. Gimble. Is there a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis? Bone 2000; 27:177-184.
93. L. F. Liu, W. J. Shen, Z. H. Zhang, L. J. Wang and F. B. Kraemer. Adipocytes decrease Runx2 expression in osteoblastic cells: roles of PPARgamma and adiponectin. J Cell Physiol 2010; 225:837-845.
94. A. V. Schwartz, S. Sigurdsson, T. F. Hue, T. F. Lang, T. B. Harris, C. J. Rosen, E. Vittinghoff, K. Siggeirsdottir, G. Sigurdsson, D. Oskarsdottir, K. Shet, L. Palermo, V. Gudnason and X. Li. Vertebral Bone Marrow Fat Associated With Lower Trabecular BMD and Prevalent Vertebral Fracture in Older Adults. J Clin Endocrinol Metab 2013; 98:2294-2300.
95. E. L. Scheller and C. J. Rosen. What's the matter with MAT? Marrow adipose tissue, metabolism, and skeletal health. Annals of the New York Academy of Sciences 2014; 1311:14-30.
96. C. J. Rosen, C. Ackert-Bicknell, J. P. Rodriguez and A. M. Pino. Marrow fat and the bone microenvironment: developmental, functional, and pathological implications. Crit Rev Eukaryot Gene Expr 2009; 19:109-124.
97. S. Botolin and L. R. McCabe. Inhibition of PPARgamma prevents type I diabetic bone marrow adiposity but not bone loss. Journal of cellular physiology 2006; 209:967-976.
98. U. T. Iwaniec and R. T. Turner. Failure to generate bone marrow adipocytes does not protect mice from ovariectomy-induced osteopenia. Bone 2013; 53:145-153.
99. M. Misra and A. Klibanski. Anorexia nervosa and osteoporosis. Reviews in endocrine & metabolic disorders 2006; 7:91-99.

FIGURE LEGENDS

Figure 1 – Circulating adiponectin does not increase during moderate CR in rabbits. Adult male rabbits were fed a control or 30% CR diet from 15 to 22 weeks of age, as described in *Materials and Methods*. **(A)** Body mass was measured weekly and is presented relative to body mass at 15 weeks of age. **(B-G)** After 7 weeks on CR or control diet, rabbits were euthanized and fat pads, serum, WAT, and MAT were isolated. **(B)** WAT masses were recorded at necropsy. **(C)** Serum leptin concentrations, as determined by ELISA. **(D-E)** Total RNA was isolated from iWAT (D) and tibial MAT (E). Expression of the indicated transcripts was determined by qPCR and normalized to *Ppia* expression. **(F)** Immunoblot of total adiponectin in sera from 22-week-old rabbits. **(E)** Densitometry was used to quantify serum adiponectin from (F). Data in (A) are reported as mean ± SD of 5 control and 6 CR rabbits. All other graphs are box and whisker plots. Statistically significant differences between control and CR rabbits are indicated as follows: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Figure 2 – Bone marrow adiposity does not increase during moderate CR in rabbits. Control and moderate CR rabbits (*described in Figure 1*) were euthanized and humeri, radii, ulnae, tibiae, and femurs were removed. **(A, B)** Representative images of bisected humeri (A) or femurs (B); scale bar, 1 cm. **(C-E)** Whole, intact BM was isolated from one femur of each rabbit, followed by isolation of total RNA (C), protein (D), or lipid (E). In (C), expression of the indicated transcripts was determined by qPCR and normalized to *Ppia* expression. In (D), perilipin A expression was determined by immunoblotting, with alpha-tubulin used as a loading control. In (E), total triacylglycerol was isolated by TLC and the concentration determined by an enzymatic assay. **(F)** Schematic showing the sites from which each sample of RM or MAT was isolated. **(G)** Adipocyte size distribution in the indicated RM, MAT, or WAT samples was determined by quantitative histomorphometry; median adipocyte size was then determined. Data in (C) and (E) represent 5 control and 6 CR rabbits, and are shown as box and whisker plots. In (C), statistically significant differences between control and CR rabbits are indicated by * ($P < 0.05$). Data in

(G) are reported as mean ±SD of the following numbers of samples: femur RM - 5 control, 6 CR; humerus RM - 5 control, 6 CR; tibia RM - 3 control, 5 CR; femur MAT - 5 control, 5 CR; humerus MAT - 4 control, 6 CR; tibia MAT - 5 control, 6 CR; radius MAT - 5 control, 6 CR; ulna MAT - 4 control, 6 CR; gWAT, iWAT, or pWAT - 5 control and 6 CR. In (G), statistically significant differences in median adipocyte size were assessed by two-way ANOVA. Lower-case letters indicate statistical significance for the control tissues, while upper-case letters are used for the CR tissues; samples that do not share a common letter are significantly different from each other ($P < 0.05$). Significant effects of CR, within each tissue type, are indicated in Supplemental Figure 2.

Figure 3 – Circulating adiponectin does not increase during extensive CR in rabbits. Male rabbits were fed *ad libitum* (control) or at 40% of *ad libitum* food intake (CR) from 6 to 13 weeks of age. **(A)** Body mass was measured weekly. **(B-F)** After 7 weeks of CR or control diet, rabbits were euthanized and fat pads, serum, and bones were isolated. **(B)** WAT masses were recorded at necropsy. **(C)** Serum leptin concentrations, as determined by ELISA. **(D)** Lengths of the indicated bones were recorded at necropsy. **(E)** Immunoblot of total adiponectin in sera from 13-week-old rabbits. **(F)** Densitometry was used to quantify serum adiponectin from (E). Data in (A) are reported as mean ± SD of 6 control and 6 CR rabbits. All other graphs are box and whisker plots. Statistically significant differences between control and CR rabbits are indicated as described for Figure 1.

Figure 4 – BM adipocyte size is decreased during extensive CR in rabbits. Control and extensive CR rabbits (*described in Figure 3*) were euthanized and humeri, radii, ulnae, tibiae, and femurs were removed. **(A, B)** Representative images of bisected humeri (A) or tibiae (B); scale bar, 1 cm. **(C)** Median adipocyte size in the indicated RM, MAT, or WAT samples was determined by quantitative histomorphometry, as described for Figure 2G. Because of the extent of MAT and WAT loss, from some CR rabbits we were unable to detect any MAT for further analysis. Thus, data in (C) are reported as mean

866 ±SD of the following numbers of samples: femur RM - 6 control, 4 CR; humerus RM - 5 control, 4 CR;
867 tibia RM - 5 control, 4 CR; femur MAT - 4 control, 2 CR; humerus MAT - 4 control, 4 CR; tibia MAT - 6
868 control, 4 CR; radius MAT - 5 control, 5 CR; ulna MAT - 6 control, 5 CR; gWAT - 5 control, 3 CR;
869 iWAT - 6 control, 1 CR; pWAT, 5 control, 1 CR. Because femur MAT for the CR group is from only two
870 rabbits, the SD of this group represents 0.7071 times the range of the two data points. Significant
871 differences are indicated as described for Figure 2G. Data for iWAT and pWAT are from only one CR
872 rabbit, and data for femur MAT are from only two CR rabbits; hence, ANOVA could not be used to
873 assess statistical significance for these samples owing to uncertainty over the normality of data
874 distribution.
875
876 **Figure 5 – In female mice CR increases MAT without decreasing circulating leptin.** Male and female
877 C57BL/6J mice were fed *ad libitum* or a 30% CR diet from 9-15 weeks of age. **(A,B)** Tibiae from 15-
878 week-old mice were stained with osmium tetroxide followed by μCT analysis. **(A)** Representative μCT
879 scans of osmium tetroxide-stained tibiae. MAT appears as darker regions within each bone. **(B)** MAT
880 volume within each tibial region was determined from μCT scans. **(C,F)** Body composition of 15-week-
881 old live mice was determined by NMR. **(D,G)** Masses of the indicated tissues were recorded at necropsy.
882 **(E,H)** Blood was sampled from the lateral tail vein of 15-week-old live mice. Serum was isolated and
883 leptin concentrations were determined by ELISA. Data in (C-D) and (F-G) are reported as mean ±SD of
884 the following numbers of mice: male control, n = 6; male CR, n = 7; female control, n = 6; female CR, n
885 = 5. All other graphs are box and whisker plots. For each sex, statistically significant differences between
886 control and CR animals are reported as described for Figure 1.
887
888 **Figure 6 – MAT expansion during CR is associated with changes in circulating glucocorticoids.**
889 C57BL/6J mice (A,B) or New Zealand White rabbits (C-F) were fed control or CR diets, as described in
890 Figures 1-5. Blood was sampled at the end of the CR protocols and concentrations of total corticosterone
891 and cortisol were determined by ELISA. Data are presented as box and whisker plots. Within each group

(male mice; female mice; moderate CR rabbits; extensive CR rabbits) statistically significant differences between control and CR animals are reported as described for Figure 1.

Click here to download Table MAT expansion in CR - Table 1.docx

TABLES

	Control	CR	*p*-value
TV (mm^3)	35.91 ± 0.83	39.87 ± 3.59	0.352
BV (mm^3)	7.07 ± 3.32	7.05 ± 1.7	0.167
BVF (%)	27.1 ± 2.0	21.6 ± 1.0	**0.032**
Conn. Dens	9.62 ± 0.81	7.46 ± 0.47	**0.041**
SMI	0.61 ± 0.10	0.88 ± 0.17	0.211
Tb.N	2.60 ± 0.09	2.48 ± 0.05	0.271
Tb.Th	0.174 ± 0.01	0.170 ± 0.01	0.513
Tb.Sp	0.366 ± 0.02	0.40 ± 0.01	0.082
BMC (mg HA)	286.70 ± 15.14	231.49 ± 10.85	**0.014**

Table 1 – Characteristics of femoral heads of control and CR rabbits, as assessed by *μ*CT. Abbreviations are as follows: TV, trabecular volume; BV, bone volume; BVF, bone volume fraction; Conn. Dens, connectivity density; SMI, structure model index; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular spacing; BMC, bone mineral content; mg HA, milligrams of hydroxyapatite.

Figure 1 – Circulating adiponectin does not increase during moderate CR in rabbits

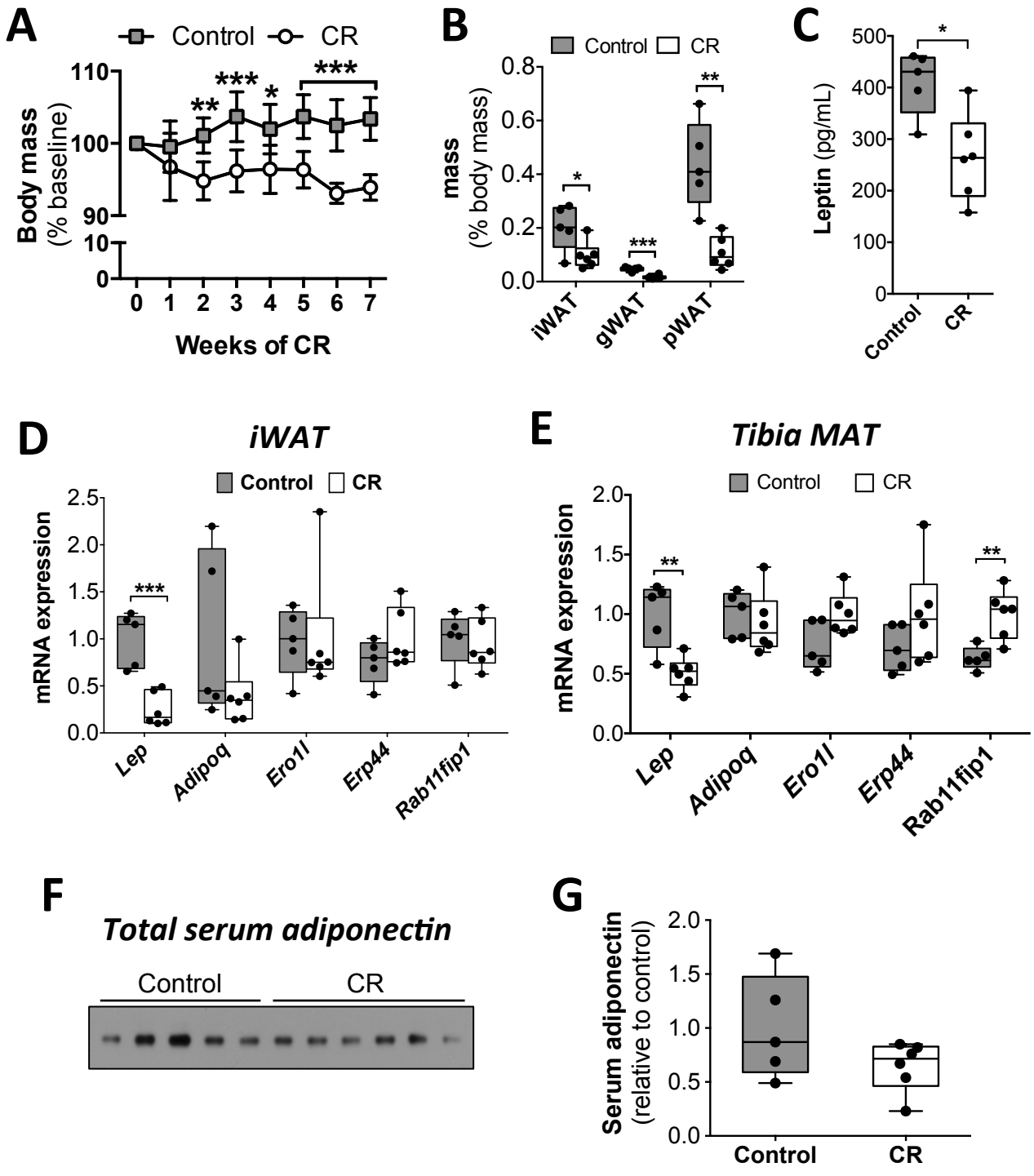


Figure 2 – Bone marrow adiposity does not increase during moderate CR in rabbits

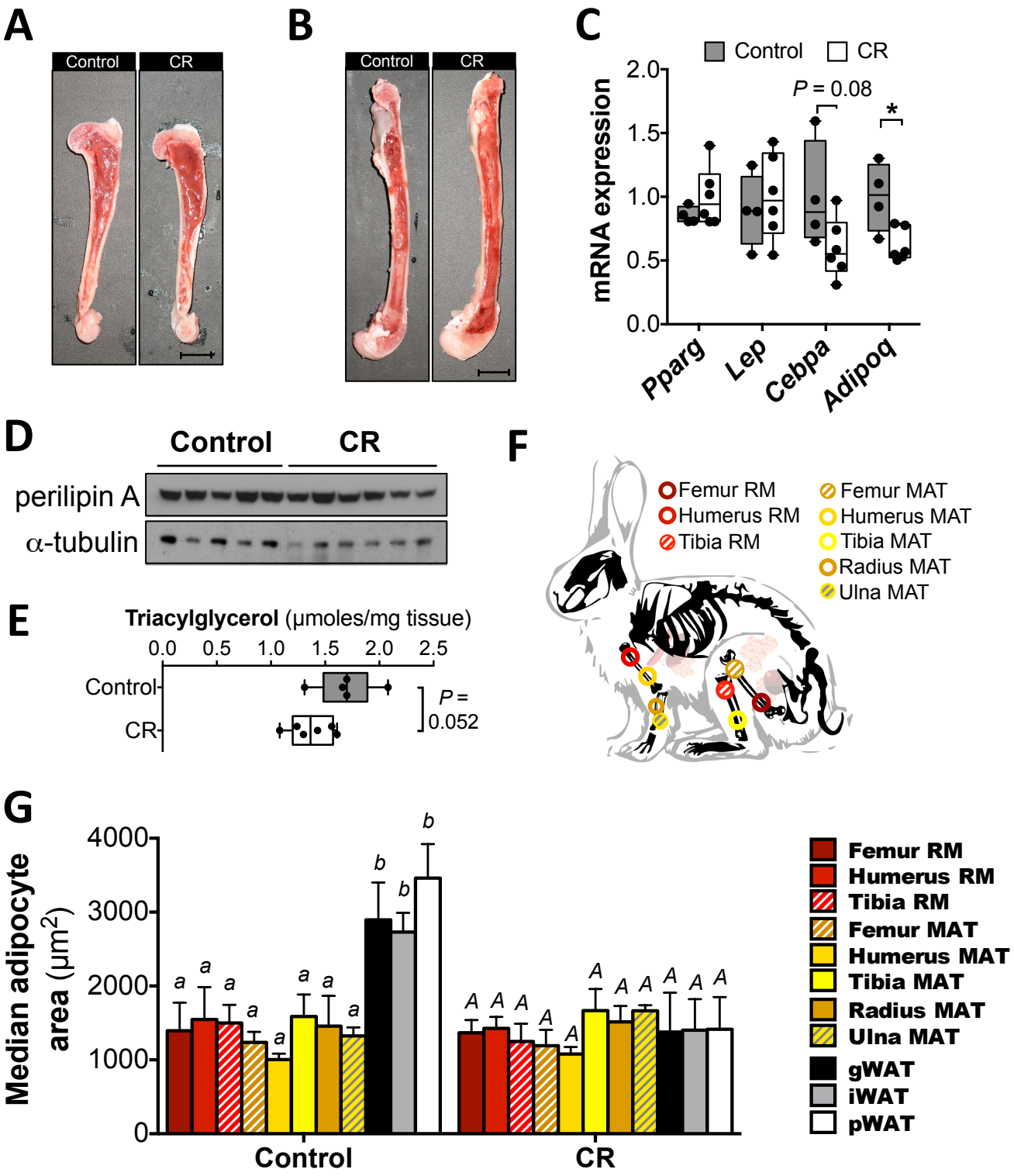


Figure 3 – Circulating adiponectin does not increase during extensive CR in rabbits

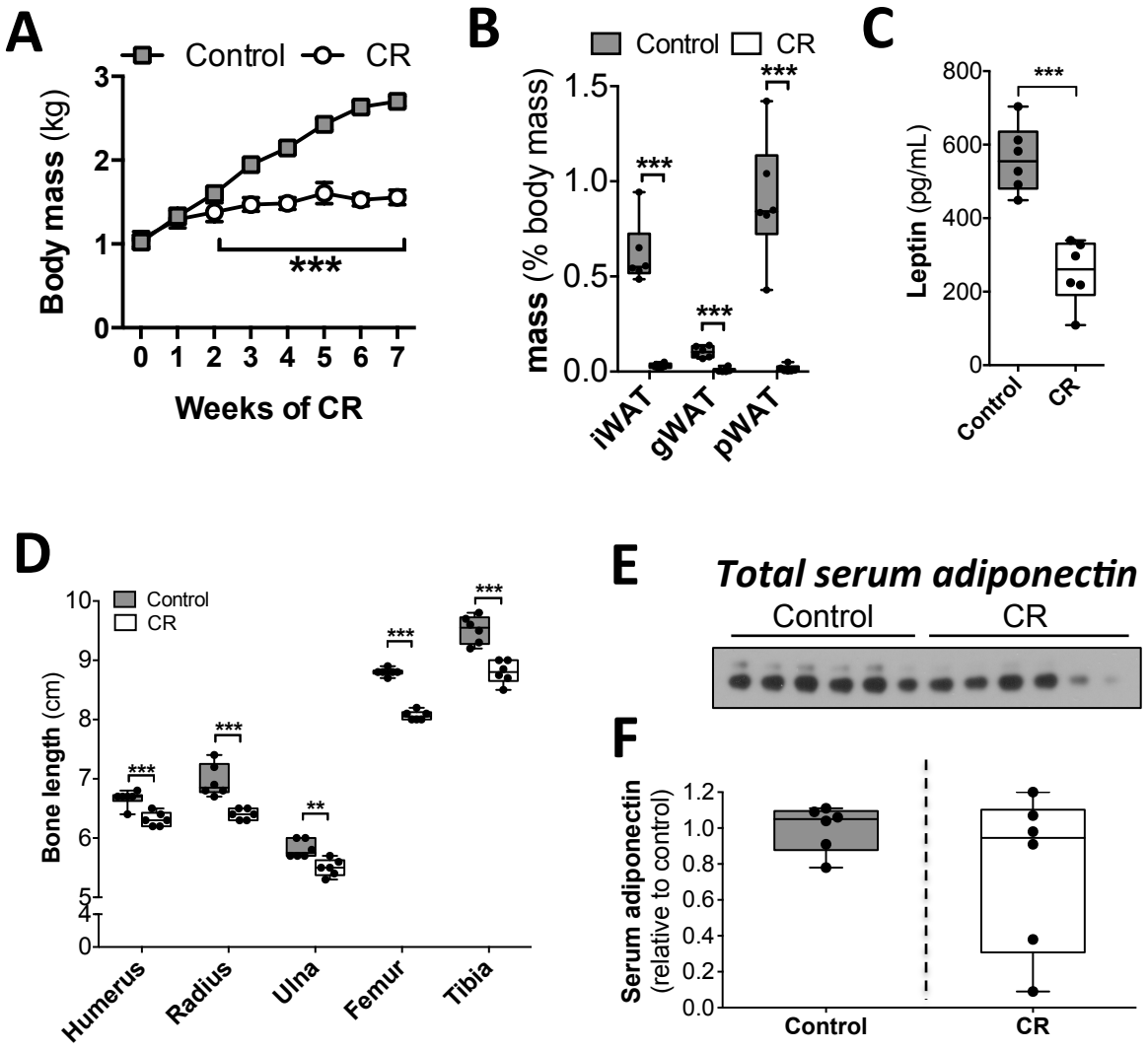


Figure 4 – BM adipocyte size is decreased during extensive CR in rabbits

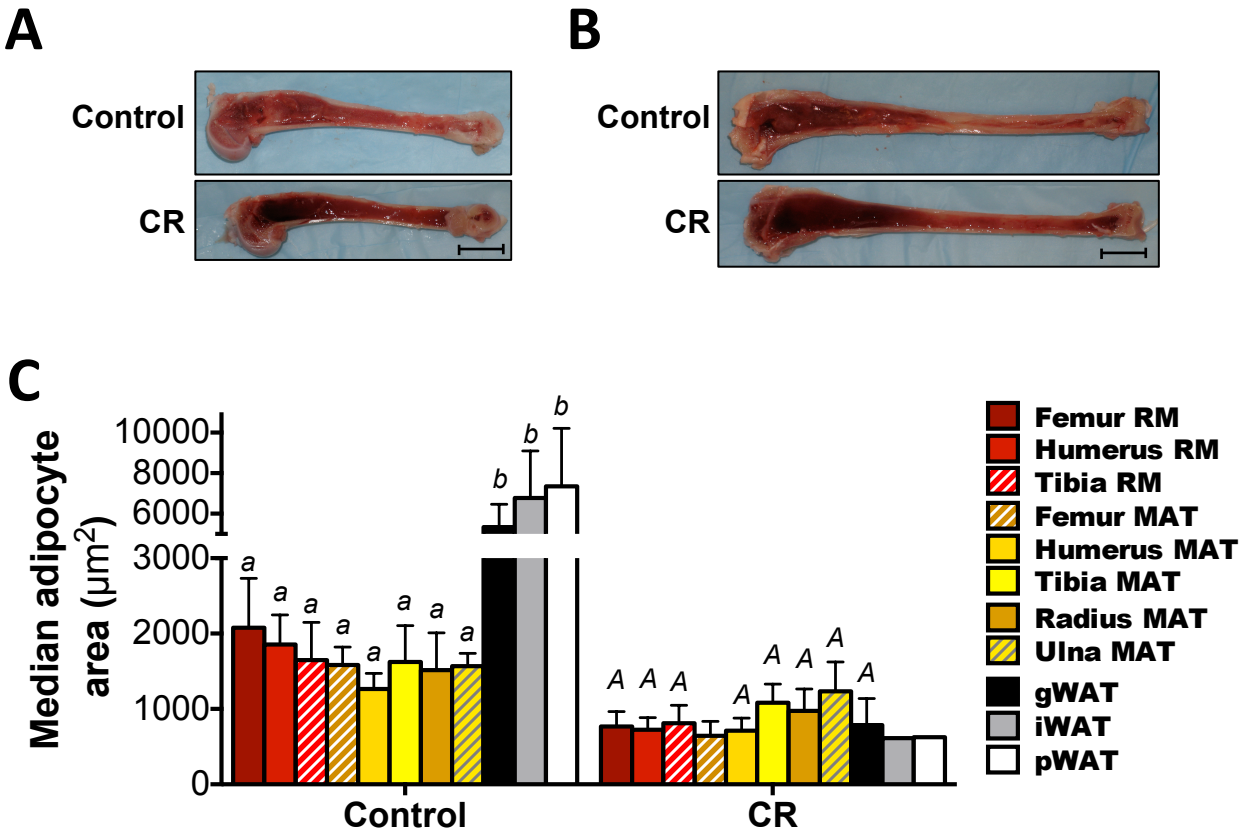


Figure 5 – In female mice CR increases MAT without decreasing circulating leptin

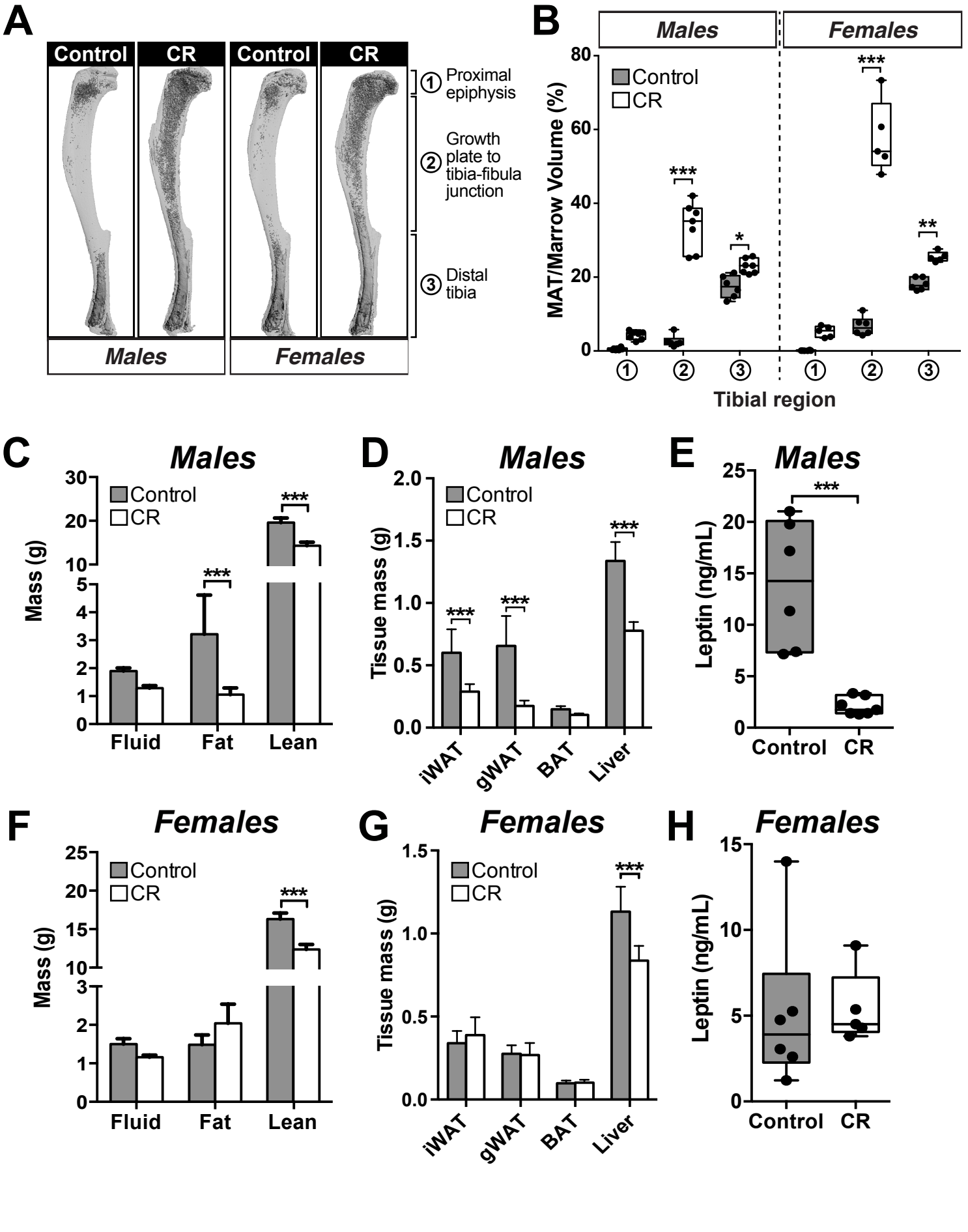
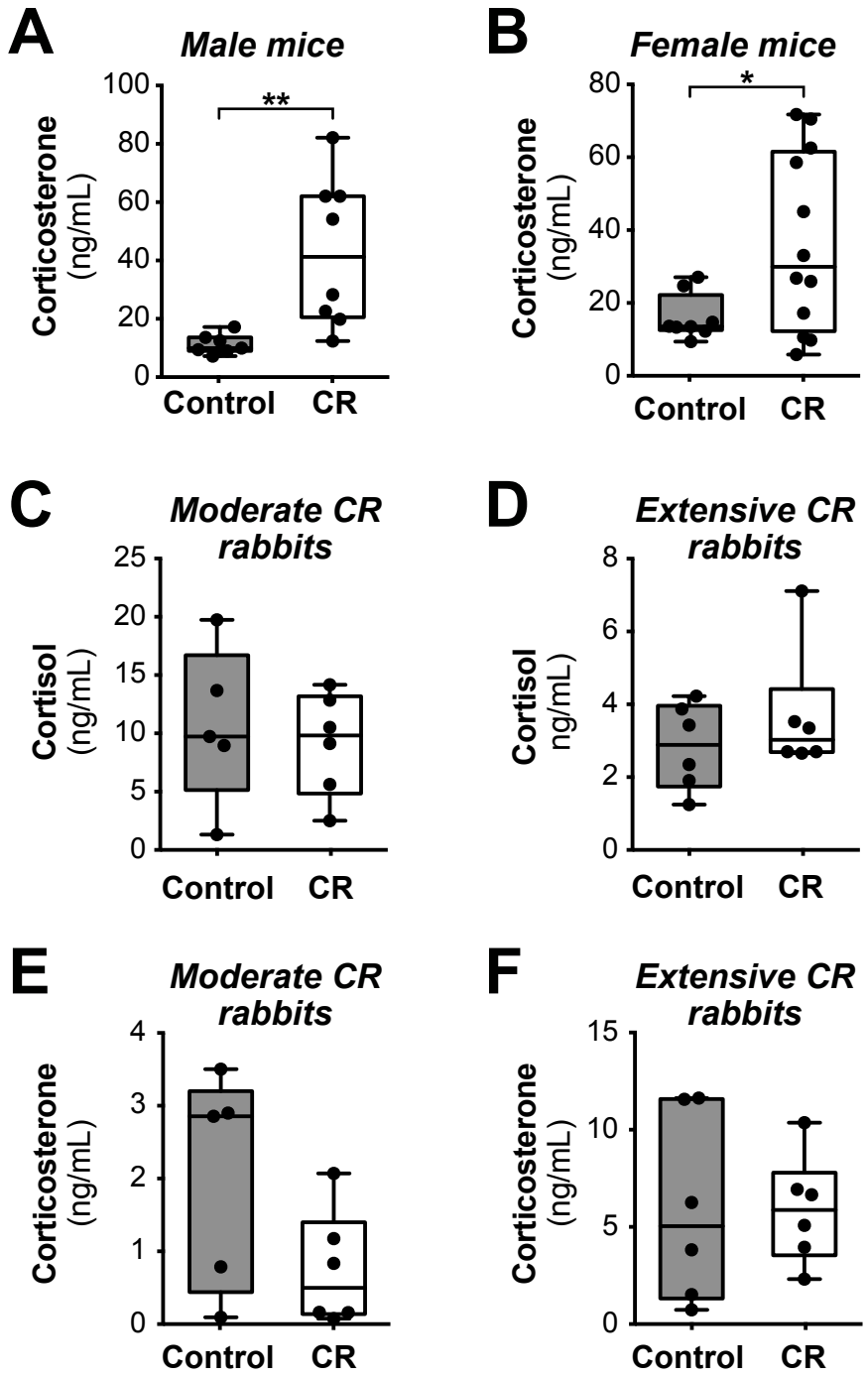
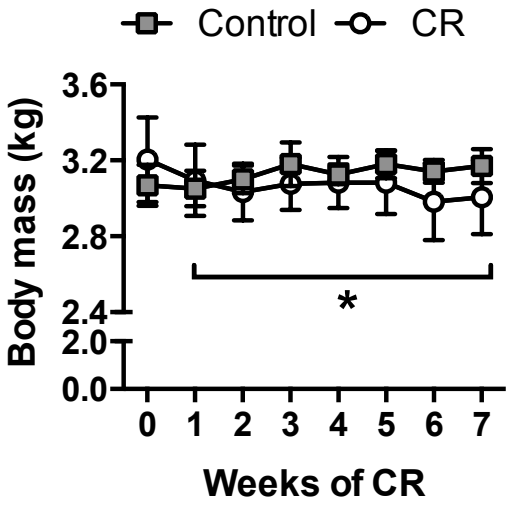


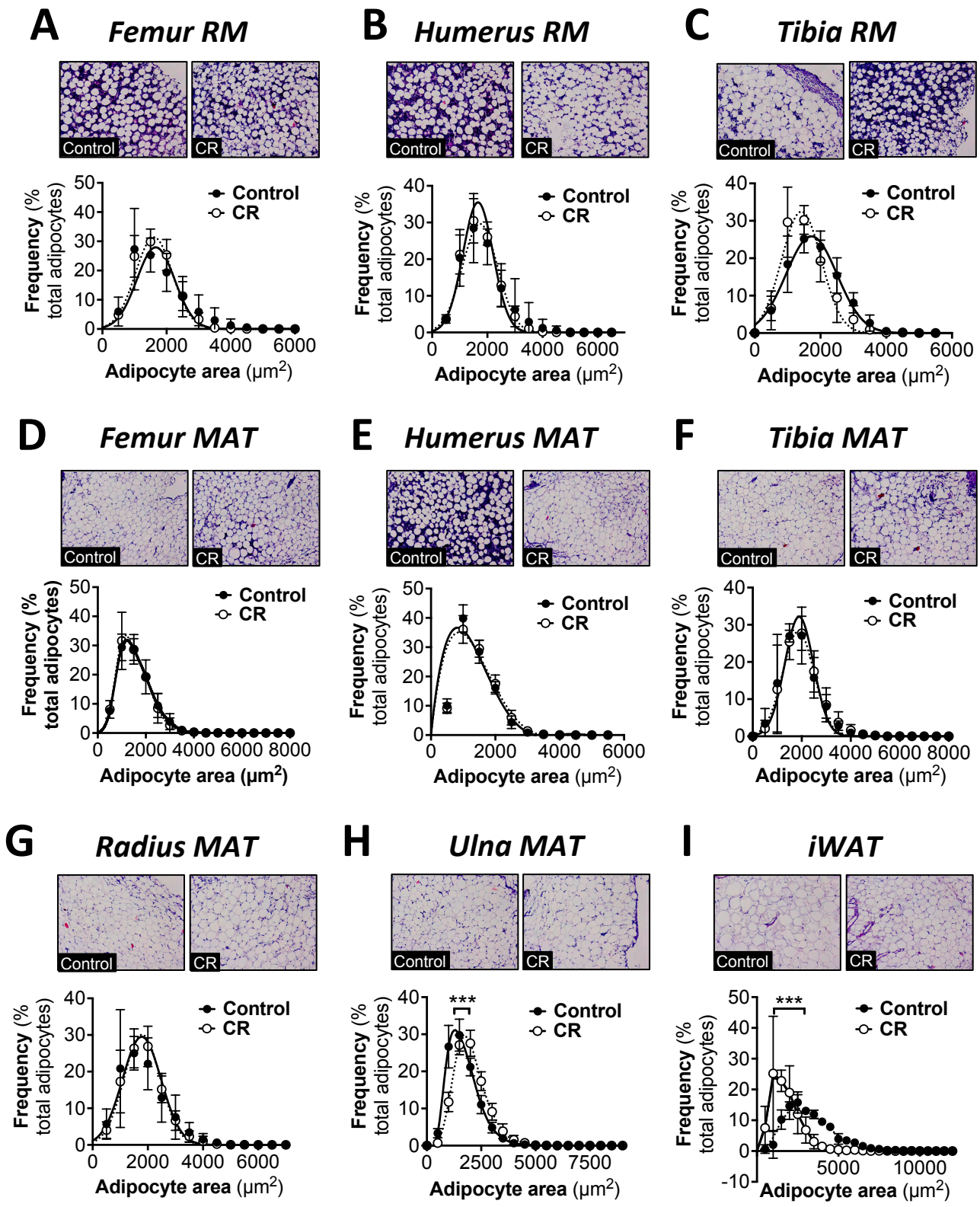
Figure 6 – MAT expansion during CR is associated with changes in circulating glucocorticoids



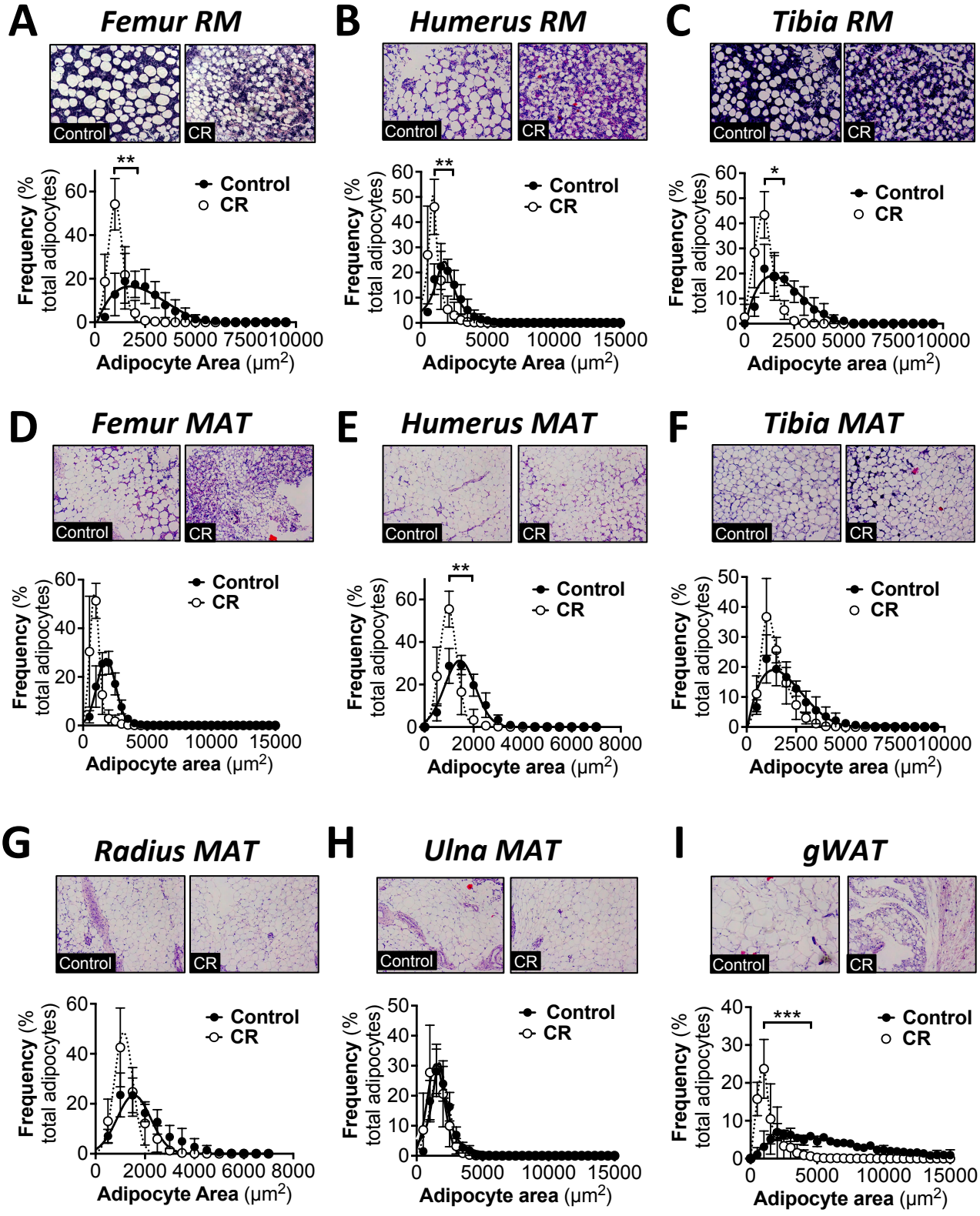
Supplemental Figure 1 – Body mass decreases during moderate CR in rabbits



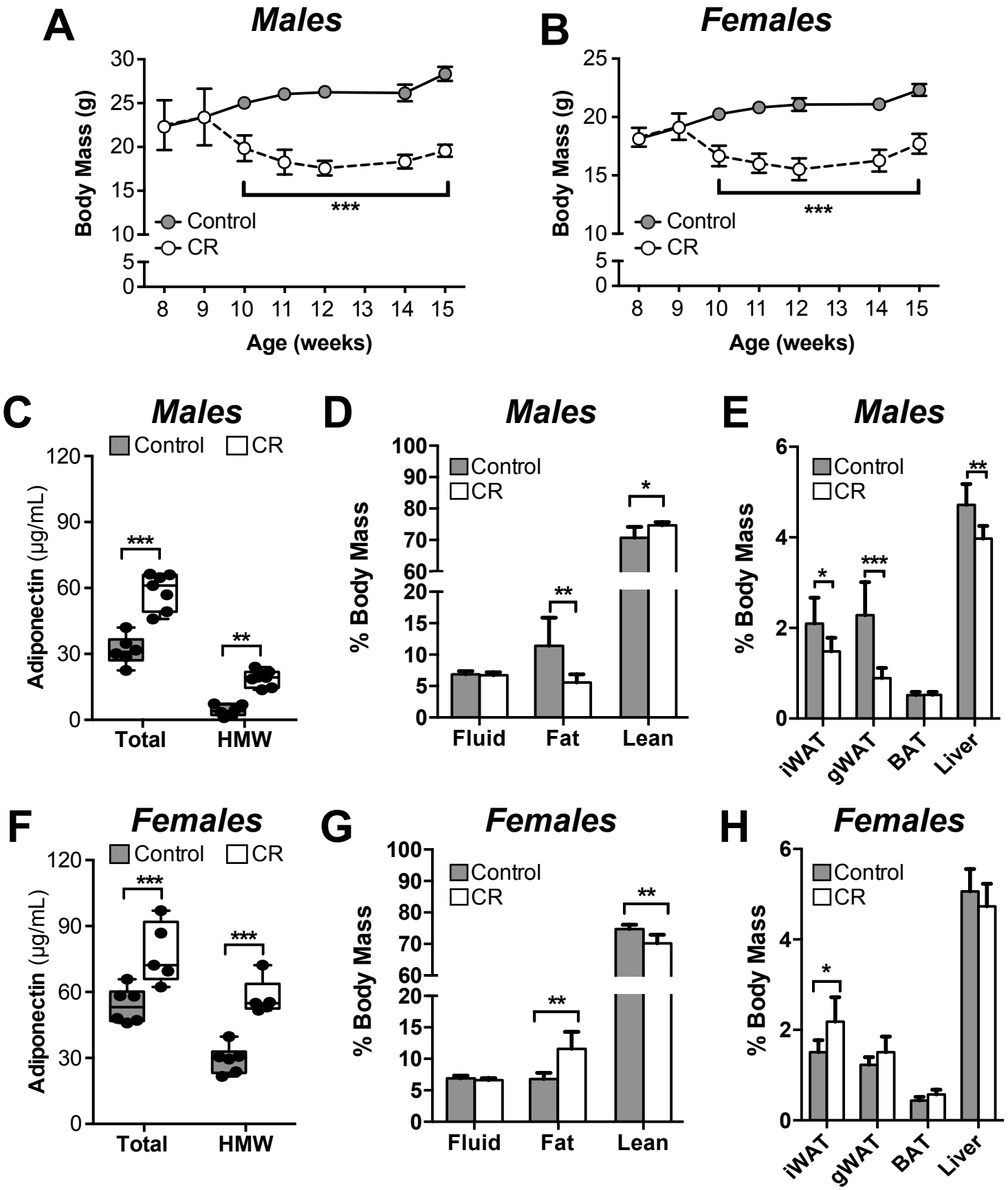
Supplemental Figure 2 – Effects of Moderate CR on adipocyte sizes in BM and iWAT



Supplemental Figure 3 – Effects of extensive CR on adipocyte sizes in BM and gWAT



Supplemental Figure 4 – Changes in body mass, body composition, tissue mass and circulating adiponectin during CR in mice



Supplemental Data – Figure legends

Supplemental Figure 1 – Body mass decreases during moderate CR in rabbits. Adult male rabbits were fed a control or 30% CR diet from 15 to 22 weeks of age, as described for Figure 1 and in the *Materials and Methods*. Body mass was measured weekly and is reported as mean ± SD of 5 control and 6 CR rabbits. Statistically significant differences between control and CR-fed rabbits were determined by 2-way ANOVA. The average body mass of control rabbits did not significantly differ to that of CR-fed rabbits at each time point; however, body mass of CR-fed rabbits was significantly lower at weeks 1-7 of CR than at baseline (week 0), whereas body mass of control-fed rabbits did not differ over the course of the study.

Supplemental Figure 2 – Effects of moderate CR on adipocyte sizes in BM and iWAT. RM, MAT, and WAT were sampled from the indicated depots and processed for histological analysis. Representative micrographs of H&E-stained sections of each tissue are shown. For each tissue, adipocyte sizes were quantified by histomorphometry. Corresponding graphs of adipocyte size distribution are shown, with the frequency of adipocytes within each size range presented as mean ± SD of the following number of rabbits: femur RM - 5 control, 6 CR; humerus RM - 5 control, 6 CR; tibia RM - 3 control, 5 CR; femur MAT - 5 control, 5 CR; humerus MAT - 4 control, 6 CR; tibia MAT - 5 control, 6 CR; radius MAT - 5 control, 6 CR; ulna MAT - 4 control, 6 CR; iWAT - 5 control, 6 CR. Significant differences in median adipocyte size between control and CR rabbits are indicated as follows: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Supplemental Figure 3 – Effects of extensive CR on adipocyte sizes in BM and gWAT. Samples were processed and data are presented as described for Supplemental Figure 1. For each tissue, data are presented as mean ± SD of the following numbers of rabbits: femur RM - 6 control, 4 CR; humerus RM - 5 control, 4 CR; tibia RM - 5 control, 4 CR; femur MAT - 4 control, 2 CR; humerus MAT - 4 control, 4 CR; tibia MAT - 6 control, 4 CR; radius MAT - 5 control, 5 CR; ulna MAT - 6 control, 5 CR; gWAT - 5 control, 3 CR. Because femur MAT for the CR group is from only two rabbits, the SD of this group represents 0.7071 times the range of the two data points. Significant differences in median adipocyte size between control and CR rabbits are indicated as described for Supplemental Figure 2. Data for femoral MAT is from only two CR rabbits, and therefore ANOVA could not be used to assess statistical significance for these samples owing to uncertainty over the normality of data distribution.

Supplemental Figure 4 – Changes in body mass, body composition, tissue mass and circulating adiponectin during CR in mice. Male and female C57BL/6J mice were fed *ad libitum* or a 30% CR diet from 9-15 weeks of age, as described for Figure 5. **(A,B)** Body mass was recorded weekly. **(C,F)** Blood was sampled from the lateral tail vein of 15-week-old live mice. Serum was isolated and concentrations of total and HMW adiponectin were determined by ELISA. **(D,G)** Body composition of 15-week-old live mice was determined by NMR. **(E,H)** Masses of the indicated tissues were recorded at necropsy and their percentage of total body mass was determined. Data in **(A-B)**, **(D-E)** and **(G-H)** are reported as mean ±SD of the following numbers of mice: male control, n = 6; male CR, n = 7; female control, n = 6; female CR, n = 5. Data in **(C)** and **(F)** are box and whisker plots. For each sex, statistically significant differences between control and CR animals are reported as described for Figure 1.