Mechanical and material properties of cortical and trabecular bone from cannabinoid receptor-1-null (*Cnr1*$^{-/-}$) mice

Aysha B. Khalid[a1], Simon R. Goodyear[a], Ruth A. Ross[b2], Richard M. Aspden[a]

[a]Arthritis and Musculoskeletal Medicine, [b]Kosterlitz Centre for Therapeutcs, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD, Scotland, UK

Present address: [1]Department of Orthopaedic Surgery, University of Tennessee Health Science Center, Memphis, TN 38163, USA. [2]Department of Pharmacology and Toxicology, University of Toronto, Toronto, Canada.

Address for correspondence:

Professor R.M. Aspden

Musculoskeletal Research Programme

University of Aberdeen

Institute of Medical Sciences

Foresterhill

Aberdeen AB25 2ZD

UK

Tel: +44 1224 437445

Email: r.aspden@abdn.ac.uk

Abstract

The endocannabinoid system is known for its regulatory effects on bone metabolism through the cannabinoid receptors, *Cnr1* and *Cnr2*. In this study we analysed the mechanical and material properties of long bones from *Cnr1*$^{-/-}$ mice on a C57BL/6 background. Tibiae and femora from 5- and 12-week-old mice were subjected to three-point bending to measure bending stiffness and yield strength. Elastic modulus, density and mineral content were measured in the diaphyses. Second moment of area (MOA_2), inner and outer perimeters of the cortical shaft and trabecular fractional bone volume *(BV/TV)* were measured using micro-CT. In *Cnr1*$^{-/-}$ males and females at both ages the bending stiffness was reduced due to a smaller MOA_2. Bone from *Cnr1*$^{-/-}$ females had a greater modulus than wild-type controls, although no differences were observed in males. *BV/TV* of 12-week-old *Cnr1*$^{-/-}$ females was greater than controls, although no difference was seen at 5-weeks. On the contrary, *Cnr1*$^{-/-}$ males had the same *BV/TV* as controls at 12-weeks while they had significantly lower values at 5-weeks. This study shows that deleting *Cnr1* decreases the amount of cortical bone in both males and females at 12-weeks, but increases the amount of trabecular bone only in females.

Keywords

Bone; cannabinoid receptor 1; mechanical properties; material properties; cortical bone; trabecular bone

1. **Introduction**

The strength of a bone is determined by the material properties of the matrix and the shape of the bone. These, in turn, are determined by a cellular process of modelling and remodelling involving matrix resorption and formation; osteoclasts resorb bone while osteoblasts lay down new bone [1]. The balance between formation and resorption determines the overall amount of bone, with osteoporosis resulting from resorption outweighing formation during remodelling [2] and high bone mass disorders when formation exceeds resorption, either because of increased anabolic activity [3] or defective osteoclastic resorption as in osteopetrosis [4,5] due to genetic abnormalities. These processes are governed centrally, *e.g.* via leptin and the hypothalamus [6], via the autonomic nervous system [7,8], as well as by local signals such as mechanical loads. Central and local regulation are coordinated to ensure that gross imbalances do not occur in bone deposition or resorption at different sites in the body.

Among many factors now identified, recent studies have uncovered a role for cannabinoid signalling in the regulation of bone [9-14]. The endogenous cannabinoid (endocannabinoid) system is widely studied for its regulatory effects on numerous physiological functions, including appetite, pain sensitivity and immune function [15-18]. In addition, it is being increasingly recognised as having a complex regulatory role in bone metabolism [10,19-21]. There are two classical cannabinoid receptors, *Cnr1* and *Cnr2* and these belong to the family of G-protein coupled receptors that, when activated, inhibit adenyl cyclase activity, and activate the MAPK signalling cascade [22]. *Cnr1* is expressed ubiquitously throughout the brain [23] and also on immune cells, in vascular tissue and adipocytes [16,24]. *Cnr2*, on the other hand, is predominately located in peripheral immune tissue such as macrophages [24,25]. Both *Cnr1* and *Cnr2* have been reported in osteoblasts and osteoclasts [11,13].

Several studies have indicated a role for the cannabinoid receptor *Cnr1* in bone metabolism [9-11,14,19,21,26] but, in trabecular bone, the phenotype resulting from deleting *Cnr1* in mice has been found to depend on mouse strain and sex. On a CD1 background, *Cnr1*$^{-/-}$ male mice exhibited a high trabecular bone mass, while the females had normal trabecular bone with slight cortical expansion [11]. Another group suggested that females also had a high bone mass and loss of *Cnr1* protected against ovariectomy-induced bone loss [10]. They later extended this to show that trabecular bone volume fraction, *BV/TV,* was significantly greater at 3 months of age in both male and female *Cnr1*$^{-/-}$ mice compared with wild-type animals, although it had fallen to become significantly lower by 12 months [26]. Different results were reported from mice on a C57BL/6 background; both male and female *Cnr1*$^{-/-}$ mice at 9-12 weeks of age exhibited a low bone mass phenotype, accompanied by an increase in osteoclast number and a reduction in bone formation rate [11].

The effects of *Cnr1* deletion on bone properties have mostly been investigated within the trabecular compartment. This is commonly done because the larger surface area of trabeculae results in a higher turnover rate and greater sensitivity to manipulation. It does not, however, reflect fully the range of bone properties. Bones can balance the quantity and quality of bone matrix and factors regulating bone properties could feasibly affect either or both of these; for instance, a weaker matrix may be compensated by increased geometrical properties. The purpose of this paper is to characterize cortical and trabecular bone from the tibia and femur of *Cnr1*$^{-/-}$ mice on a C57BL/6 background to address the discrepancies found in previous studies and extend our knowledge of bone regulation by *Cnr1*.

2. **Materials and Methods**

2.1 Animals

C57BL/6 *Cnr1*$^{-/-}$ mice were available from a previous study in which they were generated by homologous recombination, as described previously [27]. For studying the effect of knocking out *Cnr1* on the mechanical, material and geometrical properties of bone, 5 and 12-week-old male (5 Wild-type (WT), 7 knockouts (KO) and 5 WT, 4 KO) and female mice (5 WT, 6 KO and 5 WT, 9 KO) were euthanized and the hind limbs cleaned and stored in phosphate-buffered saline (PBS) at -20 °C until measurements were made. A series of mechanical and material measurements were made on all the bones, carefully ordered so that each bone was kept as intact as possible for as long as possible [28].

2.2 Mechanical Properties

The lengths of the tibiae and femora were measured using a Mitutoyo digital micrometer (Mitutoyo, Kanagawa, Japan). The longest tibia and femur were selected from each animal and the mechanical properties measured by subjecting each bone to 3-point bending using an Instron 5564 materials testing machine (Instron, High Wycombe) fitted with a 2 kN load cell. The cross-head speed was set to descend at 1.00 mm min^{-1} and the span between the supports was adjusted to 9.93 mm to accommodate the shortest bone. The tibia was positioned with the fibula insertion pointing upwards and femur was loaded with the anterior surface upwards. The bending stiffness, failure load and work to fracture were calculated from the load-displacement graph as described previously [29,30] and summarized in the supplementary information. In brief, the bending stiffness was calculated from the maximum slope of the load-displacement curve, failure load as the maximum load supported by the bone and fracture as the point at which the load decreased suddenly; work to fracture being the area under the curve to this point.

2.3 Material properties

The modulus of the mineralized bone matrix was determined from the ultrasonic speed of sound and the Archimedean density. A Panametrics Pulser Receiver model 5052PR (Panametrics Inc, Waltham, MA, USA) with a V211BA piezoelectric transducer were used to generate pulses of 10 MHz ultrasound. A slice of cortical bone, ~1.5 mm thick, cut from the knee-end of the diaphysis of each bone, was placed on the wear-plate of the transducer, with a drop of conductivity gel to ensure acoustic coupling, and the transit time, t, of a pulse measured in pulse-echo mode using a dual beam oscilloscope (Hitachi V-665A, Tokyo, Japan) and the oscilloscope's internal callipers. The thickness of the slice, d, was measured using an electronic micrometer screw-gauge (Mitutoya, RS Ltd, Corby, Northamptonshire). The longitudinal sonic plesio-velocity, v, was calculated from $2d/t$. The path length of sound in an inhomogeneous medium such as bone is unknown and will generally be longer than the specimen. Thus the calculated longitudinal velocity will be an underestimate and hence it has been termed the sonic plesio-velocity [31]. The density of the cortical bone was measured using Archimedes' principle by weighing the remaining diaphysis in distilled water, W_f, and in air, W_a [28]. Care was taken to ensure that water filled the medullary canal prior to weighing in water and all water was removed prior to weighing in air. Each measurement was performed three times and the density, ρ, calculated from $\rho_f W_a / (W_a - W_f)$ where ρ_f is the density of the water. The elastic modulus, E, was then calculated from $E = \rho v^2$. The mineral content of the bone matrix, expressed as a fraction of the dry weight, was determined by ashing the dried bones at 600 °C for 24 h [32].

2.4 Micro-Computed Tomography

A Skyscan 1072 x-ray Microtomograph (Skyscan, Aartselaar, Belgium) was used to obtain images of the proximal tibia and distal femur from each mouse. Voltage and current settings used were 50 kV and 197 µA and a 0.5 mm Al filter was inserted.

Magnification was adjusted to x58 which gave a pixel size of 5 µm. The rotation step size between each image was 0.67°. A 3D image was reconstructed from the image stack using NRecon version 1.4.4 (Skyscan) and the trabecular and cortical parameters measured using methods recommended by Skyscan [33]. A reference level was set at the level of the growth plate by scrolling down the image stack until the spots where the growth plate crossed the slice could be seen. Four spots were apparent in femora and two in tibiae. Following recommendations by Skyscan, an offset was determined as 50 slices from that reference level and the trabecular region was defined as the following 200 slices (Fig. 1) (using CTan version 1.7.0.2 software (Skyscan)). A region of interest (ROI) was drawn on the first slice to segment cortical and trabecular bone and 7-8 more drawn at regular intervals throughout the stack to ensure that in each slice this segmentation was maintained. Images were thresholded by adjusting the gray level to separate bone, shown by pixels with high gray levels, from non-bone until most of the image noise (speckle) was removed. The threshold gray level finally used in all images was 74. Measures of fractional bone volume (*BV/TV*), trabecular number (*Tb.N*), trabecular spacing (*Tb.Sp*) and trabecular thickness (*Tb.Th*) were calculated by the software. Volumetric Bone Mineral Density (BMD_v) values were calculated using calibration phantoms from primary micro-CT measurements from trabecular and cortical bone. The percentage porosity of cortical bone (5-week old mice) was calculated by measuring the difference between calculated bone volumes at high threshold values (160 for femora and 170 for tibiae) to enable identification of pores and low threshold values as above to identify total cortical bone area.

Micro-CT images were also used to measure the geometrical properties of each diaphysis from the lowest complete slice closest to the centre of the shaft of the bone (Fig. 1). A binary image representing this slice was imported to Image-J (version 1.42q) where a custom-written macro was used to compute the axial second moment of area,

cross-sectional area, and the outer and inner perimeters and cortical thickness. Results are quoted using American Society for Bone and Mineral Research nomenclature [34].

2.5 Statistics

Data analysis and statistical comparisons were made using SigmaPlot (version 11.0 Systat Software Inc., Hounslow). All data were checked for normality and equal variance. Normally distributed data are described by mean (standard deviation) and were analyzed using 2-way Analysis of Variance. Pair-wise comparisons following ANOVA were done using the Holm-Sidak test. Comparisons between two groups were done using Student's *t*-test, or a Mann-Whitney Rank sum test if not normally distributed. Effect size is represented by Cohen's *d* as the difference between the means of *Cnr1*$^{-/-}$ and wild-type divided by the pooled standard deviation so positive values indicate an increase and negative values a decrease following deletion of the gene.

3. Results

Both tibiae and femora from *Cnr1*$^{-/-}$ mice were significantly shorter than WT in young (5-week-old) mice (Fig. 2). By 12 weeks of age, however, the lengths of the KO femora were not significantly different from those of the WT animals.

3.1 Cortical bone

Deleting the *Cnr1* receptor in both male and female mice had detrimental effects on most of the measured mechanical and material properties of cortical bone. Although differences between *Cnr1*$^{-/-}$ and WT were most marked in tibiae from 12-week-old mice,

similar, but smaller, effects were seen in 5–week-old mice. Femora generally followed a similar pattern but differences were less marked so only the results from tibiae are shown here. Data from the femur may be found in Supplementary Data.

Three-point bending showed that the bending stiffness and failure load were significantly lower in *Cnr1*$^{-/-}$ mice compared with WT and that this loss of stiffness and strength was evident by 5 weeks of age (Fig. 3) (Tibial bending stiffness: male *d* = -3.9, female *d* = -3.4, tibial strength: male *d* = -4.1, female *d* = -2.5). By 12 weeks, while these properties had increased considerably in both groups, the KO group appeared to be catching up as the effect sizes were smaller (Tibia bending stiffness: male *d* = -2.6, female *d* = -2.6, tibia strength: male *d* = -2.4, female *d* = -2.3). Deleting *Cnr1* appeared to have no effect, however, on the work to fracture in the tibia, although it was significantly lower in both male (*d* =-2.9) and female (*d* = -2.3) femora at 5 weeks but not at 12 weeks (Supplementary data).

The bending stiffness of a beam arises from a combination of the material and the geometrical properties. The material properties, represented by the modulus and density, of both tibia and femur were unaffected by age or within groups for both males and females. Increases in density (*d* = 3.1) and modulus (*d* = 2.5) were apparent in the *Cnr1*$^{-/-}$ female mice by week 12 compared with *WT*, although a possible trend may be seen by 5 weeks in the female mice which failed to reach significance (modulus, *d* = 1.3, *P* = 0.09). The material properties of bone from *KO* males were unaffected by the deletion (Fig. 4).

The geometrical property that most affects bending stiffness is the second moment of area. Micro-CT showed that this was lower in the *Cnr1*$^{-/-}$ mice at 5 and 12 weeks (Fig. 5). This reduction was more pronounced in males, where it was evident at 5 weeks old (d = -1.6) and reached almost 50% at 12 weeks (*d* = -5.2), than in females where it only became significant at 12 weeks and amounted to a reduction of about 30% compared

with WT (*d* = -4.5). Corresponding to this, both the outer and inner perimeters were smaller in *Cnr1*$^{-/-}$ mice than WT, although this only reached significance at 12 weeks (Fig. 6). The outer perimeter in males appeared to be most strongly affected with a reduction of 20% (*d* = -7.5) compared with 13% (*d* = -1.6) for the inner perimeter. In the female tibiae it was the other way around with the outer perimeter being reduced by 9% (*d* = -3.8) while the inner perimeter was 16% smaller (*d* = -2.38). In the femur, both inner and outer perimeters were significantly smaller in both male female mice at 5 weeks. In the males the reduction was by 15%, for both inner (*d* = -3.3) and outer perimeters (*d* = -2.5) and in the females the reduction in the outer perimeter was by 22% (*d* = -4.7) and the inner by 27% (*d* =-4.9). However, the difference between groups had disappeared by 12 weeks of age (Supplementary data).

There was no difference between the mineral contents of the cortical bone from the female mice at either 5 weeks (mean w/w wet mass 24.4 (3.9)%) or 12 weeks (38.6 (2.2)%). The mineral content of the bone in 5 week-old males was significantly lower in the KO than the WT (15.5 (3.1)% versus 22.8 (6.0)%, *d* = -1.57, *P* = 0.028) but this difference had disappeared by the age of 12 weeks. Because the mineral contents did not appear to reflect the differences in modulus and density in the tibiae from 5-week-old animals we measured the BMD_v of *Cnr1*$^{-/-}$ and found it was slightly, but significantly, greater in the KO (KO: 1.762 (0.016) g cm^{-3}, WT: 1.730 (0.022) g cm^{-3}, *P*=0.021, *d* = 1.69*)* while the percentage porosity was smaller in KO mice than WT (KO: 1.13 (0.47)%, WT: 2.80 (0.75)%, *P* = 0.0015, *d* = -2.27).

3.2 Trabecular bone

Micro-CT was used to obtain volumetric and absorptiometric measures of bone quantity in areas of trabecular bone at the metaphyses. Different responses were found in males and females to deletion of *Cnr1* (Fig. 7). At 5 weeks of age, male *Cnr1*$^{-/-}$ had a

lower *BV/TV* (*d* = -4.2) but caught up with WT by the age of 12 weeks. This was not reflected in BMD_v, which increased with age but showed no differences between groups. Female mice, however, showed no difference between groups at 5 weeks old but by 12 weeks of age *Cnr1*$^{-/-}$ both measures in tibiae were significantly larger than in WT (*P*<0.001). The patterns, however, were different: *BV/TV* increased from 9.6% (1.7%) at 5 weeks to 14.2% (2.5%) at 12 weeks while WT remained unchanged, whereas BMD_v remained unchanged from 5 to 12 weeks in *Cnr1*$^{-/-}$ but in WT it decreased leading to a difference between the groups. The difference in appearance can be seen in micro-CT images of trabecular bone in (Fig. 8).

Both male and female *Cnr1*$^{-/-}$ mice had thinner trabeculae than WT animals and Tb.Th increased more slowly from 5 to 12 weeks of age (Fig. 9). The effect was greater in males (*d* = -2.9 and -3.0) than females (*d* = -1.2 and -3.1). In male mice at 5 weeks there were fewer, more widely spaced trabeculae but this was reversed by 12 weeks. In female mice there were no differences in Tb.N or Tb.Sp between KO and WT at 5 weeks but by 12 weeks Tb.N had increased and Tb.Sp decreased significantly in the KO animals but had not changed in the WT mice.

4. **Discussion**

Studies using animal models to investigate the regulation of bone commonly make measurements only on trabecular bone at only one site, frequently the proximal tibia. Reports, however, have indicated that not only can trabecular bone behave differently to cortical bone, but that even femur and tibia may show different responses [35-37]. Here, using an array of mechanical and material testing techniques on both tibia and femur, we have demonstrated that deleting the gene for the *Cnr1* cannabinoid receptor in C57BL/6 mice affects both cortical and trabecular bone in a sex-dependent manner. We previously showed that skeletal maturity for most factors in C57BL/6 mice is reached by about 4

months of age [38]. In this study we have used two age groups, 5 week (young mice) and 12 week old mice approaching skeletal maturity [39].

Deleting *Cnr1* resulted in diaphyses that were shorter and about 20% weaker in 12–week-old animals, as shown by reduced failure loads. Bending stiffness was also reduced, by about 40% in males and 30% in females compared with WT animals. The bending stiffness is proportional to the product of the modulus and the second moment of area and further examination suggests the reduction in bending stiffness is due more to geometrical factors than to changes in modulus. The *Cnr1*$^{-/-}$ mice appear to be slower growing than WT so that at each time-point the inner and outer cortical perimeters are smaller leading to a reduction in the second moment of area. In females, a small increase in modulus was found (about 10%), although this was not enough to offset the approximately 30% reduction in second moment of area and may represent an attempt to compensate for the loss of size. These data support a previous study by Tam *et al*. who reported that in C57BL/6 mice with a targeted deletion of the *Cnr1* receptor the medullary cavity diameter was smaller than the control animals [11].

The effect on material properties was also different in males and females. In males there was no significant difference between WT and KO for the modulus or density of tibial bone at either age whereas in females these were both increased by gene deletion. Because we found a reduction in the mineral content in tibiae from 5-week-old males while the elastic modulus and density were little altered, or even possibly increased, we also measured the percentage porosity and BMD_v of the cortical bone. The measured decrease in porosity and small increase in bone mineral density suggest a possible compensatory mechanism to maintain material stiffness in the face of the lower mineralization. In females, the modulus and density were greater in the older age group, although there were no differences in mineralization.

MicroCT measurements of trabecular bone from the tibial epiphyses also showed different patterns between male and female mice, with the effects of deleting *Cnr1* being much more pronounced in females. The amount of bone, measured as *BV/TV*, was 75% greater in female *Cnr1*$^{-/-}$ but in males was not different from WT. The measures of trabecular thickness, separation and number showed correspondingly greater differences, Tb.Th -9%, Tb.Sp -35% and Tb.N +100% in females and Tb.Th -13%, Tb.Sp -18% and Tb.N +22% in males respectively. The greater fractional bone volume in female *Cnr1*$^{-/-}$ mice then appears to be due to a greater number of more closely spaced but thinner trabeculae whereas in males, although these differences are in the same directions, they do not result in more bone. Another interesting observation was that whereas WT males had a considerably greater *BV/TV* than females, values were similar in the male and female *Cnr1*$^{-/-}$ mice, suggesting that ablating *Cnr1* has a greater effect on trabecular bone in males than in females. Other studies have reported that genes regulating BMD act in a sex- and site-specific manner; for example Lagerholm *et al* suggested that about 70% of the variance in BMD in humans is genetically determined [40].

Taken together, these data indicate that deleting *Cnr1* slows the growth of the bones and reduces their geometrical dimensions in both sexes. However, it appears to enhance the cortical material properties in females and stimulates more trabecular bone growth in the epiphyses. Previous studies have indicated that bone properties, including size and shape, are inherited differently in males and females due to physiological differences and different responses to bone regulating hormones in osteoblasts [41] and macrophages [42]. This could indicate an interaction between cannabinoid and steroid signalling in bone regulation. It is tempting to speculate on what might happen in older animals and, although this has been little studied, osteoporosis was reported to occur in both male and female *Cnr*$^{-/-}$ mice derived on a CD1 background despite a small increase

in BV/TV in younger animals [26]. Whether the larger gain in bone found in these mice on a C57BL/6 background will protect against such severe osteoporosis is an intriguing question deserving further study.

A limitation of this study is the relatively small number of animals available. This was governed by availability from a previous study over which we had no control. However, this still presents an opportunity to obtain more detailed measurements of the effects of *Cnr1* deletion on cortical and trabecular bone properties. The magnitude of the effect size of the differences reported and the number of these that were significant, however, are sufficient to show that female C57BL/6 (*Cnr1*$^{-/-}$) mice have a high trabecular bone mass phenotype, and that there is a reduction in the rate of growth leading to smaller and weaker cortices in both males and females compared with wild-type controls. In mice, sexual maturity occurs at about 5-weeks of age, and the period of most rapid growth, just before skeletal maturity, is at about 8-12 weeks [38]. Consequently, the differences between males and females in the young group are unlikely to be due to hormonal differences.

These data are in broad agreement and extend previous studies [10,11] reporting that deleting *Cnr1* results in a high bone mass, albeit in CD1-strain mice. The data do not support, however, the previous findings in C57BL/6 mice in which a low bone mass phenotype was reported [11]. It remains unclear whether there is a strain dependency in the effect of inactivating *Cnr1* or whether these differences could result from different breeding colonies or differences in breeding conditions. Based on the CD1 strain data, because *Cnr1* receptors are rare in osteoblasts, Tam *et al.* suggested that *Cnr1* regulation of bone is mediated through the sympathetic nervous system [11]. The normally high expression of *Cnr1* receptors on sympathetic nerve endings, compared with

their scarcity on osteoblasts, was believed to indicate that the lack of suppression of norepinephrine resulting from deleting *Cnr1* could result in increased bone formation.

In conclusion we have shown that knocking out *Cnr1* in C57BL/6 mice has the effect of reducing the dimensions of the cortex, hence the amount of cortical bone, in both males and females at 12-weeks. This reduces the resistance to bending in both males and females. A small compensatory increase in the elastic modulus was found only in females. The effects on trabecular bone were more pronounced in females with a significant increase in the amount of bone at 12 weeks compared with WT animals. Deleting *Cnr1* appears to slow the growth of the bones in both sexes. This study would benefit from further studies comparing the effect of receptor deletion in mice of different strains, to try to resolve the factors underlying the differences reported in this and previous studies, and to study the effects of ageing as it is here that modulation of the cannabinoid system may have greatest benefit in help to prevent bone loss. The ubiquitous nature of *CNR1* in humans, however, makes it a less-likely target than other receptors due to possible off-target effects.

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Figure captions

Fig. 1 A section through a tibia showing the reference level (growth plate). Trabecular parameters were measured in the outlined region, which was offset from the reference level by 50 slices. Cortical geometry was taken from the last complete slice above the fracture site in 3-point-bending (identified in 3D reconstructions). Femora were processed in a similar way.

Fig. 2 The lengths of tibiae from (a) male and (b) female *Cnr*$^{-/-}$ and WT mice showed a slower rate of growth of bone with KOs having smaller bones at 5 weeks of age and almost catching up by 12 weeks. Results are shown with individual values with the mean value shown as a bar.

Fig. 3 Mechanical properties of tibiae from mice measured by 3-point bending. The bending stiffness (a) and (b) and failure load (c) and (d) were lower in both 5 and 12 week old *Cnr1*$^{-/-}$ male and female mice. Deletion of *Cnr1* had no effect on the work to fracture (e) and (f). Data are represented by circles while the mean is shown as a bar.

Fig. 4 At 5-weeks of age there were no significant differences between groups in either (a) elastic modulus or (c) density in tibiae from males. In contrast, those from females had a significantly larger modulus (b) and density (d) at 12 weeks of age, which did not quite reach significance at 5 weeks. Data are represented by circles while the mean is shown as a bar

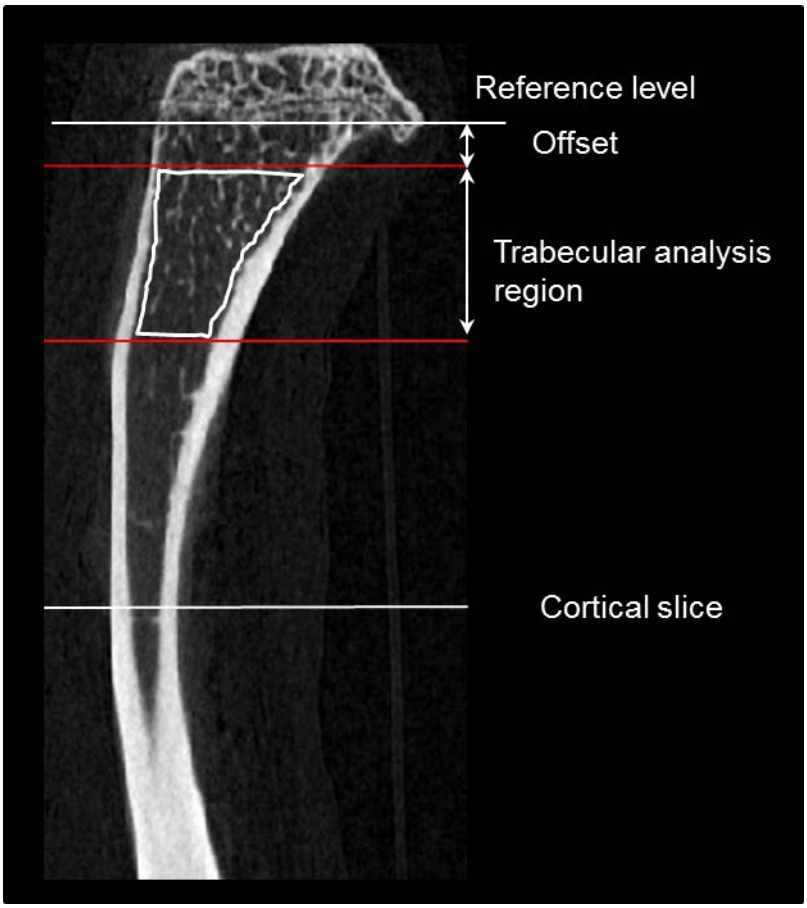
Fig. 5 The cortical second moment of area was consistently smaller in KO than WT animals but in both it increased with age. Data are represented by circles while the mean is shown as a bar

Fig. 6. Cortical geometry was measured from μCT images. Similar patterns were seen in males and females with little difference at 5 weeks and a reduction in both outer (a) and (b) and inner perimeters (c) and (d) at 12 weeks in KO mice. All differences within groups with age were highly significant (P <0.001). Data are represented by circles while the mean is shown as a bar

Fig. 7 Percentage bone volume was lower in (a) male and but not in (b) female *Cnr1*$^{-/-}$ mice at 5 weeks old but had caught up in males and overtaken WT values in females by 12 weeks. This was reflected partially in volumetric bone mineral density with no differences at either age in (c) males but a difference in (d) females due to an apparent loss of BMD_v in WT mice. Data are represented by circles while the mean is shown as a bar

Fig. 8 Representative μCT images of the proximal tibia from 12-week-old *Cnr1*$^{-/-}$ mice. It is evident that *Cnr1*$^{-/-}$ females had significantly more trabecular bone than WT, whereas there was no difference between males.

Fig. 9 Micro-CT measurements from trabecular bone of the epiphysis from 5 and 12 week old *Cnr1*$^{-/-}$ mice tibiae. Trabecular thickness in (a) *Cnr1*$^{-/-}$ males was lower at both time points but in (b) females was lower only at 12 weeks old. Trabecular spacing was greater in (c) males but not (d) females at 5 weeks but by 12 weeks it was significantly smaller then WT in both sexes. The number density of trabeculae in (e) males and (f) females showed a reversal of this pattern being greater then WT at 12 weeks of age. Data are represented by circles while the mean is shown as a bar.



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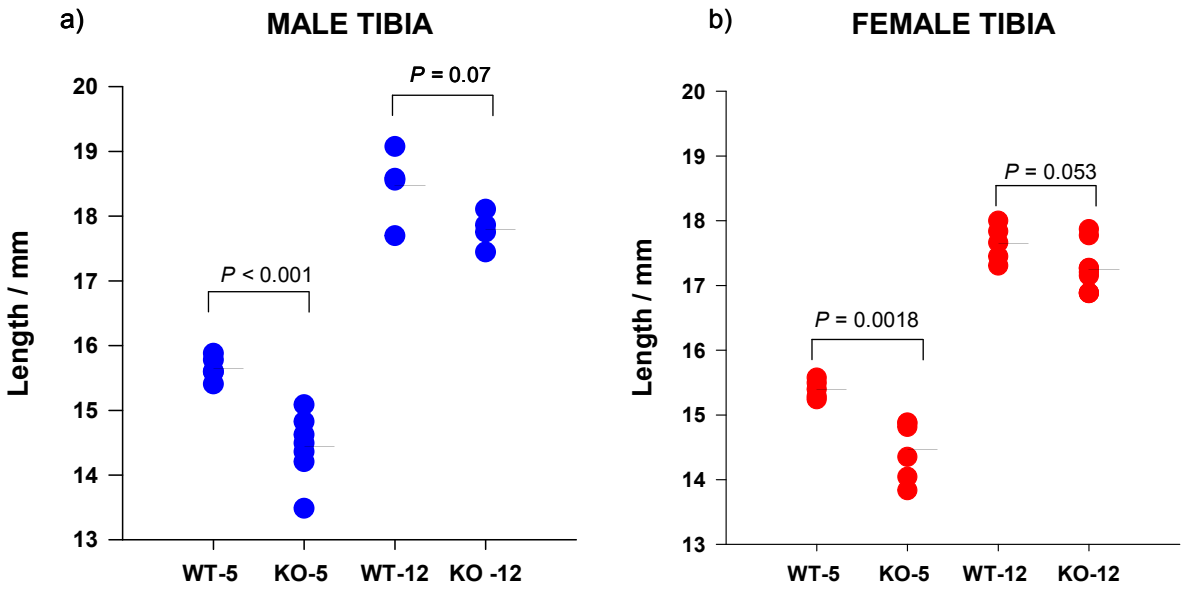

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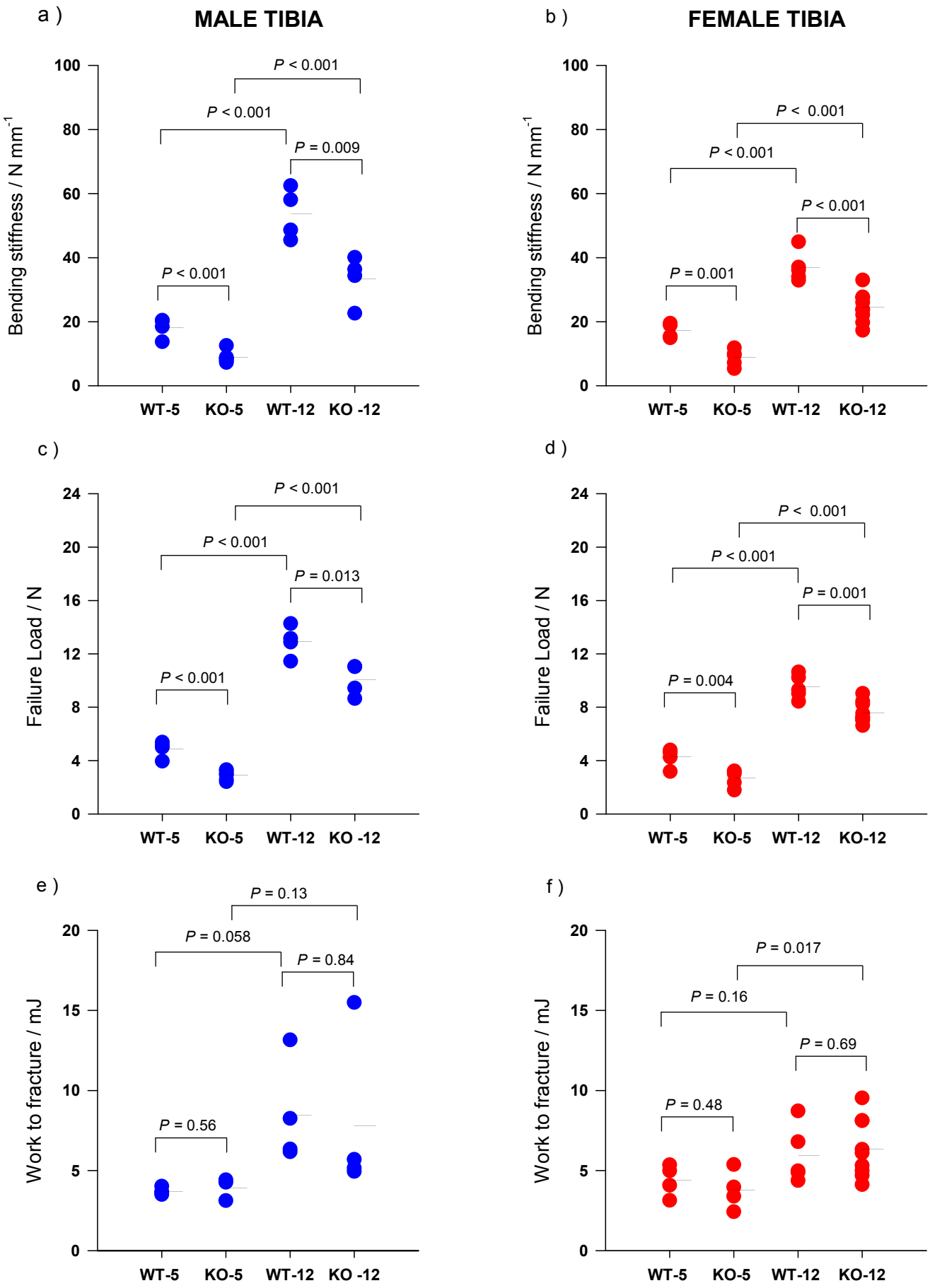

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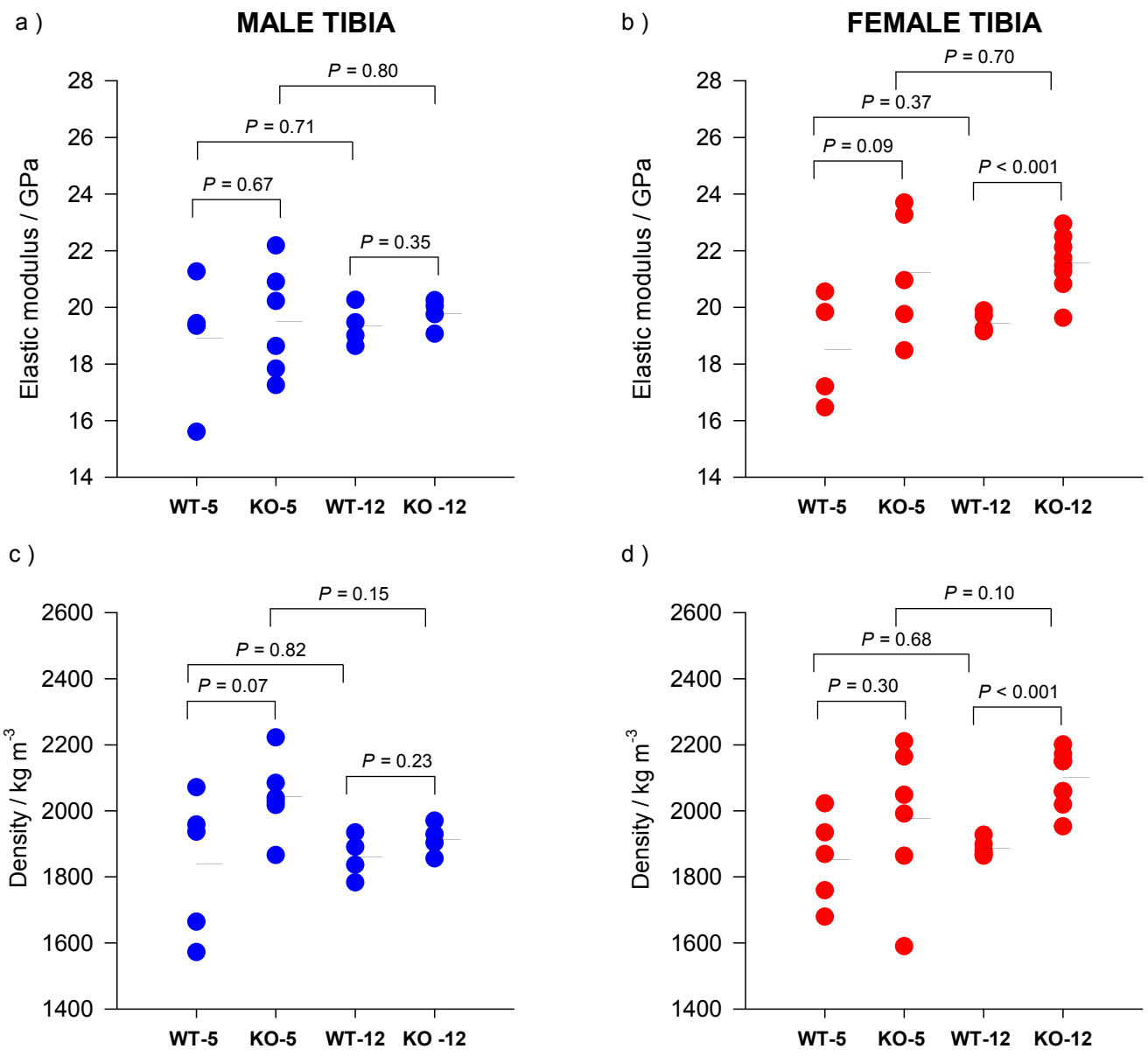

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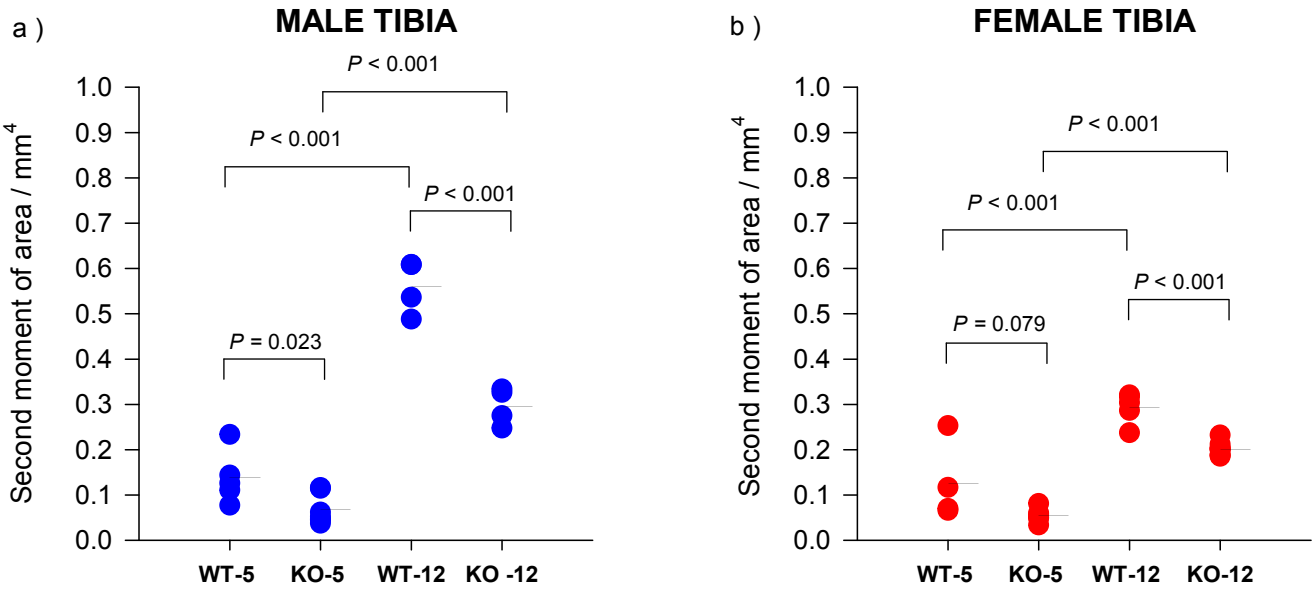

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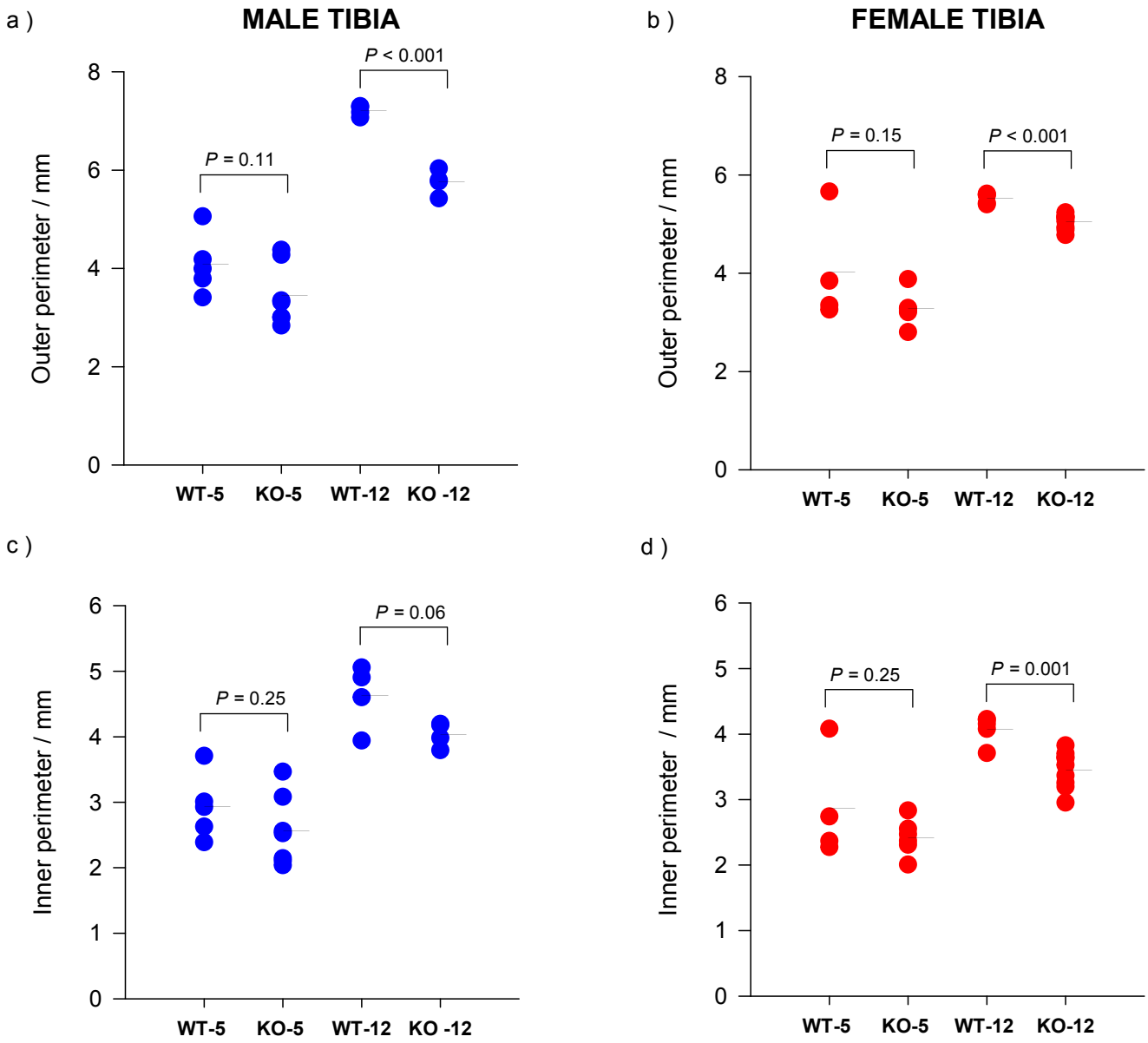

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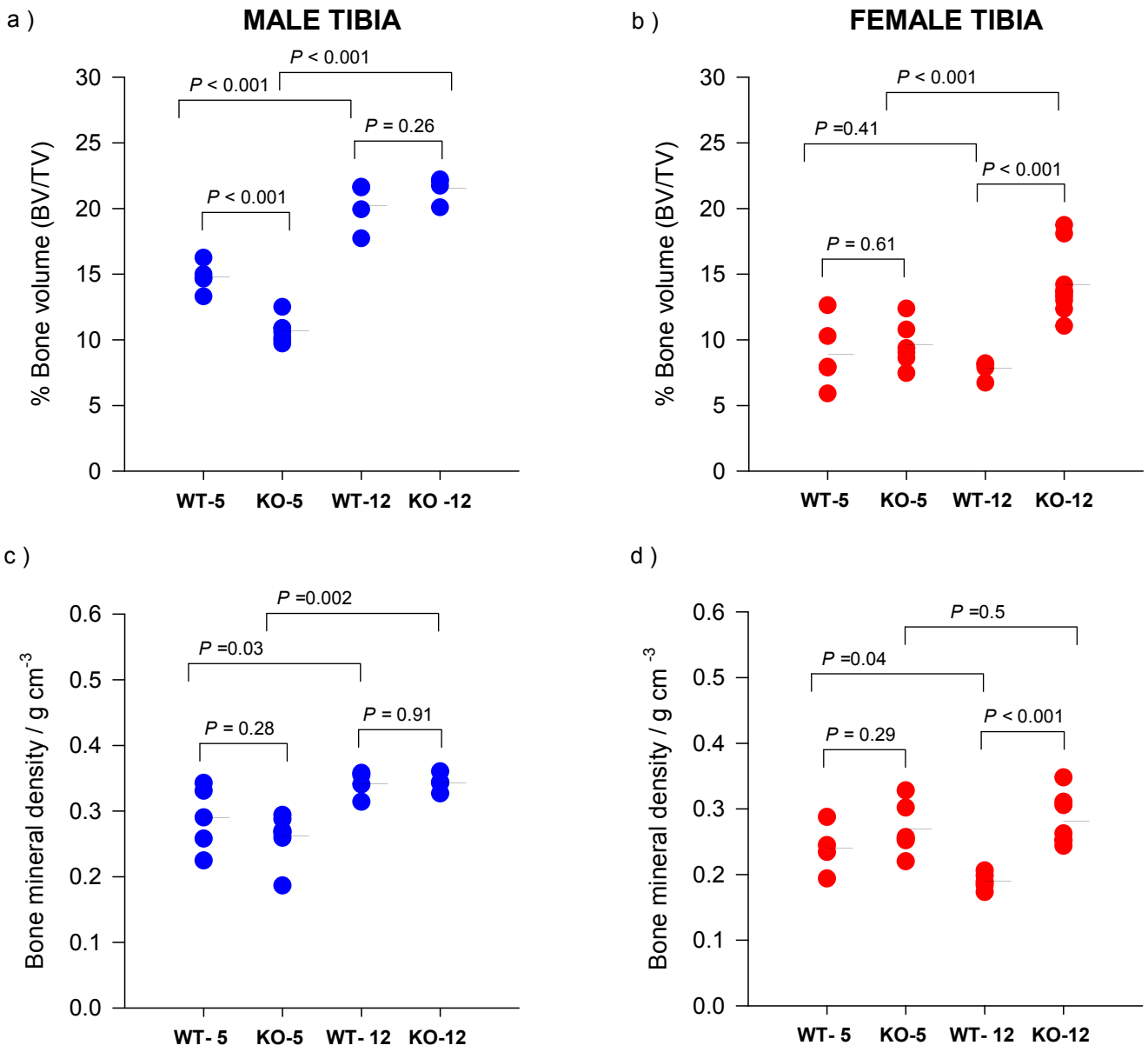

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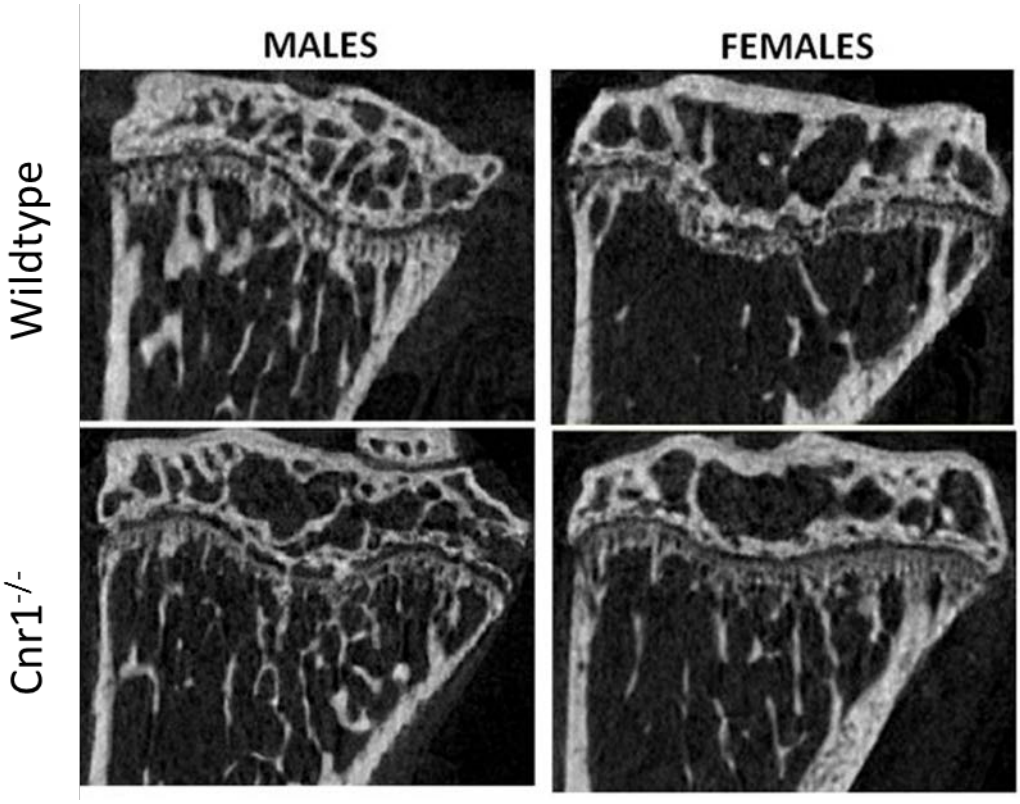

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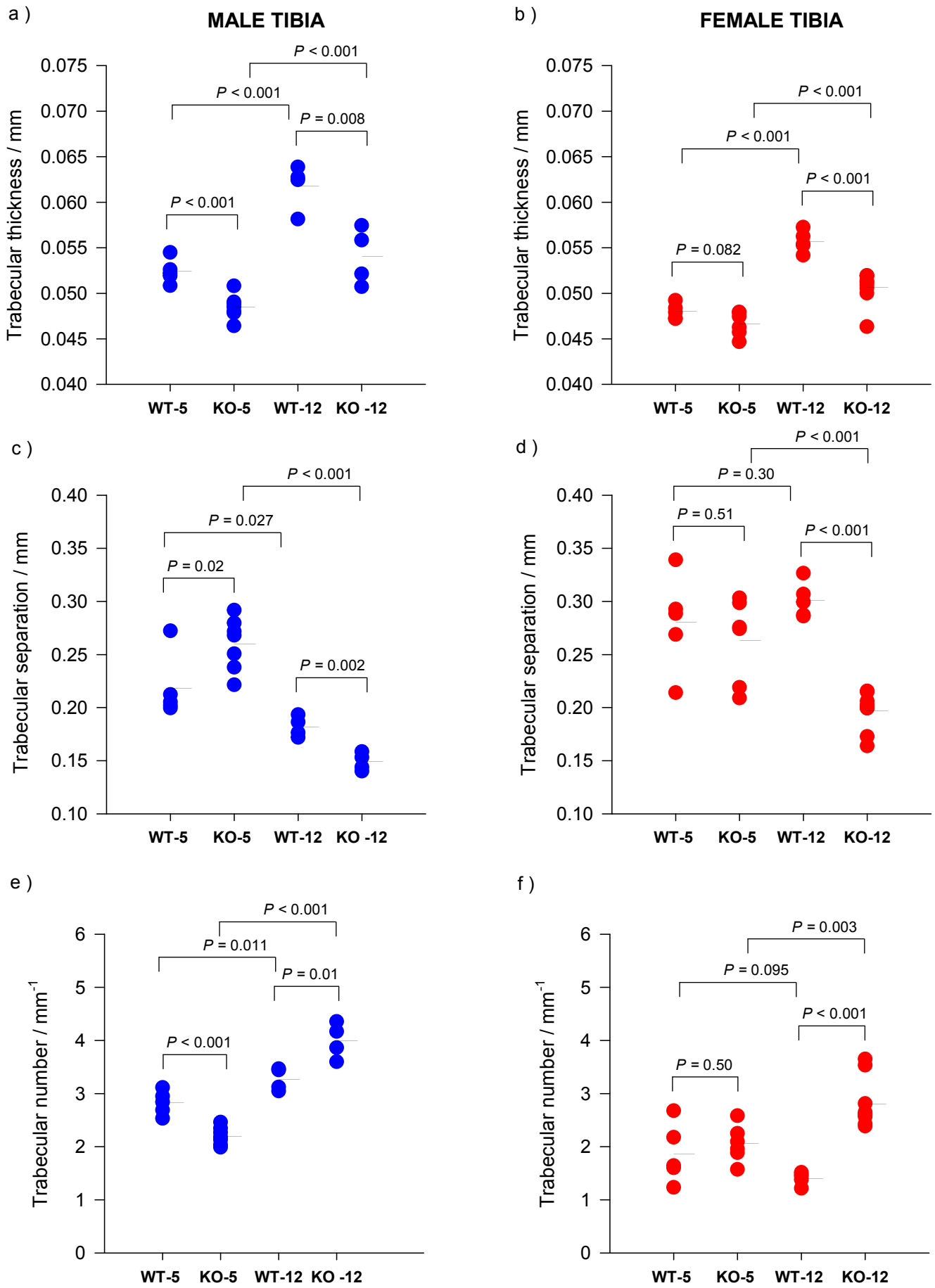

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ELECTRONIC SUPPLEMENTARY MATERIAL

Mechanical and material properties of cortical and trabecular bone from cannabinoid receptor-1 null mice

Aysha B. Khalid, Simon R. Goodyear, Ruth A. Ross, Richard M. Aspden

Corresponding author

Professor R.M. Aspden
Musculoskeletal Research Programme
University of Aberdeen
Institute of Medical Sciences
Foresterhill
Aberdeen
AB25 2ZD
UK
Tel: +44 1224 437445
Email: r.aspden@abdn.ac.uk

In this study we measured the mechanical and material properties of the tibiae and femora from mice from a C57BL/6 background in which the *Cnr1* cannabinoid receptor gene had been inactivated. Mechanical properties measured were the bending stiffness and strength by three-point bending (Fig. S1). The material properties were elastic modulus, density and composition. Micro-CT was used to analyze the cortical geometry and, in trabecular bone, the fractional bone volume *BV/TV*, BMD_v, trabecular number, separation and thickness. Because of the large number of graphs and the similarities with data from the tibia, data from the femora are presented here.

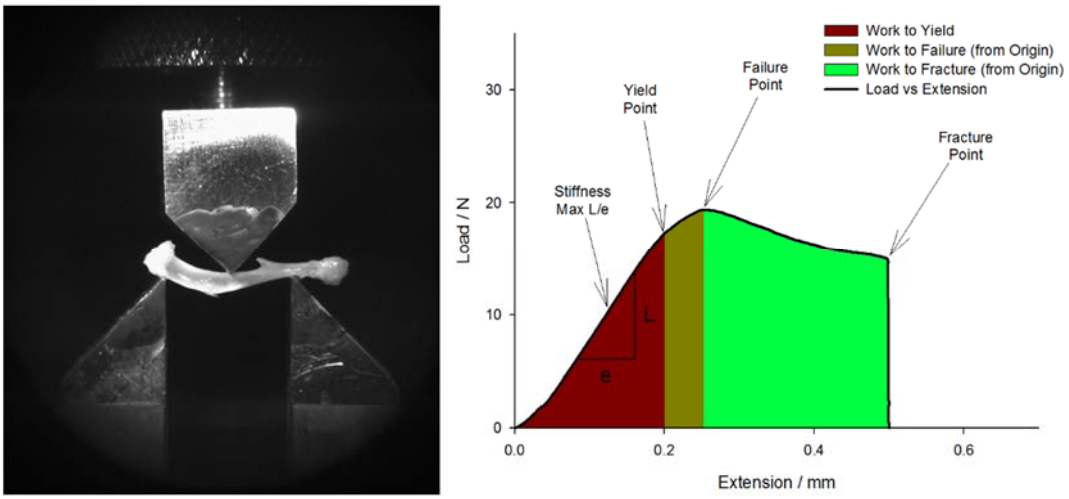

Figure A1. Photograph of a mouse tibia ready for a three-point bending test. The supports were radiused to prevent stress concentrations. The resulting load-displacement curve was analysed for the steepest gradient to define the bending stiffness. The yield stress was defined as the stress at which the gradient had decreased by 5% from its maximum. The area under the curve to each point is a measure of the work done to that point.

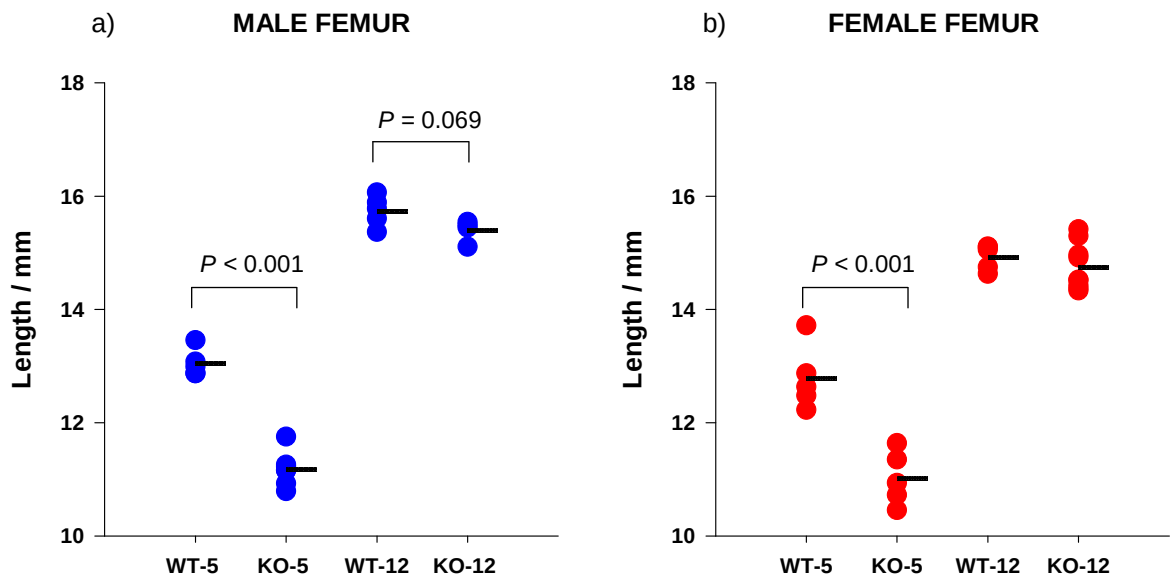

Fig. S2. The lengths of bones from (a) male and (b) female femora of Cnr$^{-/-}$ mice showed a slower rate of growth of bone with KOs having smaller bones at 5 weeks of age and almost catching up by 12 weeks. Results are shown with individual values with the mean value shown as a bar

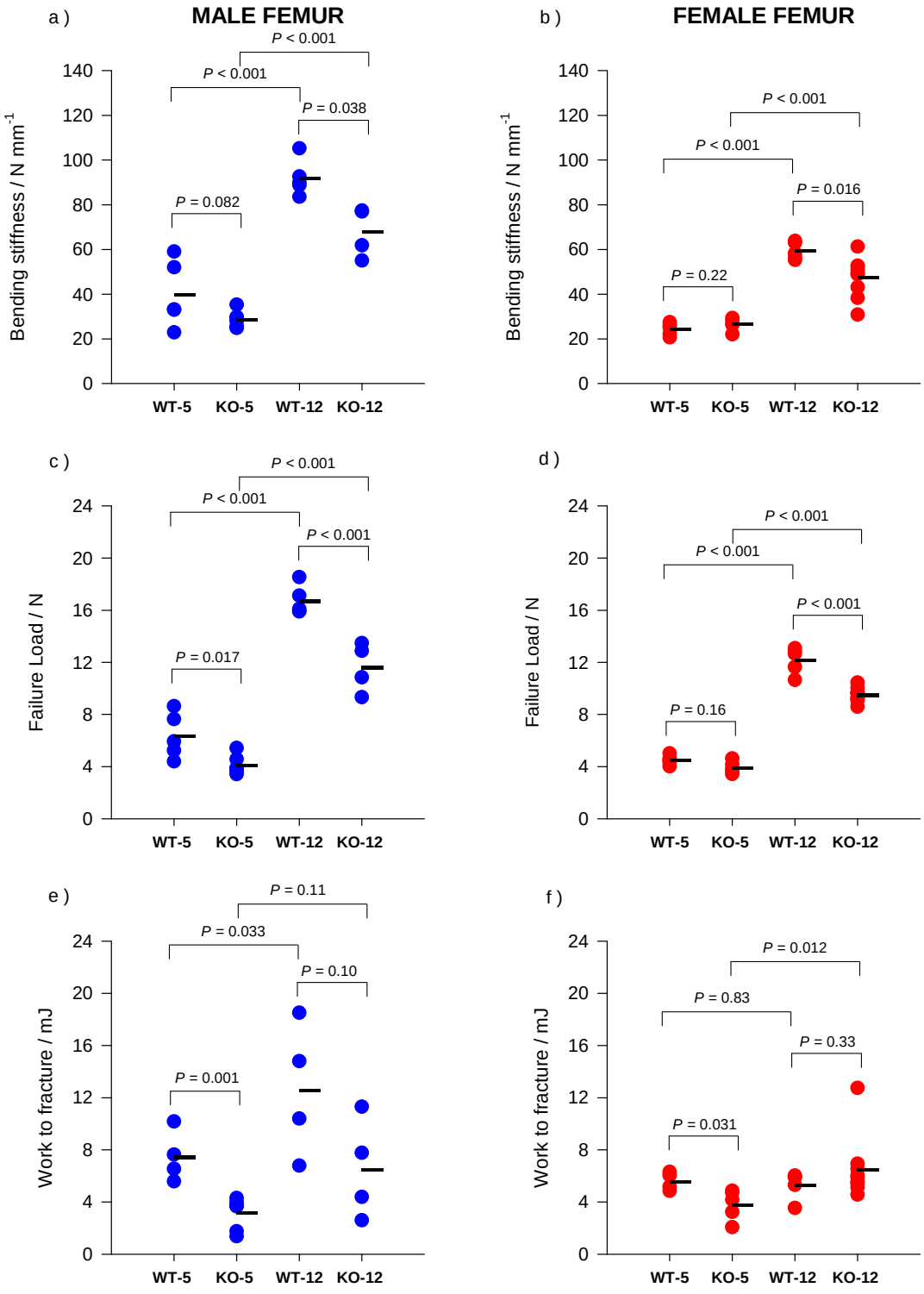

Fig. S3. Mechanical properties of femora from mice measured by 3-point bending showed similar patterns to those in the tibia. The bending stiffness (a), failure load (c) and work to fracture (e) were lower in 5 and 12 week old *Cnr1*$^{-/-}$ male mice. Bending stiffness (b) and failure load (d) were lower in 12-week-old female *Cnr1*$^{-/-}$ femora but there was no difference in work to fracture (f). Individual measurements are represented as circles while the mean is shown as a bar

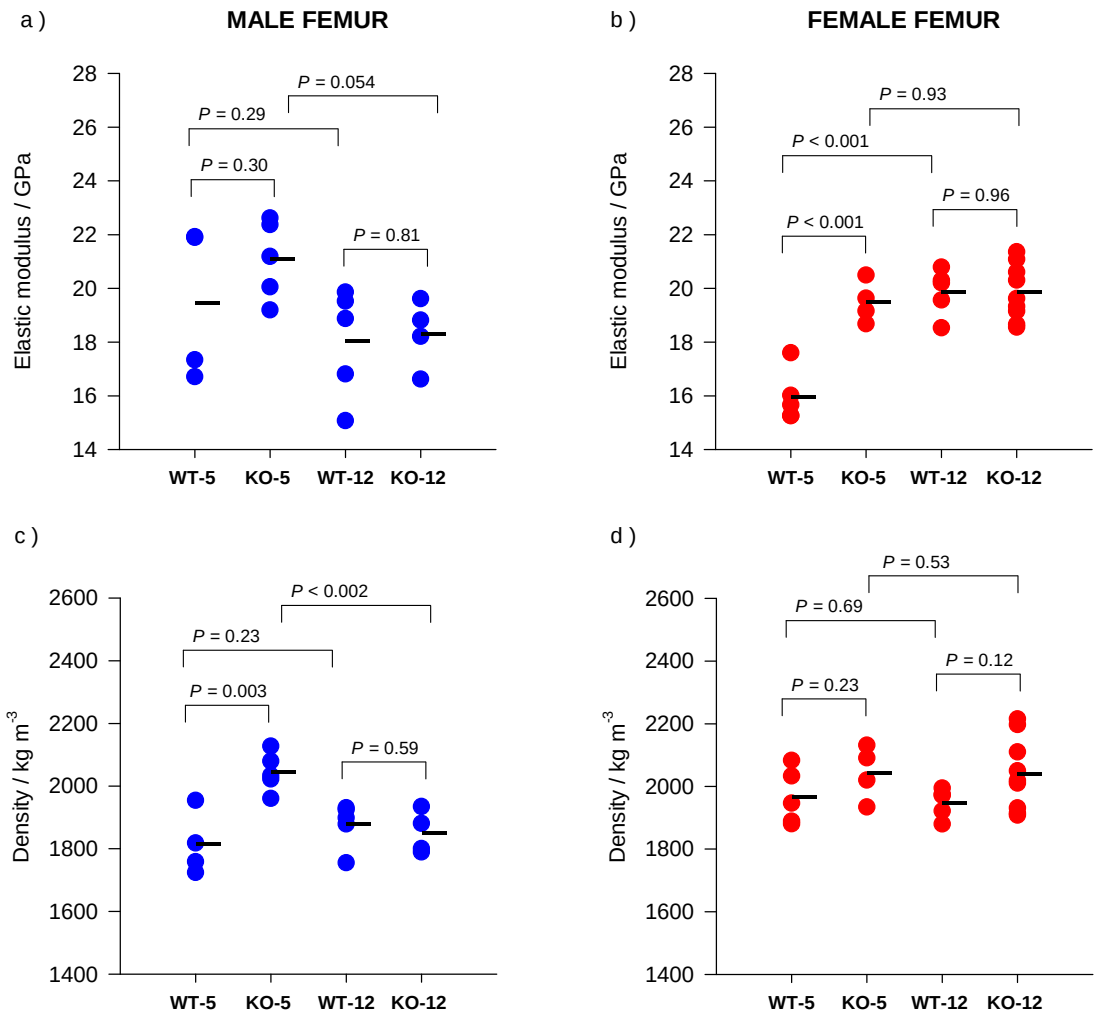

Fig. S4. Bone from femora from *Cnr1*$^{-/-}$ male mice had a greater density, but not modulus, at 5 weeks of age. By 12 weeks, however, there was no difference between the groups in either of these parameters. These data are similar to measurements in tibiae. In females the modulus was greater in *Cnr1*$^{-/-}$ mice at 5 weeks but the WT had caught up by 12 weeks such that no difference was apparent between groups in either modulus or density. This contrasts with the tibia in which both modulus and density were significantly larger in the KO animals.

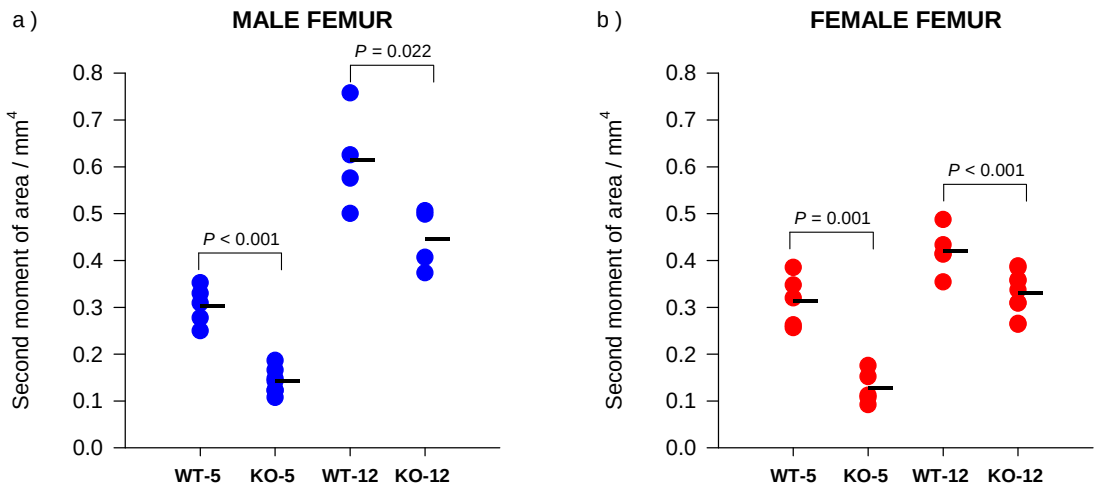

Fig. S5. Similar to the tibia, deleting *Cnr1* resulted in the cortical second moment of area being significantly smaller in male (a) and female (b) femora. Values increased significantly with age in both sex/groups, all $P < 0.001$ except between WT females where $P = 0.002$ between age groups.

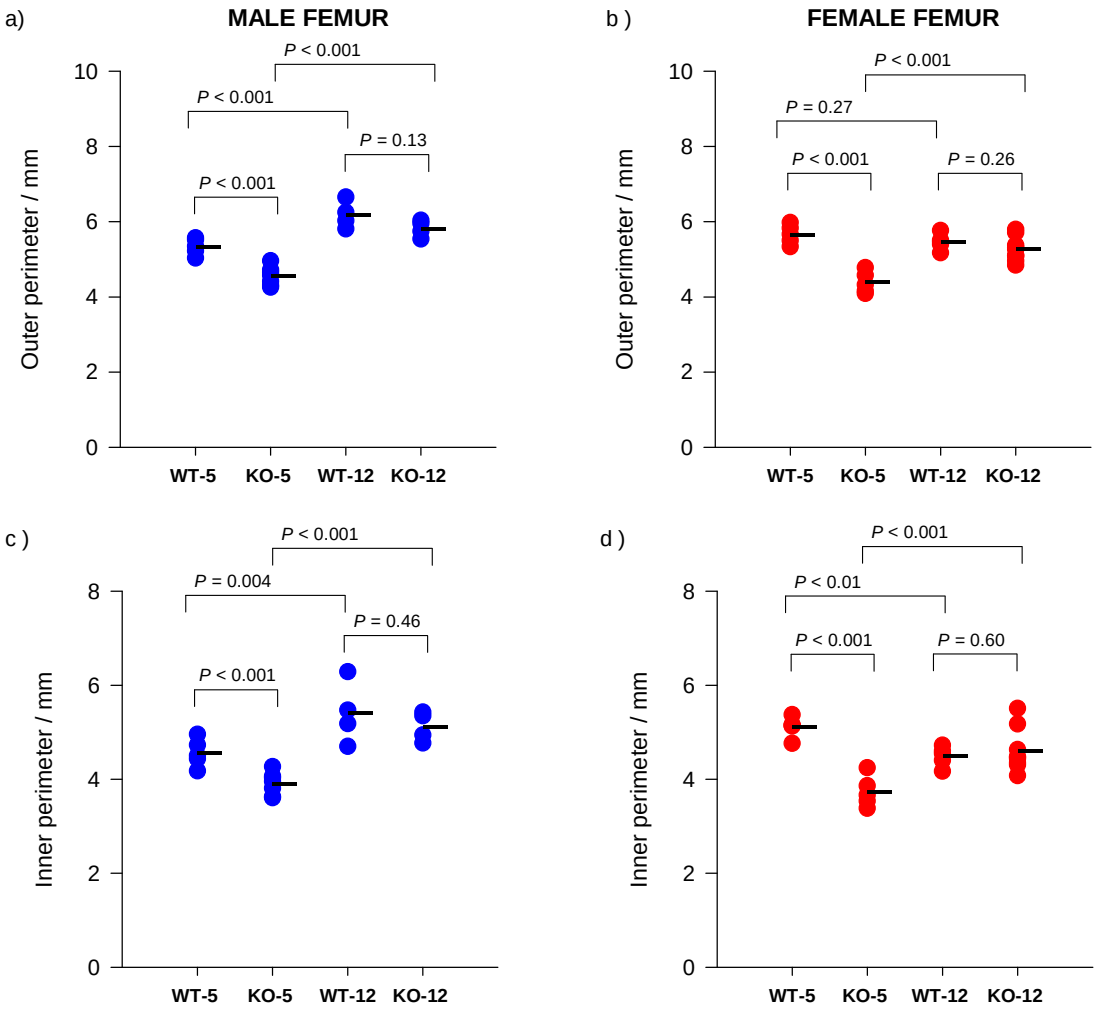

Fig. S6. Cortical geometry measured from micro-CT images showing. Unlike in the tibia, neither the males nor the females showed any reduction in the outer or the inner perimeter at 12 weeks, although both were smaller at 5 weeks of age. This suggests that growth of femur might start more slowly than in the tibia but progresses at a faster rate so that the dimensions have 'caught up' with WT by 12 weeks. (a) male outer perimeter, (b) female outer perimeter, (c) male inner perimeter and (d) female inner perimeter.

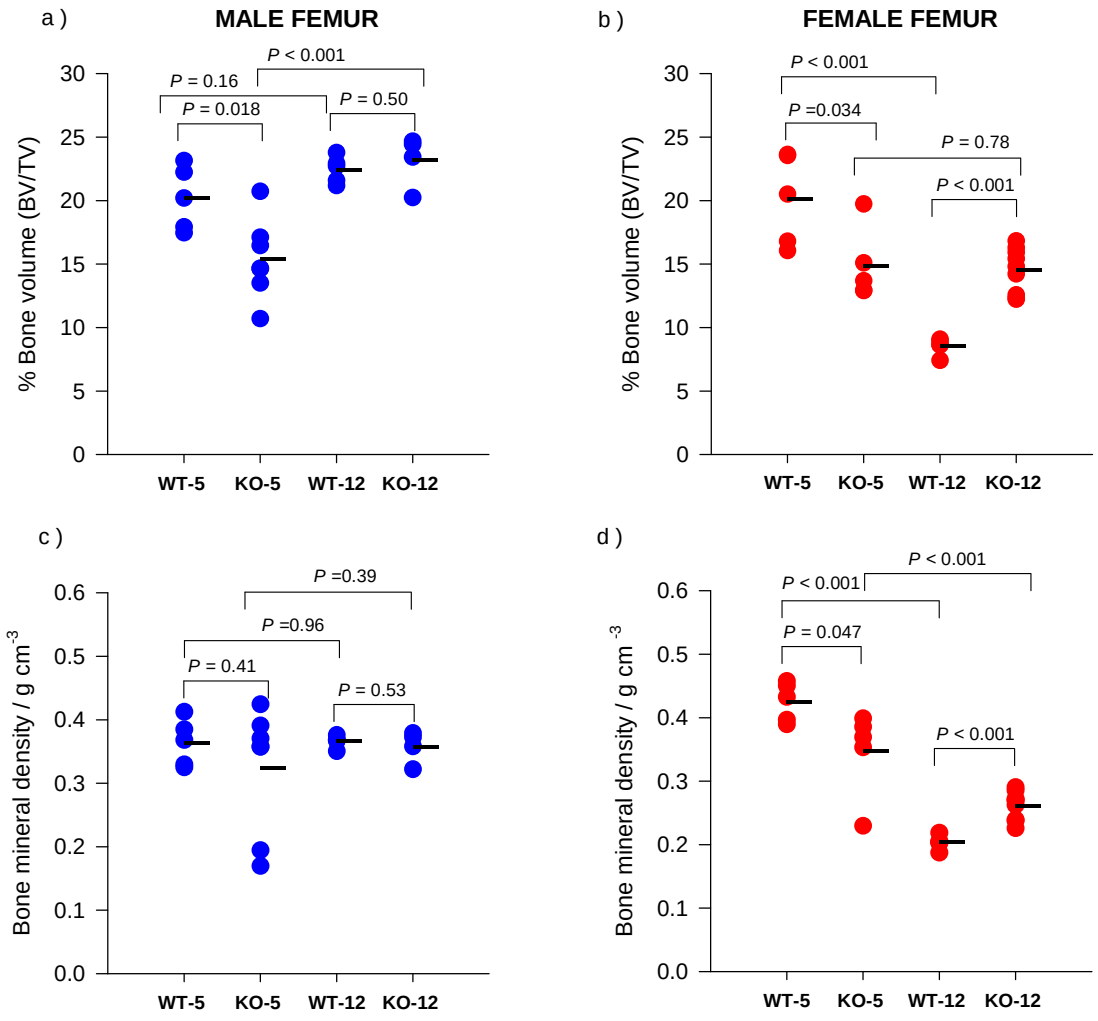

Fig. S7. The fractional bone volume of trabecular bone was lower in (a) male and (b) female *Cnr1*$^{-/-}$ mice at 5 weeks old but had caught up in males by age 12 weeks. In females The WT had lost significant bone volume by 12 weeks old whereas the KO maintained the same BV/TV resulting in a greater value for the KO at 12 weeks. This was not fully reflected in the volumetric bone mineral density as no difference was found in (c) males between groups at either time point but (d) a similar loss of bone was found in WT, and a smaller loss in KO, females.

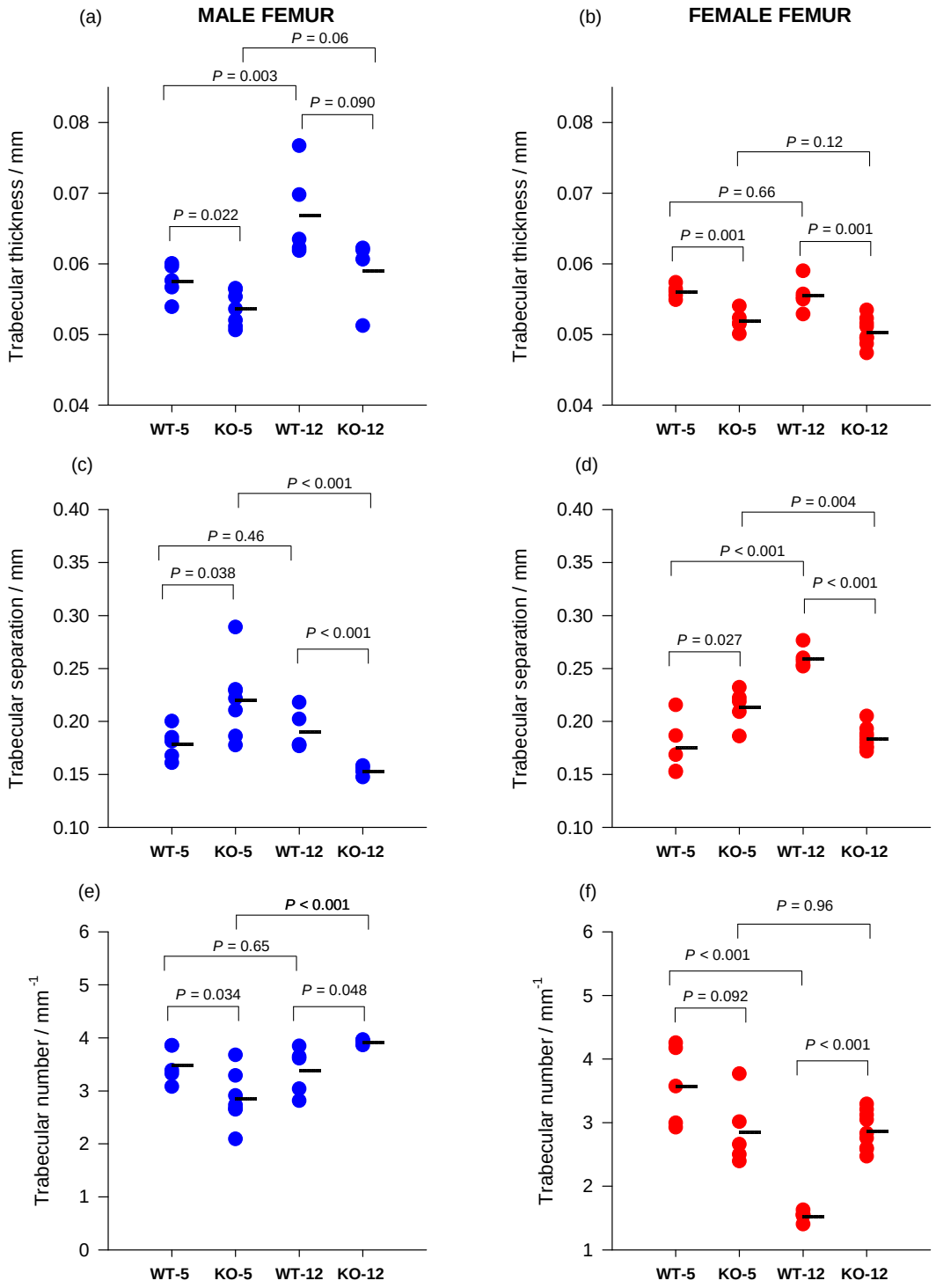

Fig. S8. Micro-CT measurements from trabecular bone of 5 and 12 week old *Cnr1*$^{-/-}$ mice femora showed that the trabeculae were thinner in both (a) males and (b) females. Although this difference was not significant in 12 week old males. Trabecular spacing in (c) males and (d) females was greater than WT in 5-week-old mice but by 12 weeks it was significantly smaller, and the number density of trabeculae in (e) males and (f) females were lower in 5-week-old mice but greater at 12 weeks of age. Trabecular number and spacing remained constant in WT males and all the changes with age were in the KO mice, whereas in female mice the separation increased and the number decreased with age.